NK1.1 receptor ligation or Tumour interactions prime NK cells for IL2-mediated metabolic and functional responses

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

School of Biochemistry and Immunology
Trinity College, Dublin

March 2019

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Declaration

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Nidhi Kedia-Mehta

For Aaba, and Pamma Aaji,

For loving me unconditionally..

Publications

Glucose represses Dendritic Cell-induced T cell responses. (Nature communications, 2017)

Simon Lawless*, **Nidhi Kedia-Mehta***, Jessica Walls, Ryan McGarrigle, Orla Convery, Linda V. Sinclair, Maria N. Navarro, James Murray, David K. Finlay

***equal contributions**

Amino acid-dependent cMyc expression is essential for NK cell metabolic and functional responses in mice (Nature Communications , 2018)

Roisin Loftus, Nadine Assmann, **Nidhi Kedia-Mehta,** Katie L. O'Brien, Arianne Garcia, Conor Gillespie, Jens L. Hukelmann, Peter J. Oefner, Angus Lamond, Clair M. Gardiner, Katja Dettmer, Doreen A. Cantrell, Linda V. Sinclair, David K. Finlay.

Competition for nutrients and its role in controlling immune responses (Nature communications, 2019)

Nidhi Kedia-Mehta and David Finlay

Summary

Natural Killer (NK) cells are innate cytotoxic lymphocytes that are essential for immune response against viral infections and cancer. NK cells are known for their ability to recognize and directly kill target cells through the release of cytotoxic granules. In addition, they can orchestrate the adaptive immune response through their ability to secrete pro-inflammatory cytokines.

The immunometabolic requirements of cytokine activated NK cells have been characterized in detail with glucose described as the main fuel to sustain NK cell metabolism. However, nothing is known about the metabolic changes associated with receptor-mediated stimulation of NK cells. In the present study, we show the metabolic changes that ensue following NK cell stimulation via receptor ligation and the associated functional responses. An interesting finding of this research was high induction of the high affinity IL2 receptor CD25. Our findings reveal that innate immune responses work in conjunction with the adaptive cytokine IL2, where addition of IL2 enhances metabolic responses induced by receptor ligation. Activation of NK cells through NK1.1+IL2 results in a robust induction of glycolysis and OxPhos and this metabolic response is accompanied by a substantial increase in NK cell effector responses like IFN-γ and Granzyme B production. This metabolic response was found to be vital for receptor ligation induced effector responses as any interruption in these metabolic pathways resulted in a loss of NK cell effector responses.

This study revealed the involvement of three key metabolic regulators in the control of receptor ligation mediated metabolic responses- mTORC1, cMyc and Srebp. All these metabolic regulators are sustained by IL2 signalling and therefore IL2 is demonstrated to be the key player in the execution of NK cell metabolic response. Loss of any of these metabolic regulators resulted in a failure in the induction of the metabolic pathways upon NK1.1+IL2 stimulation and

this eventually affected NK1.1+IL2 mediated functional response. Not only did IL2-induced metabolic pathways sustain NK cell metabolism and function; IL2 also contributed to NK cell survival. A key finding of this study was that NK cells interacting with tumour cells also increase the expression of CD25, thus sensitizing them to IL2 mediated metabolic responses that induce potent anti-tumour effects and promote NK cell survival. This study reveals the role of integration of adaptive and innate responses in orchestrating an effective immune response against cancer cells.

Additionally, this report investigated the importance of NK cell metabolic responses for NK cell training. Initial activation of NK cells under metabolic obstruction abrogated training in these cells with diminished ability to produce IFN-γ and kill K562 target cells upon re-stimulation. This study emphasizes the importance of optimal metabolic response in NK cells not just for NK cell effector functions but also for NK cell trained responses.

In conclusion, this report has broadened our understanding about cellular metabolism in NK cells and its role in controlling NK cell effector functions. Data in this report may provide a reason as to why NK cells are often dysfunctional inside a tumour microenvironment and this might underpin the low efficacy rates of most of the current NK cell based Cancer immunotherapies in solid tumours. This report provides a glimpse into the cellular metabolism in NK cells that are directly in contact with tumour cells and this knowledge will lead us towards development of more effective immunotherapies.

Acknowledgements

I would like to extend my deepest gratitude to Dr David Finlay for giving me this wonderful opportunity. Dr Finlay has been extremely supportive and encouraging throughout the PhD, not only guiding my project but giving me numerous opportunities to present at conferences and forums. Secondly, I would like to thank Dr Clair Gardiner who has been a mentor to me. Not only has she provided professional support but has also generously supported me emotionally through the ups and downs of PhD. Clair, your friendship has been invaluable.

I am endlessly grateful to all the past and present members of the Finlay lab. Ray, Roisin and Simon, you guys welcomed me to the lab so graciously, and I have had the most amazing fun with you guys. Thanks for helping me out so much in the beginning of my PhD. You guys came to my rescue over and over again without complaints. I am so thankful that we got along so well and my initial experience was so enjoyable. Simon, it was an absolute pleasure working with you on the DC paper. I can never forget your suggestion on making me the joint first author. That was definitely one of the best moments for me.

Roisin, I think I can never thank you enough for your friendship and support over the years. You have been an abolsute sunshine in my life in TBSI. I am always so refreshed after our chats. Your positivity and energy is infectious. You are a shining star in science and I am so proud of how well you are doing. I am so looking forward to you big day!

Jess, I absolutely adore you! You are one of the brightest people I know, yet so humble and kind. I respect you so much for that. I was so sad that you had to leave for the States after your transfer but I couldn't be prouder of the amazing person you've grown into. I am infinitely grateful for your friendship. We don't see each other often but whenever we chat, it's like you never left. Thank you for always being so kind, supportive and a wonderful friend.

Katie, Thank you for always showing me the bright side. It's been amazing having you in the lab, your company was especially appreciated when the lab was sparse. I am so thankful that you were there. You have grown so wonderfully as a scientist and I am sure you will nail your PhD. I will never forget our San Antonio trip and it wouldn't have been half as much fun without you, even with all the missed flights and the detours!

Chloe, when I met you as my summer student, I never thought we would strike a friendship for life. Not only are you the best student I have ever had, you were also so honest and kind. Thanks for the numerous times you offered help,

especially during the last few weeks. I knew I could always trust you. I am sure you will have an amazing PhD. You are a rare mix of hard work and talent, and I am sure you will excel in your career. I am always there for you.

I have been immensely lucky to have such amazing bunch of girls in the lab, Diana and Elena, you both are so brilliant that it's inspiring and I can't thank you enough for all the help you have so selflessly offered, especially over the last few months. Karen, it's been so fun working alongside you. I am so amazed by all the multitasking you do. More strength to you! Elisabeth and Aisling, you guys are amazing additions to the lab and I am so glad I got to know you before I left. You have been ever so helpful and kind. Alhanouf, you have been so sweet and kind. Thanks for all the sweet treats you got for us. A very special thanks to Sinead Keating who trained me in the lab and was extremely patient with me during my very initial days.

Vanessa and Ashanty, We all clicked the moment we met and it's been amazing doing this PhD with you both around. It was always fun to discuss our lab things that no one else would be interested in! Vanessa, I am always inspired by how strong you are. Thank you for teaching me how to deal with everything that life throws at you with a smile on your face.

Ji, Niki and Aisling A. you girls have been my rock throughout this journey. I could always share my worries with you and that kept me going. Your words of encouragement steadied me when I was falling apart. You all are doing amazing and I am so so proud of you all. Gavin and Lydia, I have had the most fun chats with you two, especially because they are mostly non-sensical but so wise. You guys have brightened up "this" side of the reading room for me. Lydia, it's great to have you as my neighbour, both at work and home. This co-incidence will always blow my mind. Aoife, I don't know a more helpful person than you, thanks for everything. Louise, thanks for answering my random microsoft questions so patiently, it was great doing that evening Philosophy course with you.

I would like to thank all the past and present inhabitants of the 5th floor. Paul, Ryan, Natalie, Darren, Laura, Jenny, Dan, Sarah Power, Sarah Gomez, James, Ana Hutanu, Ana Hastings, Livia, Antoine, Clara, Sadia, Sahar, Ellie, Emma, Peter, Deiter, Daire, Salma, Yong Jing, Ola, Arshad, Pancham, you all have been so much fun to work with and have amazing conversations. You all are top people and I wish you all the best. I think I will never get a more fun environment to work in.

I am grateful to Barry Moran, for helping out with FACS, and to everyone in the animal research unit, especially Rustom and Ciaran for helping me out innumerable times. I am really grateful to Liam Cross and Noel for their help

with any tech problems and Liam McCarthy for his help with the finances. I would also like to thank Laavanya, and everyone in the school office.

Pedro, Daine, Lenka, Eadair, my Ireland fam! You guys have been there when no one else was and when my family was so far away and out of reach. You have looked out for me when Ajinkya was away and even when he wasn't, especially when I was dying sick towards the end of my PhD. I would never have been able to get where I am today without your love and friendship. I knew you guys always had my back and that kept me going.

I would like to thank all my friends back home - Anushree, Aniket, Mona, Gayu, Suraj and Pranoti and all my family who were always rooting for me especially, Sana and Arjun. But most of all I want to thank my cousin, Aakanksha, my brother, Ankit and his wife, Priya for being so supportive and loving. Bhaiya, Bhabhi, I knew I could always come to you guys and you would look out for me.

I am extremely grateful to my parents Mr Santosh and Mrs Sangita Kedia, and my in-laws Dr Suchitra and Dr Ajay Mehta for their unwavering support and enthusiasm for my PhD. You guys were always so proud of me and never let me give up. Your encouragement and care kept me going and so did your happiness and celebration at my smallest achievement. I am blessed to have not one but two sets of loving parents. This PhD would not happen without your belief in me.

Last but never the least, my wonderful husband, Ajinkya. I am not exaggerating when I say that this PhD would not have been possible without you. I have shared each and every day of my PhD with you and you have been there through it all- my complaints, my failed experiments, my successes, my good days and bad days. Your love, support, friendship, companionship made this journey so easy for me. You took care of everything else so that I could focus on my PhD. The only reason I haven't gone crazy yet is because you were there for me at the end of every day.

Chapter 1

Introduction

Natural Killer Cells or NK cells are a population of lymphocytes that were initially classified as innate immune cells but are rapidly proving to be important for the adaptive arm of the immune system as well. NK cells are one of the three major cell types derived from common lymphoid progenitors (CLP) in the bone marrow; the other two cell types being B and T cells of the adaptive immune system. They belong to the extended lymphocyte population known as innate lymphoid cells or ILCs, and are also called ILC1 (Cording et al., 2016; Spits et al., 2013). NK cells are best described to have a role in tumour immune-surveillance and early responsiveness to viral infections. They are the innate counterparts of cytotoxic T cells or CTLs (Sun and Lanier, 2011). NK cells, like CTLs are cytotoxic cells and significant producers of the pro-inflammatory cytokine, Interferon-γ (IFN-γ). However, unlike the highly specific receptors of the CTLs, NK cell receptors do not undergo 'recombination activating gene' (RAG)-dependent gene rearrangement but are encoded by germ line genes.

NK cell effector functions

Natural killer cells, as their name implies, kill target cells directly, without the need of immunization or antigen presentation. They can rapidly produce soluble factors such as chemokines and cytokines that can alert other immune effectors as well as having cytotoxic effects. NK cells also have myriad receptors that can respond to cytokines and viral proteins. These receptors can also distinguish target cells from self-cells by virtue of stress ligands and abnormal presentation of major histocompatibility complex (MHC) class I ligands (Kärre et al., 1986). This highly evolved arsenal of defence makes NK cells an indispensable player in the innate immune system.

Cytotoxicity

NK cells contain secretary granules that can be released when an NK cell encounters a target cell. These secretory granules are modified lysosomes that

contain two different types of cytotoxic proteins- Perforin and Granzyme (Kägi et al., 1994; Lieberman, 2003). Perforin is a pore-forming protein that perforates the target cell, not only damaging it, but also creating a channel through which the granzymes can enter the target cell. Granzymes are serine proteases that induce apoptosis once they enter the cytosol of the target cell. Granzyme B is one of the most abundant granzymes and brings about apoptosis either by directly initiating the caspase proteolytic cascade through the activation of caspase 3 or by cleaving a protein called BID, which brings about the initiation of intrinsic apoptotic pathway via the release of cytochrome c from the mitochondria into the cytosol (Packard et al., 2007). Another strategy that NK cells use to bring about target cell killing involves TNF family members- FAS ligand (FASL), Tumour necrosis factor (TNF) and TNF related apoptosis inducing ligand (TRAIL) (Di Santo, 2006; Wiley et al., 1995; Zamai et al., 1998). These ligands interact with death receptors DR4 and DR5 on the surface of target cells, which triggers the activity of pro enzyme caspase 8, causing apoptosis (Ashkenazi and Dixit, 1998; Pan et al., 1997; Screpanti et al., 2001). NK cells also express Fc receptors that recognize the Fc portion of antibodies bound to cells. This triggers NK cells to release cytotoxic granules and bring about antibody dependent cellular cytotoxicity (ADCC) (Clynes et al., 2000).

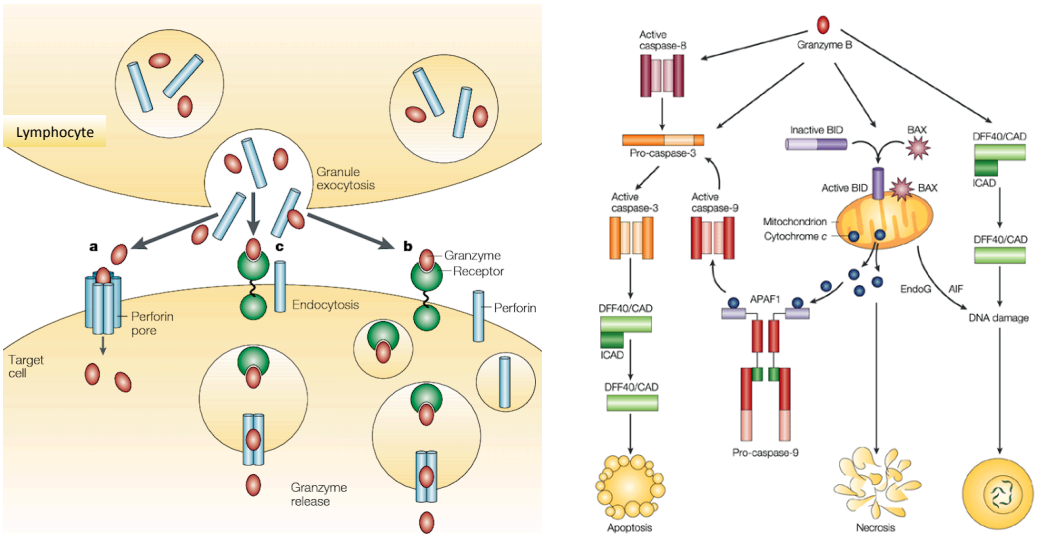


Figure 1.1 Lymphocyte mediated Cytotoxicity

On being activated, cytotoxic lymphocytes such as CD8+ T cells and NK cells release lysosomes containing cytotoxic proteins. Perforin is a pore forming protein that assembles together on the surface of a target cell and creates a port through which Granzymes can gain entry into the target cell (left). Granzymes then initiate a chain of reactions in the target cell, resulting in target cell death by apoptosis (right) (Barry and Bleackley, 2002).

Cytokine release

Activation of NK cells by some of the innate cytokines like IL12 and IL18 can induce the production of the pro-inflammatory cytokine IFN-γ. This early IFN-γ is crucial in the innate response to viral and bacterial infections. As well as boosting innate immunity by stimulating macrophages to enhance their killing capacity, this early IFN-γ produced from NK cells is pivotal in orchestrating the adaptive immune response. It does so by inducing antigen presenting cells to express MHC class II molecules and by polarizing the CD4 T cell differentiation towards the pro-inflammatory T helper type I (Th1) subset that produce IFN-γ (Morvan and Lanier, 2016). NK cells also produce other pro-inflammatory cytokines such as TNFα and granulocyte macrophage colony stimulating factor (GM-CSF).

Chemokines- NK cells also work to mobilize and activate other immune cells to the site of inflammation by secreting chemokines such as CCL3 (MIF1α), CCL4 and CCL5 (RANTES).

1.2 NK cell activation

NK cells express a plethora of receptors that recognize and respond to cytokines and chemokines secreted from other immune cells, MHC class I molecules and stress ligands expressed on the target cells. Engagement of these receptors results in NK cell activation (Chan et al., 2014). NK cell activation induces proliferation, production of pro-inflammatory cytokines and chemokines and upon identifying a target cell, the release of cytotoxic granules. NK cells can be activated by cytokines released from other immune cells in the local microenvironment and this is known as the bystander activation of NK cells (Walzer et al., 2005). Alternatively, NK cells carry out immune surveillance (Iannello and Raulet, 2013), whereby their receptors detect stress ligands on aberrant cells or altered expression of MHC class I molecules, which leads to formation of an immunological synapse and eventual cytotoxicity (Grakoui et al., 1999).

Cytokine mediated NK cell activation

In the event of an infection, phagocytes and antigen presenting cells such as macrophages and DCs are the first to encounter pathogens. In response, these cells secrete soluble factors that can in turn, activate NK cells. As already mentioned, IFN-γ, released from NK cells early in an infection, is the key mediator of innate and adaptive immune responses (Biron et al., 1999). One of the most potent inducers of IFN-γ production in NK cells is IL12. The importance of IL12 is evident from the fact that neutralization of this cytokine in mice resulted in significant reduction in IFN-γ production in response to MCMV infection (Orange and Biron, 1996). Apart from IL12, DCs also release

type I Interferons, IFN-α and IFN-β. These cytokines signal through IFNAR on NK cells and trigger NK cell cytotoxicity (Biron, 1997). In addition to inducing IFN-γ production and cytotoxicity on their own, IL12 and Type I IFNs can synergize with other cytokines like IL2, IL18 and IL15, resulting in robust induction of IFN-γ. For instance, IL12 induces the expression of CD25; a functional high affinity IL2 receptor (IL2Rαβγ) that makes NK cells more responsive to T cell derived IL2 (Donnelly et al., 2014; Lauwerys et al., 1999; Leong et al., 2014; Malmgaard and Paludan, 2003). IL12 is also essential for IL18 mediated responses. IL18, which is a potent inducer of IFN-γ cannot exert its full effect without IL12 signalling (Nakahira et al., 2002).

Most of these cytokines signal through receptors that contain the common γ chain (γc), a highly conserved receptor subunit, which associates with the Janus Kinases or JAK proteins (Miyazaki et al., 1994). These proteins phosphorylate tyrosine residues that initiate different signalling cascades including the JAK/STAT pathway, the PI3K/Akt pathway and the MAPK pathway. All these pathways result in the induction of transcription factors that induce the expression of genes involved in NK cell proliferation, cytotoxicity and cytokine production (Marçais et al., 2013) **(Fig 1.2).** IL12 signals through the JAK/STAT pathway and specifically activates STAT4. Interestingly, IL12 dependent induction of IFN-γ and Th1 polarization is a result of STAT 4 activation (Kaplan et al., 1996). IL2 and IL15 mainly signal through STAT5 and also activate the mTORC1 pathway, which regulates cellular metabolism (Marçais et al., 2013; Powell et al., 2012) **(Fig 1.3).**

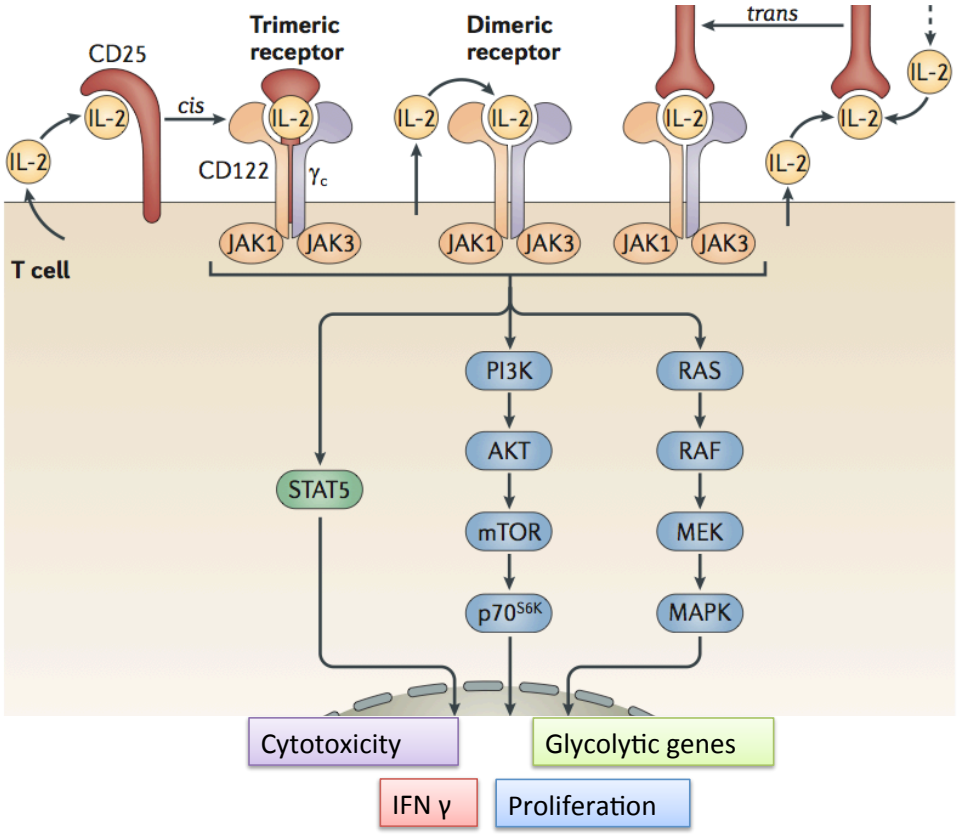


Figure 1.2 The IL-2 receptor system and IL-2 signaling

Interleukin-2 (IL-2), a 15 kDa four-α-helix bundle cytokine, is secreted as a soluble molecule by activated T cells and to a lesser extent by activated dendritic cells (DCs). IL-2 can bind to the IL-2 receptor (IL-2R) subunit CD25 (also known as IL-2Rα) and thisinteraction induces a distinct conformational change in IL-2 that increases its affinity for the IL-2R subunit CD122 (also known as IL-2Rβ). Subsequently, the IL-2–CD25 dimer recruits CD122 followed by the common cytokine receptor γ-chain (γ_c), another subunit of the IL2R. This quaternary IL-2–IL-2R complex has a *K* of~10^{-11}M. In cells that lack the expression of CD25, IL-2 can associate with the dimeric IL-2R directly (with a K_d of~10^{-9} M). Alternatively, T cell- or DC-derived IL-2 can bind to CD25 molecules expressed by DCs, and this IL-2 can then be presented in *trans* to neighbouring T cells that express CD122 and γ_c. On binding to CD122 and γ_c, IL-2 induces the transcription of target genes (such as *Cd25*) through several signalling pathways, including the Janus kinase (JAK)– signal transducer and activator of transcription (STAT) pathway, the phosphoinositide 3-kinase (PI3K)–AKT pathway and the mitogen-activated protein kinase (MAPK) pathway. MEK, MAPK/ERK kinase; mTOR, mammalian target of rapamycin; $p70^{S6K}$, p70 S6 kinase. (Boyman and Sprent, 2012)

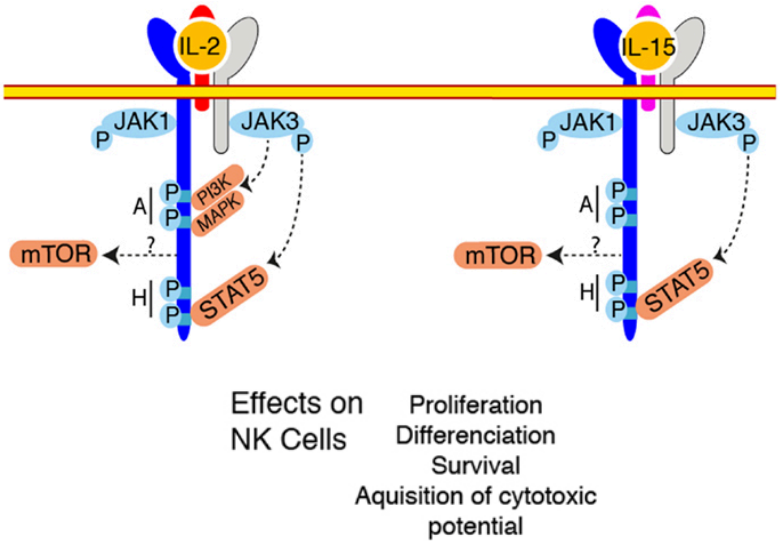


Figure 1.3 Cytokine signaling in NK cells-IL2 and IL15

IL2 and IL15 use γc as a co-receptor to regulate NK cell biology and effector functions. The different signalling pathways they trigger are shown. The effect IL2 and IL15 on NK cells is also mentioned at the bottom. These signal transduction pathways lead to the activation of STAT5 by JAK1 and JAK3. IL15 and IL2 signalling also activate the mTOR pathway. Activation of mTOR is essential to increase NK cell metabolism. Figure adopted from (Marçais et al., 2013)

Receptor mediated NK cell activation

NK cell receptors induce a cytotoxic response against tumour cells or virally infected cells by interacting with ligands present on these cells. Unlike B and T cells NK cells don't undergo recombination activating-gene dependent (RAG) recombination to generate a wide range of antigen recognition receptors that are unique to antigens (Kondo et al., 1997; Mombaerts et al., 1992; Shinkai et al., 1992). In contrast, NK cell receptors have a range of activating and inhibitory receptors that are encoded by germ line genes. NK cell responses depend on the balance between signalling through these stimulatory or inhibitory receptors. As NK cells don't have antigen specific receptors, NK cell recognition of target cells relies on the presence of abnormal ligands or absence of MHC (Major histo-

compatibility complex) class I receptors on target cells (Ljunggren and Kärre, 1990; Olsson et al., 1995). Activating receptors recognize cell surface proteins that are expressed on target cells in response to transformation and infection. These stress induced changes in the host cell surface phenotype is known as 'stress-induced-self' which is indicative of DNA damage, metabolic stress and signalling through TLRs which can result in expression of stress ligands. NK cells can also recognize the Fc portions of the antibodies secreted in response to and attached to various pathogens. The binding of Fc portion to the Fc receptor CD16 on NK cells results in the transduction of signalling pathway that leads to ADCC (Watzl and Long, 2010) **(Fig 1.1).** However, activating receptors may also recognize some constitutively expressed stress ligands on healthy host cells in which case a protective mechanism is required. This protection is afforded by a plethora of MHC class I binding receptors expressed on the surface of NK cells, which are mostly inhibitory receptors. When an inhibitory receptor recognizes and binds to self-MHC class I molecules, the signals from the inhibitory receptors dampen the signals from the activating receptors, thereby preventing host cell killing. However, when an NK cell comes across a self-cell that has either down regulated expression of MHC class I (missing-self) or overexpresses stress ligands (induced-self), signals from activating receptors overcome the signals from inhibitory receptors, allowing NK cells to effectively kill these cells **(Fig1.4).** The recognition and killing by NK cells of target cells that have down regulated their MHC class I expression is known as the "missing self-recognition". This pathway of NK cell recognition possibly evolved alongside the immune evasion strategies of aberrant cells. Both virus-infected cells and tumour cells either down-regulate the expression of MHC class I molecules or modify them as a strategy to escape antigen presentation and the eventual immune response from T cells. NK cells detect this alteration of the self on the target cells and attack them (Lanier, 2005; Murphy et al., 2008).

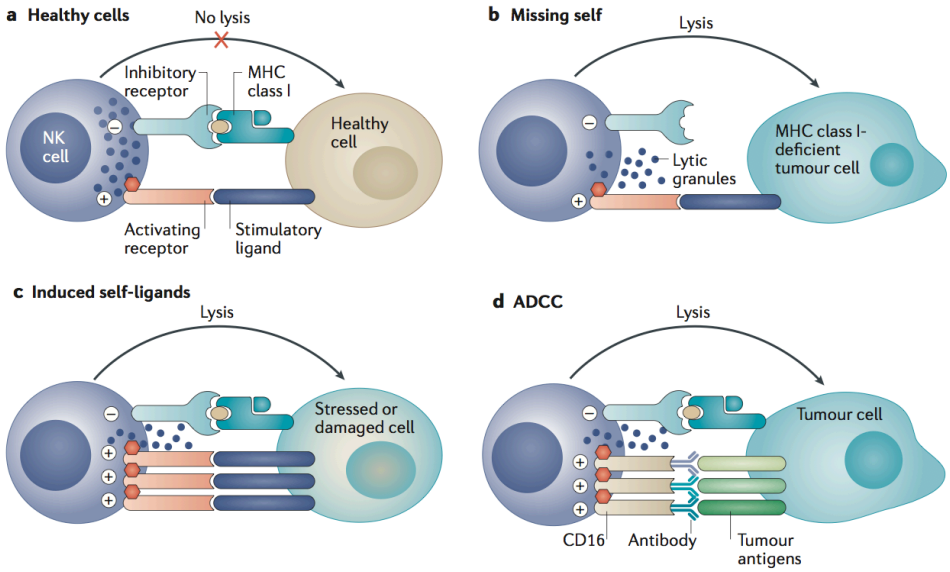


Figure 1.4 Balance of inhibitory and activating receptors governing NK cell functions

a) A balanced signal transduction from activating and inhibitory receptors regulates the recognition of healthy cells by NK cells . b) Infected or transformed cells that downregulate major histocompatibility complex (MHC) class I molecules are detected as 'missing self' and are lysed by NK cells. c) Overexpression of induced stress ligands by aberrant cells leads to recognition by activating NK cell receptors, which override the inhibitory signals and elicit target cell lysis. d) Tumour antigen-specific antibodies bind to CD16 and elicit antibody-dependent NK cell-mediated cytotoxicity. Figure adapted from (Morvan and Lanier, 2016)

Activating receptors signal through immunoreceptor tyrosine based activating motifs or ITAMs. When these receptors are engaged, the tyrosine residue in the tail gets phosphorylated and recruits protein tyrosine kinases of the Syk family such as Syk and ZAP70 (Kong et al., 1995; van Oers and Weiss, 1995). Syk and ZAP70 associate with various transmembrane adapters like linker of activated T cells (LAT) and cytosolic adapters like SH2 domain containing leukocyte phosphorylation of 76kD (SLP-76) that are crucial for the pathways leading to the induction of NK cell cytotoxicity and cytokine production. LAT also activates Phospholipases Cγ1 and 2 (PLCγ1/2), which controls the intracellular

levels of Calcium ions (Ca^{2}) (Cassatella et al., 1989; Tassi et al., 2005). Ca^{2+} activates the Calmodulin/calcenurin signalling which leads to the phosphorylation and activation of nuclear factor of activated T cells or NFAT (Feske et al., 2001; Hogan et al., 2003). NFAT traverses through the nucleus and induces transcription of various cytokines. Other NK cell activating receptors signal through adaptors known as DAP12 and DAP10 (Wu et al., 2000). DAP12 is an adapter of ITAMs, whereas DAP10 is an adapter for NKG2D that activates PI3K(Upshaw et al., 2006). Both ITAM and DAP10 pathways activate Rac. Activation of Rac leads to activation of mitogen activated protein kinase (MAPK) followed by activation of mitogen activated or extracellular signal regulated protein kinase (MEK) and p21-activated kinase (PAK) leading to the induction of extracellular regulated kinase (ERK). All these pathways result in NK cell cytotoxicity and cytokine production(Pegram et al., 2011).

Inhibitory receptors signal through molecules called the intracellular immunoreceptor tyrosine based inhibitory motifs or ITIMs located in the cytoplasmic tail of the receptors. Receptors attached to ITIMs are known to control various immunopathological conditions like autoimmunity, allergy, phagocytosis and graft versus host disease. ITIMs serve to control NK cell cytotoxicity and cytokine production (Long, 2008). Phosphorylation of a tyrosine residue results in the recruitment of the Src homology 2 domain containing phosphatases (SHP1 or 2) and SH2 containing inositol phosphatases (SHIP) (Nakamura et al., 2000). Signalling through ITIMs has been shown to interfere with intracellular phosphorylation in order to inhibit NK cell activating signalling pathways either through dephosphorylation of Vav1, through phosphorylation of a tyrosine adaptor, Crk (Peterson and Long, 2008; Stebbins et al., 2003) or through SHIP dependent dephosphorylation of PIP3 to PIP2 (Scharenberg et al., 1998).

1.3 NK cell receptors and their ligands

There are two main families of receptors that regulate NK cell function. The receptors of one of these families are known as the killer cell immunoglobulin-like receptors (KIRs) as they contain immunoglobulin-like domains. Another family of receptors comprises killer lectin-like receptors (KLRs) that are C-type lectin like proteins (Murphy et al., 2008). Mice express predominantly C-type lectin like Ly49 receptors, whereas, humans lack Ly49 genes and rely predominantly on KIR genes. KIRs and KLRs can be both inhibitory and activatory depending on the intracellular motif (ITAM or ITIM) they associate with (Lanier, 2005; Moretta et al., 1996).

Inhibitory receptors mainly recognize MHC class I molecules. The mouse Ly49 inhibitory receptors associate with ITIMs and signal through the phosphatases SHP-1. Another kind of inhibitory receptor is the CD94-NKG2 heterodimer that recognizes non-polymorphic MHC class I like molecules, in particular HLA-E in humans and Qa1 in mice. HLA-E and Qa1 bind to signal peptides that are derived from the MHC class-I molecules being processed in the endoplasmic reticulum, allowing them to recognize a wide range of MHC class I variants.

While inhibitory receptors recognize MHC class I molecules present on host cells in order to prevent unnecessary host cell killing by NK cells, activating receptors directly sense the presence ligands expressed on infected and transformed cells in response to cellular stress. Activating receptors comprise Ig-like receptors known as natural cytotoxicity receptors or NCRs (NKp30, NKp44 and NKp46), 2B4, DNAM1; and C-type lectin like receptors like Ly49H, NKG2D and NKR-P1. NCRs recognize viral proteins such as haemagglutinin (HA) expressed on the influenza virus and tumour associated ligands like HLA-B associated transcript-3, heparan sulphate proteoglycans (HSPGs) and B7-H6. 2B4, DNAM-1 (CD226) are co-stimulatory receptors that recognize ligands such as CD48 and CD155, which are expressed on all hematopoietic cells, but

their expression is modified in tumour cells. These receptors enable NK cells to recognize target cells that don't express tumour-associated antigens (Pegram et al., 2011).

Members of the C-type lectin family include activating receptors such as Ly49H that recognizes the viral protein m157 expressed by the murine cytomegalovirus (MCMV), and NKG2D that recognizes MHC class-I like molecules expressed in response to cellular stress. These MHC class I like molecules include the MIC molecules, MIC-A and MIC-B and the RAET1 family of proteins, also known as the UL-16 binding proteins or ULBPs. In mice these proteins are known as the retinoic acid early inducible (RAE1) protein family. NKG2D also recognizes MULT1 and H60 in mice (Pegram et al., 2011).

Another C-type lectin like receptor expressed in mice is NKR-P1. These receptors are encoded by the NKR-P1 gene family. These receptors are type II membrane glycoprotein receptors. NK1.1 also known as NKR-P1C, the well-known NK cell marker, belongs to this family, is encoded by the *nkrp1-c* gene (Pegram et al., 2011). NK1.1 has been shown to stimulate NK cell function (Arase et al., 1997; Karlhofer and Yokoyama, 1991) and proliferation (Reichlin and Yokoyama, 1998) upon crosslinking. NK1.1 ligation was shown to signal through the FcRγ associated ITAM leading to cytotoxicity through the activation of the Syk tyrosine kinases.

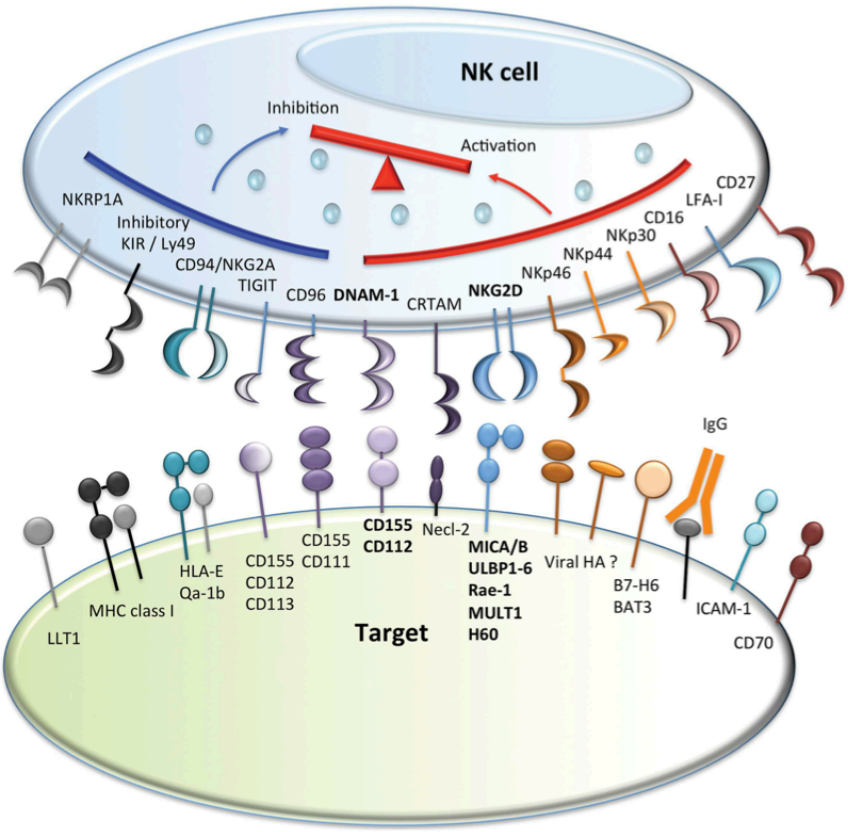


Figure 1.5 NK cell receptors and their ligands

Major inhibitory and activating receptors on NK cells and their cognate ligands on targets are depicted. BAT3, human leukocyte antigen (HLA)-B-associated transcript 3; CRTAM, class I-restricted T-cell-associated molecule; HA, hemagglutinin; HLA-E, HLA class I histocompatibility antigen, alpha chain E; IgG, immunoglobulin G; LFA-1, leukocyte function-associated antigen-1; LLT1, lectin-like transcript 1; TIGIT, T cell immunoglobulin and ITIM domain. Figure adapted from (Chan et al., 2014)

1.4 NK cells in viral infections

The importance of NK cells in viral infections is well recognized and NK cell mediated cytotoxicity has been observed in a number of viral infections including LCMV, MCMV and HSV (Biron et al., 1999). NK cells are the early responders in viral infections. They either respond to virally infected cells directly through NK cell receptors or indirectly following bystander activation (Lodoen

and Lanier, 2006). Viruses have been shown to activate IFN producing cells and DCs by TLR9 stimulation, which triggers MyD88 dependent pathways in these cells, which in turn triggers type I IFN production, and IL12 expression. These cytokines can then stimulate NK cells to produce IFN-γ and cytotoxic granules to help build up an effective defence against viral infection (Lodoen and Lanier, 2006).

In addition, NK cells can also directly kill virally infected cells when receptors on NK cells recognize viral ligands. A well-studied example is the Ly49H receptor on NK cells that recognizes the ligand m157 present on the mouse cytomegalovirus (MCMV) (Vivier et al., 2008). The importance of NK cells in MCMV infections has been demonstrated by NK cell depletion studies where mice with defects in NK cell activity or cytotoxicity were found to be more susceptible to MCMV infections (Scalzo et al., 2007). MCMV strains with a variant of the m157 ligand also rendered mice more susceptible to MCMV infections (Lam and Lanier, 2016). The significance of NK cells in MCMV infections is also evidenced by the presence of MCMV genes associated with NK cell evasion. These genes include those that down-regulate NKG2D ligands thus preventing NK cell stimulation and some that are associated with the expression of MCMV decoy ligands that hinder NK cell function (Vivier et al., 2008). There is emerging evidence of a role of NK cells in human viral infections including HIV and HCV.

1.5 NK cells in Cancer

NK cells were first recognized as cytotoxic cells that could distinguish between healthy self cells and transformed cells that exhibit physical and chemical changes, for instance, lowered expression of MHC I molecules (Vivier et al., 2012). NK cells have been shown to be important in controlling tumours and metastases in numerous studies. NK cell deficient mice were shown to have a higher frequency of lung metastases (Sathe et al., 2014). NK cell activating

receptors are especially important for NK cell mediated tumour immunosurveillace as demonstrated by NKG2D deletion (Guerra et al., 2008). Mice deficient in NK cell effector molecules were also shown to develop spontaneous cancer (Smyth et al., 2000). IFN-γ was found to be important for NK cell infiltration and tumour suppression in early stages of MCA205 fibrosarcoma (Hayakawa et al., 2011).

There have also been studies correlating elevated intra tumoral NK cell numbers to increased survival in various types of cancers in humans (Baginska et al., 2013). Increased CD57+ NK cells have been shown to be indicative of good prognosis in oral squamous carcinoma (Türkseven and Oygür, 2010). Patients of head and neck squamous cell carcinoma with increased CD56+ NK cells had better survival rates (van Herpen et al., 2005). Also, as noted earlier, NK cells are important producers of IFN-γ which has been implicated in various direct anti-tumour activities including inhibition of tumour cell proliferation and angiogenesis and the induction of apoptosis (Guillerey and Smyth, 2016). Moreover, NK cells are also able to regulate anti-tumour adaptive immunity by induction of potent anti-tumour specific cytotoxic T cells. The release of IFN-γ on NK cell activation is of particular importance for the regulation of adaptive anti-tumour immunity (Paust et al., 2010b). NK cells recognize tumour cells that have effectively evaded T cells by down-regulating MHC class I molecules, which are essential for antigen presentation to the T cells. The importance of reduced MHC class I expression in NK cell recognition of tumour cells has been demonstrated by a number of studies (Dorfman and Raulet, 1996; Olsson et al., 1995). Tumour cells may also increase the expression of certain stress molecules that are present on normal cells but in very low quantities. NK cell receptors like NKG2D have been shown to recognize and interact with self-molecules that are up-regulated in response to stress (Vivier et al., 2012). These mechanisms of immune-surveillance suggest that NK cells have evolved to have an important role in cancer cell recognition.

1.6 NK cell training/memory

It is now well known that NK cells, although being classically innate cells, can act alongside adaptive immune cells and show characteristics of immunological memory (Cerwenka and Lanier, 2016). NK cells have been described as memory like NK cells, trained NK cells or adaptive NK cells in both human and murine systems (Cooper et al., 2009b; Romee et al., 2012). The memory like property of cytokine pre-activated NK cells was shown to be intrinsic and was inherited by daughter cells that had not been exposed to cytokines (Keppel et al., 2013). This indicates that there might be an epigenetic reprogramming event involved in the formation of memory. Indeed, the CNS1 at the *Ifng* gene locus was shown to be demethylated in cytokine activated NK cells (Luetke-Eversloh et al., 2014; Ni et al., 2016). Memory like NK cells in humans that were generated by activating NK cells with IL12/IL15/IL18 cytokines showed promising results in acute myeloid leukaemia upon re-administration into patients (Romee et al., 2016).

1.7 NK cells in Cancer Immunotherapy

NK cells have been shown to successfully kill tumour cells *in vitro* in several studies and have been shown to eradicate experimentally induced or spontaneous tumours in mice (Wu and Lanier, 2003). Due to the fact that cancers can be immunosuppressive and may escape NK cell mediated killing, several approaches to enhance NK cell activity are being studied. One of these approaches is activating endogenous NK cells, where NK cell activating cytokines such as IL2 are administered into cancer patients. However, IL2 is often toxic due to the inevitable cytokine and small molecule secretion it induces in other immune cells and the attempts to reduce its toxicity have unfortunately, been unsuccessful (Ljunggren and Malmberg, 2007; Smyth et al., 2002). Also, IL2 therapy has low efficacy rates due to its effect on Tregs that compete with NK and T cells for IL2. This results in a decrease in anti-tumour response (Sakaguchi et al., 2001).

Although there have been recent advancements in this area with the advent of new mutated versions of IL2 cytokine, such as 'super-2', which has more affinity towards IL2Rβ, therefore it avoids the activation of Tregs. Another genetically modified version of IL2, IL2cx which when administered in conjunction with anti-CTLA-4 has been shown to increase the NK cell/Treg ratio (Caudana et al., 2019; Levin et al., 2012). There have also been successful attempts at creating novel fusion proteins that combine NK cell activation receptors and IL2 so that IL2 only acts on NKG2D expressing cells, thus bypassing Treg activation (Ghasemi et al., 2016). Other immune-modulatory drugs and substances like Thalidomide and *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) have been shown to enhance antitumor activity by way of NK cell activation (Ljunggren and Malmberg, 2007).

Another strategy is adoptive transfer of autologous NK cells, where NK cells isolated from the patients themselves are treated *ex-vivo* with cytokines and transferred back into the patient. However this strategy has had mixed results due to inhibition by self-MHC (Li et al., 2015). This led to the advent of allogeneic NK cell adoptive transfer. Transfer of NK cells from donors bearing different MHC molecules can induce a cytotoxic response against tumour cells without causing Graft vs Host disease (GvHD). Allogeneic transplant of haematopoietic cells from haplo-identical donors into T cell depleted patients with AML have shown successful control of relapse in an NK cell dependent manner (Li et al., 2015). In spite of the success achieved in treating haematological malignancies, NK cell based immunotherapies haven't had desirable effects in solid tumours, for instance, breast and ovarian cancers (Geller et al., 2011).

One thing that distinguishes solid tumours from haematological malignancies is the tumour microenvironment (TME), which can be severely nutrient deprived (Vander Heiden et al., 2009). This nutrient deprivation in the TME is a result of

tumour cells adopting aerobic glycolysis so as to circumvent energy crisis due to hypoxia (Vander Heiden et al., 2009). However, this change in tumour cell metabolism serves them a dual purpose by making the TME extremely immunosuppressive. There has been an emerging body of evidence suggesting that cellular metabolism is not just limited to fulfilling the energy demands of immune cells, but also regulating their effector responses (Donnelly et al., 2014; Pearce and Pearce, 2013). Immune cells require undergoing a metabolic shift in response to activation, in order to elicit an effective defence. Due to the acute nutrient deprivation caused by tumour cells, immune cells become metabolically distressed and dysfunctional (Kedia-Mehta and Finlay, 2019). Tumour infiltrating NK cells in non small cell lung carcinoma show an altered phenotype with reduced expression of receptors and decreased degranulation and IFN-γ expression (Platonova et al., 2011). CD56 bright cells in the tumour microenvironment were also shown to be defective at killing (Carrega et al., 2008). Similar alterations in NK cell phenotype were observed in tumour associated NK cells in colorectal carcinoma (Rocca et al., 2013). Myeloid derived suppressor cells or MDSCs home into TME and are responsible for immunosuppression by producing anti-inflammatory cytokines like IL10. IL10 suppresses IL12 production by macrophages that are required for NK cell activation. All these defects in NK cell function are possibly due to the metabolic distress caused by the hostile conditions in the TME.

Therefore, it is imperative to understand mechanisms linking cellular metabolism to immune cell effector responses, in order to devise effective therapeutic strategies against solid tumours.

1.8 Immunometabolism

Every cell needs adenosyl triphosphate (ATP) as a source of energy whether it is activated or quiescent. The energy demands of a cell are met by the workings of three main pathways, which are integrally linked with each other- Glycolysis,

TCA cycle and Oxidative Phosphorylation (OxPhos). When glucose enters the cells via glucose transporters, it is converted to Pyruvate through Glycolysis. In normoxic conditions of homeostasis, pyruvate obtained from glycolysis is broken down into acetyl coenzyme A (acetyl CoA) and carbon dioxide. Acetyl CoA enters the tricarboxylic acid (TCA) cycle, which then produces NADH to fuel OxPhos, which in turn produces large amounts of ATP. However, under hypoxic conditions, cells obtain ATP solely from the breakdown of glucose to pyruvate, which is then converted into lactate and NAD+. Lactate is released in the extracellular milieu. This is known as anaerobic glycolysis. Glycolysis is an inefficient way to produce ATP (2xATP/ molecule of glucose) as compared to OxPhos (36xATP/molecule of glucose). However, many activated immune cells switch their metabolism to glycolysis as a preferred way of metabolism even under normoxic conditions. This is known as aerobic glycolysis and it is adopted by activated immune cells to facilitate cellular biosynthetic pathways.

1.9 Aerobic glycolysis fuels Cellular biosynthesis

When immune cells are quiescent, their biosynthetic demands are minimal, but they are required to maintain normal cellular functions so as to live long. These modest energy requirements of quiescent immune cells are met by OxPhos. Following immune activation, due to infections or inflammation, immune cells acquire new functions and switch on a whole new set of genes required to produce effector cytokines, chemokines, reactive oxygen and nitrogen species, actin/cytoskeleton remodelling enzymes etc. in high numbers and at a high speed (Pearce and Pearce, 2013). In order to sustain these effector responses, immune cells need to increase the uptake of nutrients, glucose, amino acids etc. from their microenvironment as there is an increased demand for biosynthetic precursors and energy production (Ganeshan and Chawla, 2014). This is achieved by a switch in the metabolic profile whereby activated immune cells adopt aerobic

glycolysis as their principal means of energy production(Pearce and Pearce, 2013).

Activated immune cells take up significantly increased amounts of glucose and up-regulate their expression of glucose transporters (Donnelly et al., 2014; Loftus and Finlay, 2016; Macintyre et al., 2014). This results in increased rates of glycolysis. As the TCA cycle cannot utilize the increased amounts of pyruvate so produced, the pyruvate is pumped out of the cells in the form of lactate so as not to cause product inhibition due to pyruvate accumulation (Tan et al., 2015). Apart from preventing glycolytic inhibition, conversion of pyruvate to lactate by lactate dehydrogenase (LDha) also results in the oxidation of NADH to NAD+(Cheng et al., 2014). This causes the replenishment of NAD+ that is required for the action of Glyceraldehide-3-phosphate dehydrogenase or GAPDH, a glycolytic enzyme that catalyses the sixth step of glycolysis (Nagy and Rigby, 1995; Palamalai and Miyagi, 2010). All these mechanisms enable continuation of aerobic glycolysis at a high rate. Increased rates of aerobic glycolysis may seem redundant in the presence of oxygen and it might seem futile that immune cells switch to a less efficient pathway with respect to energy production. However, activated immune cells need to produce large amounts of effector molecules like cytokines and chemokines as well as biosynthetic precursors which they need to proliferate and divide into effector and memory cells. This increases the energy demands of the cell and need for macromolecules synthesis. Aerobic glycolysis does not only produce energy at a faster rate but also facilitates biosynthesis of macromolecules (Lunt and Vander Heiden, 2011; Pfeiffer et al., 2001). Glycolytic intermediates generated during glycolysis are fed into various biosynthetic pathways (Hume and Weidemann, 1979; Vander Heiden et al., 2009). For instance, Glucose-6-phosphate is shunted into the Pentose phosphate Pathway (PPP) that leads to the production of biosynthetic precursors like ribose sugars and nucleotides (Lunt and Vander Heiden, 2011). This in turn enables growth, proliferation and effector functions in immune cells.

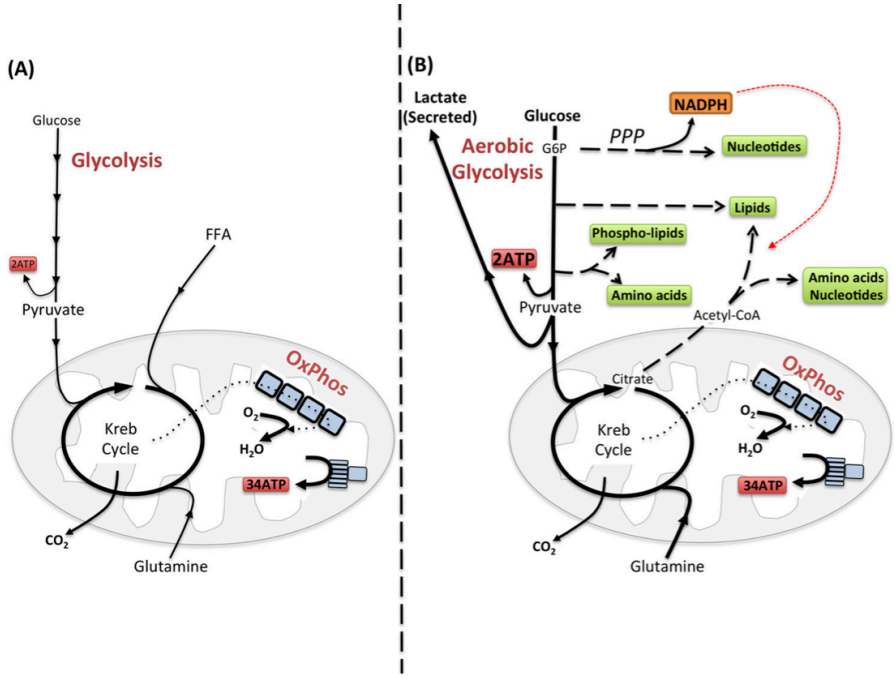


Figure 1.6 Metabolic reprogramming facilitates Cellular biosynthesis

A)-Quiescent cells are functionally inert and engage in oxidative phosphorylation for efficient generation of ATP. B)- Activated cells engage in high rates of aerobic glycolysis that supports biosynthetic processes of the cell as it allows the uptake of larger amounts of glucose and the maintenance of elevated glycolytic flux. Glycolytic intermediates are then diverted into various pathways for the synthesis of biomolecules that support biosynthetic processes. For instance, glucose 6-phosphate (*G6P*), generated by the first step in glycolysis, can feed into the pentose phosphate pathway (*PPP*) to support nucleotide synthesis. Figure adapted from (Loftus and Finlay, 2016)

1.10 Cellular metabolism facilitates Immune cell function

Immune cells seem to adopt a metabolic profile that is tailored according to the functional requirements of the cell. Quiescent immune cells, owing to their limited biosynthetic demands, mainly utilize OxPhos to efficiently generate ATP. On being activated, lymphoid cells such as T cells, B cells and NK cells increase the rates of both aerobic glycolysis and OxPhos. High rates of aerobic

glycolysis facilitate clonal expansion in activated lymphocytes by means of its capacity to enable cellular biosynthesis. Effector T cells subsets such as CD4+ and CD8+ T cells maintain high rates of aerobic glycolysis. In contrast, longer lived T cells such as regulatory T cells and memory T cells essentially utilize oxidative metabolism (Loftus and Finlay, 2016). However, not much is known about the metabolic profiles of different B cell and NK cell subsets.

Myeloid cells such as M1 macrophages, dendritic cells and granulocytes switch to an exclusively glycolytic metabolism, with no flux through OxPhos. In M1 macrophages and DCs, OxPhos is actively inhibited by nitric oxide produced in these cells. Recent studies have shown that in M1 macrophages, OxPhos inhibition serves the purpose of Kreb's cycle intermediates - citrate and succinate - being alternatively used for the synthesis of important inflammatory mediators like reactive oxygen and nitrogen species, prostaglandins and IL1β respectively (O'Neill and Pearce, 2016). In case of neutrophils, even though there is an increase in oxygen consumption when they are activated and perform phagocytosis, they use glycolysis for ATP generation (Borregaard and Herlin, 1982; SBARRA and KARNOVSKY, 1959). The oxygen is instead utilized for the generation of NADPH, which is important for the production of microbicidal effectors of neutrophils (Dale et al., 2008).

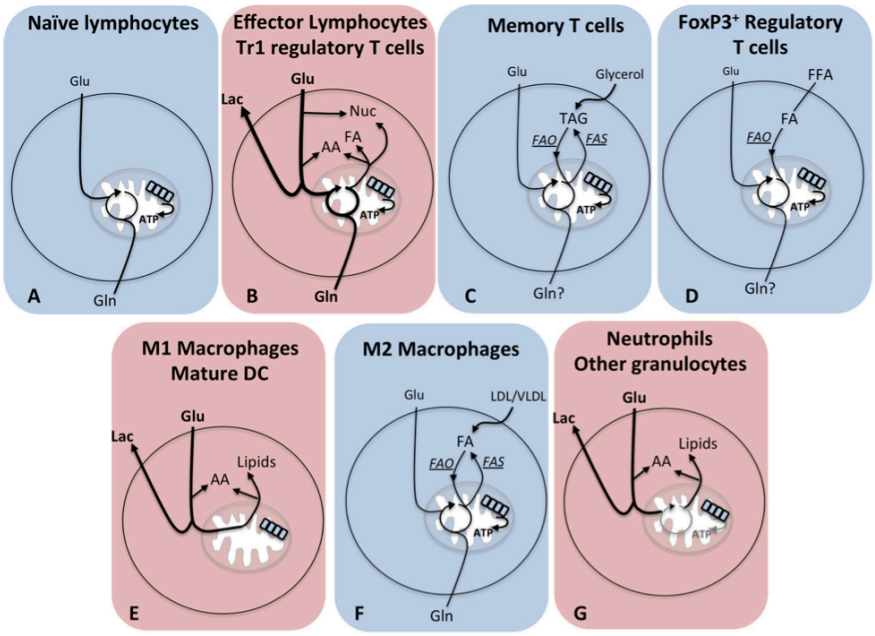


Figure 1.7 Cellular metabolism facilitates immune cell function

Blue panels represent cells with oxidative metabolism, and *red panels* represent cells with glycolytic metabolism. *A*) Naïve T cells use glucose and glutamine and OxPhos. *B*) effector lymphocytes such as T and B cells have high rates of both glycolysis and OxPhos, metabolize glucose to lactate, and use metabolic intermediates to support biosynthetic processes. *Nuc*, nucleotides; *FA*, fatty acids; *AA*, amino acids; *Lac,* Lactate. *C*) Memory T cells use glucose to generate mitochondrial citrate, which is exported into the cytosol to support lipid synthesis. These *de novo* synthesized fatty acids are used with imported glycerol to generate and store TAGs. Acetyl-CoA generated following β-oxidation of these TAGs fuels OxPhos. *FAO*, fatty acid oxidation; FAS, fatty acid synthesis. *D*) Regulatory T cells use exogenously derived fatty acids metabolized by β-oxidation to support OxPhos. *E*) M1 macrophages and mature DC engage aerobic glycolysis for ATP synthesis and to support biosynthesis while also inactivating OxPhos. *F*) M2 macrophage metabolism is characterized by fatty acid β-oxidation and OxPhos. β-Oxidation is fuelled by lipids that are scavenged from the external microenvironment and also by lipids generated by *de novo* fatty acid synthesis. *G*) neutrophils are highly glycolytic with few functional mitochondrial and very low rates of OxPhos. (Loftus and Finlay, 2016)

In contrast, M2 macrophages, whose main function is tissue repair and tissue remodelling, adopt oxidative metabolism with uninterrupted flux through the Kreb's cycle. M2 macrophages rely on oxidative pathways such as β-oxidation of

fatty acids (FAO) and OxPhos, to produce anti-inflammatory cytokines and growth factors required for tissue repair. It has been suggested that M1 macrophages need a rapid burst of energy to produce all the effector molecules and chemokines required for the containment of microbes, whereas M2 macrophages that fight parasites over a long period of time need a sustained supply of energy which is provided by OxPhos (O'Neill and Pearce, 2016).
All these metabolic attributes of different immune cells seem to complement the functional profile of these cells, pointing towards a more sophisticated role of metabolism in immune cell function.

1.11 Metabolism in T cells

T-cell response to an immunogen is a more evolved, multifaceted process. Naïve T cells are quiescent and become activated, once presented with an antigen. They require minimum amounts of energy; just enough for survival and vigilance against any threat, therefore these cells undergo OxPhos for a sustained supply of ATP. Activated T cells are effector cells that divide every 6-8 hours and produce huge amounts of effector molecules like chemokines, cytokines and cytotoxic substances. To facilitate all these biosynthetic functions, activated T cells need to up-regulate the rate of ATP production as well as the generation of biosynthetic precursors. Therefore, activated T cells increase the rates of glycolysis, which serves both these purposes. Increased glycolysis would require an increased uptake of glucose and expression of glycolytic enzymes. Indeed, there is an increase in the expression of glucose transporters such as Glut 1 in activated T cells (Swainson et al., 2005) and genes involved in glycolysis (Wang et al., 2011). Regulatory T cells, or Treg cells are immune-suppressive that mainly engage in oxidative phosphorylation, oxidising both glucose and fatty acids for ATP (Loftus and Finlay, 2016; Michalek et al., 2011). This is reminiscent of the metabolism adopted by M2 macrophages. Indeed, like M2 macrophages, Treg cells do not require to proliferate as abundantly as effector T cells, but need to

produce anti-inflammatory and immuno-suppressive cytokines like IL-10 and TGF-β. Memory T cells, like naïve T cells are long-lived, relatively passive with minimal biosynthetic requirements. Not surprisingly, like naïve T cells, memory T cells also engage in oxidative metabolism. Though, memory T cells engage predominantly in FAO as it is a much more efficient way of ATP synthesis (106 ATP/molecule of palmitate) as compared to oxidation of glucose. In addition, memory T cells use glycolysis for de novo fatty acid synthesis. This cycle of fatty acid synthesis and oxidation might seem redundant but facilitates rapid memory and recall responses that characterises memory T cells(Loftus and Finlay, 2016).

1.12 Metabolism controls Immune cell function

With an increase in the understanding in the field of immune-metabolism, there is a growing appreciation for the role of metabolic reprogramming in immune cells that is beyond supporting cellular biosynthesis. Metabolic enzymes and metabolites have been shown to directly influence immune cell function. For instance, GAPDH has been shown to directly control IFN-γ expression in CD4 T cells. GAPDH is a known RNA binding protein and is a component of IFN-γ activated inhibitor of translation complex (GAIT). GAIT binds to the 3' UTR region of certain inflammatory genes including IFN-γ, inhibiting their activity. Up-regulation in the rate of glycolysis in activated CD4T cells engages GAPDH in glycolysis, lifting GAPDH induced regulation on IFN-γ mRNA translation (Chang et al., 2013). Another example of metabolism directly controlling immune cell function is the glycolytic intermediate phosphoenol pyruvate. It has been demonstrated that phosphoenol pyruvate is required for effective NFAT signalling. It promotes NFAT signalling by inhibiting reuptake of Ca++ ions into the endoplasmic reticulum. Ca-calcenuerin signalling activates NFAT. Mitochondrial oxidation also controls immune cell function. Defective ROS production hinders NFAT activation, IL2 expression and proliferation in response to TCR stimulation (Ho et al., 2015). Other examples of metabolites

that regulate immune cell function are succinate and citrate in M1 macrophages. These TCA cycle intermediates are accumulated in activated M1 macrophages as these macrophages preferentially utilize glycolysis and turn off OxPhos. These intermediates are instead repurposed as functional regulators. Succinate can inhibit prolyl dehydroxylases that ubiquitinate HIF1α; this results in stabilization of HIF1α and HIF1α dependent production of IL1β. Citrate accumulation promotes the production of pro-inflammatory mediators such as Nitric oxide, ROS and prostaglandins (Loftus and Finlay, 2016). This is clear evidence that metabolism directly controls immune cell function by re-purposing metabolic enzymes and metabolites as regulators of cellular effector functions.

1.13 NK cell metabolism

There has been extensive research on cellular metabolism in NK cells, especially it's requirement for NK cell effector function. Like other quiescent immune cells, freshly isolated murine NK cells have basal levels of metabolic activity with higher rates of OxPhos compared to glycolysis (Keppel et al., 2015). These low metabolic rates are sufficient to sustain short-term activation and effector responses for instance, 4-hour cytokine stimulation with IL15/IL18 or with IL15/IL12 or 6 hour receptor ligation with αNK1.1 and α-Ly49D. Moreover, there is no increase in glycolysis and OxPhos during this short term activation (Keppel et al., 2015). However, inhibition of metabolism in these cells caused impairment in IFN-γ production, indicating that metabolism, albeit at lower rates, was still important for NK cell effector functions.

It is well established that NK cells play an important role in sustained immune responses, operating alongside the adaptive immune response. Indeed, NK cells that were stimulated over a longer duration showed a robust metabolic response that was essential for the NK cell effector functions in both murine as well as

human systems (Donnelly et al., 2014; Keating et al., 2016; Marçais et al., 2014a).

These increased rates of metabolism in activated NK cells are accompanied by a change in metabolic machinery and metabolic configuration. There is an increased uptake of glucose by activated NK cells, with a concomitant increase in glycolytic gene expression (Donnelly et al., 2014). Increased rates of OxPhos and mitochondrial respiration in activated NK cells are supported by an increased mitochondrial mass (Assmann et al., 2017).

The importance of cellular metabolism in activated NK cells was studied by partially inhibiting the two main metabolic pathways glycolysis and OxPhos using different inhibitors. It was found that inhibition of glycolysis by 2-DG and inhibition of OxPhos by oligomycin inhibited IFN-γ production and expression of Granzyme B in both mice and human NK cells (Donnelly et al., 2014; Keating et al., 2016). 2DG was also shown to decrease the tumour cell killing capacity of IL15 activated human NK cells (Mah et al., 2017). Another way to limit the rate of glycolysis is by replacing the glucose in culture medium by galactose. Galactose, which is diverted to the Leloir pathway slowing down glycolysis, was shown to inhibit effector functions in both murine and human NK cells (Donnelly et al., 2014; Keating et al., 2016). 2DG treatment of mice injected with PolyI:C resulted in decreased IFN-γ production and increased viral load in mice infected with MCMV (Donnelly et al., 2014; Mah et al., 2017).

IL2/IL12 activated NK cells are mainly fuelled by glucose that is broken down into pyruvate and then lactate, which is then exported out of the cells. Some pyruvate enters the mitochondria where instead of being fully metabolised by the TCA cycle, it gets converted into citrate, which is then exported out of the mitochondria into the cytosol through the obligate antiporter Slc25A1. In the cytosol, citrate is broken down into oxaloacetate and Acetyl Co-A by the enzyme ATP citrate lyase (ACLY). Oxaloacetate eventually is converted to malate,

which then re-enters the mitochondria through Slc25A1. This cycle is known as the citrate malate shuttle (CMS) (Assmann et al., 2017). This is in contrast to what happens in other lymphocytes. While other lymphocytes, which engage in TCA cycle are fuelled by different types of energy sources, NK cells heavily rely on glucose as CMS is exclusively fuelled by glucose. Indeed, it has been shown in NK cells that inhibiting the glutaminase enzyme that breaks down glutamine to be used as fuel, does not decrease NK cell effector functions (Loftus et al., 2018).

1.14 Regulation of NK cell metabolism

mTOR- mammalian Target of Rapamycin

Mammalian target of rapamycin complex 1 (mTORC1) is an important regulator of cellular metabolism. Rapamycin was first identified as an anti-fungal agent and was isolated from a strain of Streptomyces found on the Easter Island. Rapamycin was initially thought of as a weak antibiotic, but soon recognized to have immunosuppressive and antitumor properties. It was shown to inhibit growth in yeast and was found to act on a serine-threonine kinase in yeast cells, as a result termed, target of Rapamycin or TOR and eventually the mammalian homologue was identified and called, mammalian target of rapamycin or mTOR (Powell and Delgoffe, 2010). mTOR senses several environmental cues such as nutrients, growth factors amino acids and oxygen to control growth, proliferation and metabolism in immune cells. Nutrient availability or energy stress in the environment can switch mTORC1 on or off. Of the two mTOR complexes, mTORC1 and mTORC2, mTORC1 is much more extensively studied.

mTORC1 regulates cell growth and metabolism

mTORC1 promotes anabolic pathways such as biosynthesis of macromolecules for cell growth and proliferation and inhibits catabolic processes such as

autophagy. It does so by phosphorylating components of the translation initiation machinery such as eIF4G, eIF4B and 4-E-BP1. 4E-BP1 or 4E (eIF4E) binding protein-1 regulates 4eIF4E that binds to the 5' mRNA cap structure. Phosphorylation of 4E-BP1 prevents it from binding to eIF4E, allowing it to initiate cap dependent translation. mTORC1 also activates S6K that in turn activates the components of translation initiation machinery like ribosomal protein S6 and eukaryotic elongation factor 2 kinase(eFK2K). Phosphorylation of S6K by mTORC1 at Thr389 allows it to bind to phosphoionositide dependent kinase 1 PDK-1 that then phosphorylates S6K at Thr229, activating it. Activation of S6K by mTORC1 results in mRNA biogenesis, cap dependent translation and translation of ribosomal proteins (Ma and Blenis, 2009). There is evidence that mTORC1 regulates lipid biosynthesis by activating sterol regulatory element binding protein 1 (Srebp1) which is a transcription factor for genes involved in lipid synthesis (Porstmann et al., 2008). Another molecule that mTORC1 positively regulates is peroxisome proliferator activated receptor γ (PPAR-γ) which is involved in the transcription of genes involved in cholesterol homeostasis. In addition, mTORC1, controls other transcription factors involved in lipid metabolism such as, PPARα and PGC-1 (Laplante and Sabatini, 2009).

Another important role of mTORC1 is its control of cellular metabolism. It has been reported that inhibition of mTORC1 resulted in lower ATP levels, reduced oxygen consumption and mitochondrial membrane potential (Schieke et al., 2006). mTORC1 has also been shown to be essential for glycolytic reprogramming. Inhibition of mTORC1 by rapamycin was shown to affect rates of glucose uptake, glycolysis and expression of glycolytic genes (Donnelly et al., 2014; Finlay et al., 2012).

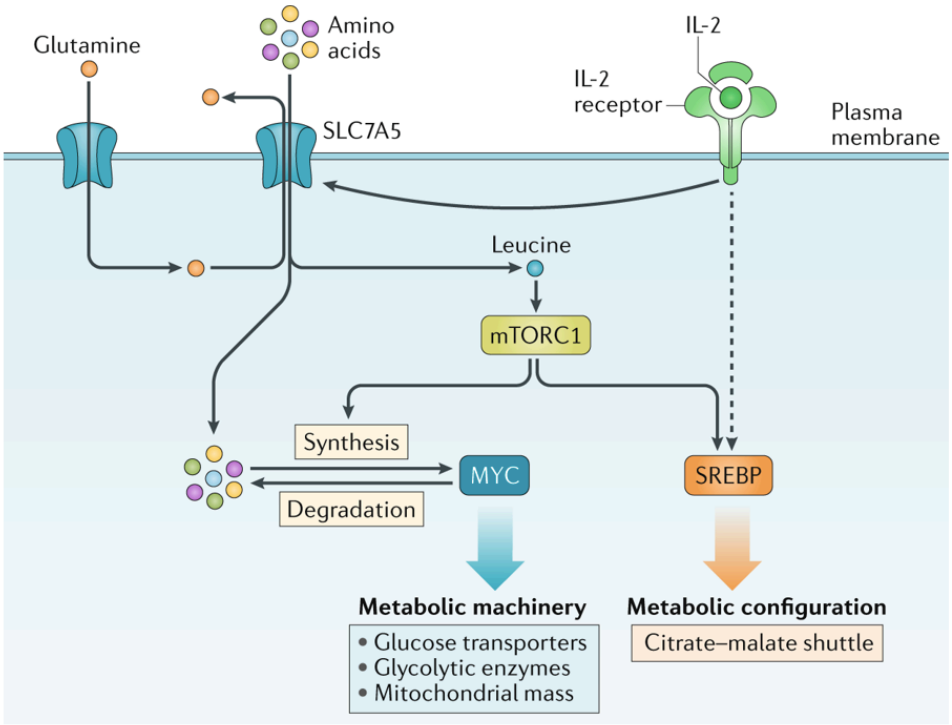


Figure 1.8 Regulation of NK cell metabolism

Two transcription factors have been identified as key metabolic regulators in mouse natural killer (NK) cells: cMyc and sterol regulatory element-binding protein (Srebp). cMyc levels are controlled by the balance of cMyc protein synthesis and glycogen synthase kinase 3 (GSK3)-targeted degradation of cMyc by the proteasome. At early time points following IL-2 plus IL-12 stimulation (minutes), cMyc protein levels accumulate in a manner dependent on mammalian target of rapamycin complex 1 (mTORC1). mTORC1 is activated by IL-2 signalling and can increase cMyc protein translation by promoting 5′ cap-dependent translation. Cytokine stimulation also induces the expression and activity of the amino acid transporter Slc7A5. At later time points following cytokine stimulation (hours), amino acid uptake through Slc7A5 is essential to sustain cMyc protein levels, whereas mTORC1 activity is not required. Withdrawal of glutamine, which is exported through Slc7a5 to sustain Slc7A5-dependent amino acid import, or the direct inhibition of Slc7A5-mediated transport results in rapid loss of cMyc protein expression within minutes. Although leucine uptake through Slc7A5 is required for mTORC1 activity at these later time points, leucine withdrawal is not sufficient to reduce cMyc expression. Therefore, the uptake of other amino acids, such as methionine, phenylalanine, tyrosine, arginine or tryptophan, through Slc7A5 is important in the regulation of cMyc. The activity of Srebp is greatly increased following stimulation with IL-2 plus IL-12 in mouse NK cells. Although mTORC1 is the key regulator of Srebp activation, other mechanisms also contribute to Srebp activity in cytokine-stimulated NK cells. cMyc is important for the expression of the metabolic machinery in NK cells, and Srebp controls the metabolic configuration, namely, glucose metabolism through the citrate–malate shuttle (O'Brien and Finlay, 2019).

SREBP

As discussed above, Srebp is a transcription factor under the control of mTORC1 and is mainly involved in lipid synthesis. However, new research has emerged highlighting a new role for Srebp in NK cell metabolism. The Srebp transcription factors encode for genes of the cholesterol synthesis pathway and those of fatty acid synthesis (Horton et al., 2002).

Srebp is embedded in the membrane of the endoplasmic reticulum in its inactive form. To be activated, the N-terminal of Srebp, which is the active transcription factor, has to be proteolytically cleaved and translocated to the nucleus. Cleavage and activation of Srebp is a multistep process that involves several enzymes. Once synthesized, Srebp molecules are inserted into the endoplasmic reticulum (ER) membrane where they associate with a membrane bound chaperone protein called SCAP through their C-terminal regulatory domain. Binding with SCAP allows Srebp to leave the ER in transport vesicles through to the Golgi apparatus (Sun et al., 2007). The Golgi apparatus is where Srebp is converted to its active form. The cleavage of Srebp to its active form is facilitated by two Golgi resident protease enzymes - site-1-protease (S1P) and site-2-protease (S2P). S1P and S2P cleave Srebp in a sequential manner to release its active N-terminal domain (Duncan et al., 1998; Sakai et al., 1998). This active form of Srebp then translocates to the nucleus where Srebp binds to the Sterol regulatory element (SRE) and initiates transcription of Srebp associated genes (Smith et al., 1990). Srebps are regulated by INSIG anchor proteins that prevent the SCAP/Srebp complex from leaving the ER. INSIG in turn is controlled by Sterols such as cholesterol and oxysterol. High sterol concentrations in cells were shown to promote binding of INSIG to the SCAP/Srebp complex and prevent Srebp activity (Yang et al., 2002).

Control of NK cell metabolism by SREBP

IL2/IL12 cytokines induce Srebp activation in murine NK cells. However, the originally known role of Srebp in controlling the genes for fatty acid synthesis and cholesterol synthesis is not important for cytokine induced responses in NK cells. Interestingly, Srebp has been recently shown to have a completely novel role in cytokine activated NK cells. Srebp activity was described to be important for the control of glycolysis and OxPhos is cytokine activated NK cells. Srebp controls the expression of key molecules involved in the CMS- the citrate-malate anti-porter Slc25A1 and ATP citrate lyase (ACLY), that in turn facilitate OxPhos and glycolysis in NK cells. Inhibition of Srebp using pharmacological inhibitors or inhibiting the CMS associated proteins in cytokine activated NK cells had severe consequences for NK cell metabolism and effector functions (Assmann et al., 2017).

Myc, Glutamine and Slc7A5

The transcription factors cMyc and HIF1α have both been shown to have an important role in the induction of metabolic pathways, such as, glycolysis in immune cells (Finlay et al., 2012; Shi et al., 2011; Wang et al., 2011). In T cells cMyc controls the early metabolic reprogramming upon TCR induced activation. cMyc regulates the transcription of genes involved in glycolysis and glutaminolysis in T cells and cMyc-linked glutamine metabolism was shown to sustain T cell metabolism and function (Donnelly and Finlay, 2015; Wang et al., 2011). The role of cMyc has also been elucidated in IL2/IL12 stimulated NK cells recently (Loftus et al., 2018). However, this study showed that HIF1α is dispensable for cytokine-stimulated NK cell metabolism. The importance of cMyc for NK cell metabolism highlights a role for glutamine in NK cells, owing to the fact that cMyc expression highly depends on glutamine availability. While glutamine is used as a fuel in T cells where it's broken down by glutaminase

enzymes and enters the TCA cycle, interestingly, inhibition of glutamine lysis in cytokine stimulated NK cells had no effect on NK cell metabolism and function. However, removing glutamine from the culture medium resulted in loss of cMyc and severely impacted metabolism and function in cytokine stimulated NK cells. This finding identified the role of glutamine in NK cells as an important signalling molecule rather than a fuel (Loftus et al., 2018).

Another player in the cMyc-glutamine axis is the large neutral amino acid transporter subunit Slc7A5, which is also a glutamine antiporter (Scalise et al., 2017). Slc7A5 was shown to be highly up-regulated in activated T cells (Sinclair et al., 2013). Apart from that, overexpression of Slc7A5 is linked to a lot of cancers (Scalise et al., 2017). One of the amino acids that Slc7A5 transports into the cell is leucine, whose importance for mTORC1 activation is well established (Hara et al., 1998; Sancak et al., 2008). Additionally, Slc7A5 has also been implicated in cMyc activation where loss of Slc7A5 resulted in decreased glutamine uptake by activated T cells and decreased cMyc protein expression in activated T cells (Sinclair et al., 2013). In cytokine activated NK cells, where glutamine withdrawal resulted in the loss of cMyc, a similar fate was observed upon Slc7A5 inhibition by a pharmacological inhibitor 2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid BCH(Loftus et al., 2018). The LNAA uptake by Slc7A5, that sustains cMyc, is only sustained by a concomitant export of glutamine as Slc7A5 is a glutamine antiporter. Therefore, amino acid uptake, glutamine and cMyc are essential for NK cell metabolism and function (Loftus et al., 2018).

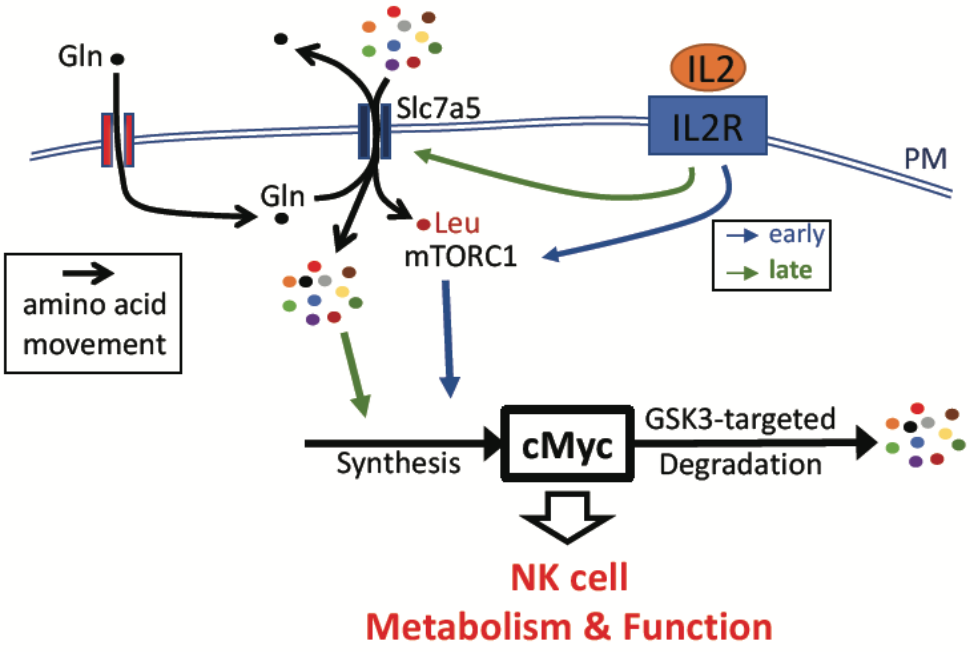


Figure 1.9 Regulation of cMyc expression in NK cells

cMyc levels are controlled by the balance of cMyc protein synthesis and GSK3-targeted degradation of cMyc in the proteasome. At early time points following IL2/IL12 stimulation (minutes) cMyc protein levels accumulated in an mTORC1 dependent manner. mTORC1 is activated by IL2 signalling and can increase cMyc protein translation through promoting 5' cap-dependent translation. IL2/IL12 stimulation also induces the expression and activity of the amino acid transporter Slc7A5. At later time points following IL2/IL12 stimulation (20 hours), amino acid uptake through Slc7A5 is essential to sustain cMyc protein levels while mTORC1 activity is not required. Withdrawal of glutamine, which is essential for Slc7A5 activity, or the direct inhibition of Slc7A5 mediated transport both result in rapid loss of cMyc protein expression. While leucine uptake through Slc7A5 is required for mTORC1 activity, at these later time points, leucine withdrawal is not sufficient to reduce cMyc expression. Therefore, the uptake of other amino acids through Slc7A5 are important in the regulation of cMyc, such as methionine, phenylalanine, tyrosine, arginine and tryptophan. Therefore, cMyc is a key regulator of NK cell metabolic and functional responses (Loftus et al., 2018).

Aims

The field of NK cell metabolism has advanced considerably in the last few years. However, most of the studies were conducted in cytokine activated NK cells and little is known about metabolic changes associated with receptor activation of NK cells. Receptor mediated stimulation of NK cells is a vital mechanism that initiates when NK cells recognize tumours or virally infected cells. This study aims to characterize the metabolic changes that ensue upon NK cell interactions with target cells.

Key objectives-

1) Examine the NK cell functional responses and changes in NK cell metabolism that occur upon NK1.1 receptor ligation.
2) Investigate the mechanisms involved in the metabolic changes associated with NK1.1 receptor ligation
3) Determine whether NK cell metabolism is linked to NK cell function in NK1.1 receptor activated NK cells
4) Investigate whether tumour interactions induce metabolic and functional responses in NK cells
5) Explore the requirement for cytokine induced metabolic changes in NK cell training

Chapter 2

Materials and Methods

2.1 Materials

2.1.1. Chemicals

Glucose, 2-Deoxyglucose (2-DG), Oxalate, β-Mercaptoethanol, Albumin from bovine serum, research grade Ethanol, Phosphate buffered saline tablets, Oligomycin, Fluoro-Carbonyl Cyanide Phenylhdrazone (FCCP), dNTPs, Rotenone, Antimycin-A, 2-aminobicycloheptane-2-carboxylic acid (BCH), Ammonium Persulphate, 40% Acrylamide A3699, TEMED, 4-Hydroxytamoxifen, Triton X, Sodium dodecyl-sulphate, Tris(hydroxymethyl)aminomethane 99%+, Agar, RedTaq, Deoxyneucleotide set, Nancy-520, Dimethyl Sulfoxide (DMSO), Tween-20, 1,4-Dithiothrotol (DTT), Proteinase K and 25-Hydroxycholesterol were purchased from Sigma-Aldrich. Phorbol myristate acetate (PMA) and Ionomycin were purchased from Fisher Scientific. PF429242 was purchased from Adoog bioscience. Go-Taq DNA Polymerase, 5x green flexi-buffer and MgCl2 were purchased from Promega. 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 were purchased from Fisher Scientific. RPMI medium 1640 (1X) [+] L-Glutamine, RPMI medium 1640 (1X) [+] L-Glutamine [-] Glucose, Hanks Buffered Saline Solution, Dulbecco's modified eagle's medium (DMEM), Iscove's modified Dulbecco medium (IMDM), Dulbecco's phosphate buffered saline (PBS), Penicillin-Streptomycin, MEM-vitamin solution (100X), L-Glutamine solution (100X), Insulin-Transferrin-Selenium-G Supplement (100X), Trypsin-EDTA (0.25%) were purchased from Biosciences. Seahorse XF media, base media and calibration buffer were purchased from Agilent technologies. Fetal Bovine Serum (FBS), dialyzed FBS was purchased from Labtech International. Sodium pyruvate was purchased from Gibco. IL-12 was purchased from Miltenyi Biotech. IL-15 was purchased from Peprotech; human IL-15 was purchased from NIH. IL-2 was purchased from NCI preclinical

repository. IL-18 was purchased from Bio-techne. Golgi plug, Cytofix/Cytoperm, Perm/Wash reagent, from BD Pharmingen and Cell-Tak was purchased from Corning. NK cell isolation kit II was purchased from Miltenyi Biotech and EasySep™ Mouse NK cell isolation kit and Robosep buffer were purchased from StemCell Technologies. Calcein AM was purchased from BD. RNeasy RNA purification mini kit was purchased from Qiagen. X-Ray films were purchased from Fujifilm. qScript cDNA supermix kit was from Quanta Biosciences. iQ SYBR Green was purchased from VWR. ECL blotting reagent was from Merck Millipore. Methanol and Absolute Ethanol were purchased from school stores. Paraformaldehyde was obtained from Sigma.

2.1.2. Equipment

Pipettes, pipette tips, filtered tips, cell culture strainers (70μM), 2ml disposable syringes, 20μM filters, tissue culture dishes (6, 12, 24, 48 and 96 well), tissue culture flasks (25cm, 75cm and 175cm) and (15ml, 50ml) falcons were obtained from Fisher.

AB-1900 High sided low profile PCR plates, Absolute qPCR seal, Scilogex MX-S Vortex and Scilogex D1008 Mini Centrifuge were from MSC. BD polystyrene 5ml FACS tubes were obtained from Unitech. Graphpad-prism software was obtained from Graphpad. FACS Canto and LSR Fortessa were from Becton Dickinson. FlowJo software was obtained from TreeStar. Seahorse machine, seahorse plates and cartridges were purchased from Agilent technologies. 7500 fast real time PCR system was from Thermo Fisher. XF 24-well microplate was from Seahorse Bioscience. 96 well flat black polystyrene plates were form Costar. LS MACS purification columns, Quadro MACS cell separator and AutoMACS pro separator were from Miltenyi Biotec. EasySep™ magnet was purchased from StemCell Technologies. Prime Thermal Cycler and Dri-Block DB-2D were purchased from Techne. Grant JB Series Water Bath was obtained from Amlab Services. Bright-Line Hemocytometer from Hausser

Scientific. Power Source 300 V was obtained from VWR International. Steri-Cycle CO_2 Incubator was from Thermo Forma. Heraeus Pico 17 centrifuge was from Thermo Scientific. 5810R centrifuge was from Eppendorf. Legend RT Centrifuge from Sorvall. 1500 Standard Fumehood from Phoenix Controls Corporation. Bioair Topsafe Fumehood from Crowthorne HiTec Services. GelDoc XR+ imaging system was purchased from BioRad. Nanodrop Spectrophotometer ND-1000 from Labtech International. Electrophoresis running system was from Atto and XCELL II Blot Module transfer system from Invitrogen.

2.1.3. Biological material

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.

cMyc$^{flox/flox}$ and **tamoxifen cre** mice were obtained from Dundee University and maintained in compliance with UK Home Office Animals (Scientific Procedures) Act 1986 guidelines. Tamoxifen cre mice were crossed with cMyc$^{flox/flox}$ mice, both of which were on a C57BL/6J background. Tamoxifen cre mice express cre-recombinase-estrogen receptor fusion protein that is maintained in the cytoplasm through interactions with HSP90. When 4-OH tamoxifen is added, this binds to the ER and displaces HSP90, resulting in the nuclear translocation of the Cre-ER fusion protein. The Cre recombinase then catalyzes the recombination of LoxP sites in the DNA resulting in the deletion of the floxed cMyc gene.

B16 melanoma cell line was kindly donated by the Kingston Mills lab.

K562 cell line was kindly provided by the Clair Gardiner Lab.

2.1.4. Antibodies

2.1.4.1. Flow cytometry antibodies

Antibodies used for flow cytometry: eFluor 450 NK1.1 (PK136), PerCP-eFluor 710 CD335 (29A1.4), FITC CD3 (145-2C11), APC CD71 (R17217) PE CD98 (RL388), PE-Cy7 Granzyme B (NGZB), were purchased from eBioscience. Flow cytometry antibodies APC-Cy7 CD25 (PC61), APC IFN-γ (XMG1.2), BV510 CD71 and Fc block anti-CD16/CD32 (2.4G2); TCRβ APC, PE were purchased from BD pharmingen. PE-C-Myc (D84C12) was purchased from Cell Signaling. 2-NBDG was purchased from Invitrogen. Western blot antibody S6 ribosomal protein (Ser235) was incubated along with a secondary antibody PE-conjugated donkey anti-rabbit immunoglobulin G purchased from Jackson ImmunoResearch for pS6 Flow cytometry analysis. CFSE, Thermo Fisher; Propidium Iodide-Sigma Aldrich and LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit was obtained from Thermo Fisher. See table 2.1.4.1

2.1.4.2. Western blot antibodies

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

2.1.4.3 Cross-linking antibody

Purified anti-mouse NK1.1 (clone PK136) was obtained from BD Pharmingen.

Table 2.1.4.1 Flow cytometry antibodies

Antibody (clone)	Source	Dilution	Location
Fc block (2.4G2)	BD Pharmingen	1/100	Extracellular
eFluor 450 NK1.1 (PK136)	eBioscience	1/200	Extracellular
PerCP-eFluor 710 CD335 (29A1.4)	eBioscience	1/200	Extracellular
FITC CD3 (145-2C11)	eBioscience	1/400	Extracellular
PE TCRβ (H57-597)	BD Pharmingen	1/200	Extracellular
APC TCRβ (H57-597)	BD Pharmingen	1/100	Extracellular
APC-Cy7 CD25 (PC61)	BD Pharmingen	1/100	Extracellular
APC CD71 (R17217)	eBioscience	1/200	Extracellular
BV510 CD71 (C2F2)	BD Horizon	1/200	Extracellular
PE CD98 (RL388),	eBioscience	1/100	Extracellular
PE cMyc (D84C12)	Cell Signaling	1/50	Intracellular
APC IFN-γ (XMG1.2),	BD Pharmingen	1/200	Intracellular
PE-Cy7 Granzyme B (NGZB),	eBioscience	1/200	Intracellular
CFSE	Thermo Fisher	1/1000	Extracellular
Propidium Iodide	Sigma-Aldrich	1/200	Extracellular
Phospho S6 ribosomal protein (Ser 235)/ PE-conjugated donkey anti-rabbit immunoglobulin G	Cell Signalling/ Jackson ImmunoResearch	1/100	Intracellular

Table 2.1.4.2 Western blot antibodies

Antibody	Source	Dilution	2^{O} Ab
S6 ribosomal protein	Cell Signaling	1/500 5% milk/PBST	Mouse
phospho-S6 S235/236	Cell Signaling	1/2,500 5% milk/PBST	Rabbit
phospho-p70 S6K Thr 389	Cell Signaling	1/2,500 5% milk/PBST	Rabbit
S6K	Cell Signaling	1/500 5% milk/PBST	Rabbit
SMC-1	Bethyl Labs	1/2500 5% milk/PBST	Rabbit

2.2. Methods

2.2.1. Cell culture

Natural Killer cell culture

Under sterile conditions, murine spleens were extracted and gently minced with a plunger from a 2ml syringe through a 70μM strainer into 4ml cold media in a 6 well plate. The cell suspension was filtered through the strainer until a homogeneous cell solution, free from any clumps, was obtained. The cell solution was centrifuged at 1400rpm for 4 minutes at room temperature. Supernatant was decanted and the cell pellet was dislodged. Erythrocytes were lysed with 3ml sterile double-distilled H_2O (dd H_2O) for 3 seconds before the addition of 10ml RPMI media to discontinue the lysis process. Cells were centrifuged down at 1400rpm for 4 minutes. Splenocytes were resuspended at $2x10^6$ cells/ml in RPMI media and cultured in 6 well dishes: 5ml per well at

37°C for 6 days unless indicated otherwise. Splenocytes were fed with IL-15 on day 0 and day 4. The biological activity of IL-15 varied between batches. For this reason, each batch of IL-15 was titrated for the optimal concentration to expand primed NK cells that were not overtly activated compared to ex vivo splenocytes (between 5-25ng/ml). Because of this variability, murine IL-15 was replaced by human IL-15 in the later experiments for which the culture concentration was 10ng/ml.

B16 cell line maintenance

B16-F10 melanoma cells were thawed and cultured in complete DMEM medium containing 10% FCS and 1%Pen/strep in a T25 flask. After 24 hours, the cells were trypsinized and $1/10^{th}$ of the cells were passaged into T75 flasks and split in the same way every 48 hours.
To freeze the cells down, the cells were resuspended at $10x10^6$ cells/ml with 5%DMSO and stored in -80°C freezer overnight before being transferred into liquid nitrogen.

K562 cell line maintenance

K562 cells were thawed and cultured in complete IMDM containing 10% FCS and 1%Pen/strep in a T25 flask. After 24 hours, the cells were counted and $0.4x10^6$ cells were seeded in a T75 flask in 15 ml fresh IMDM. The cells were observed and passaged after 48 hours.
To freeze the cells down, the cells were resuspended at $10x10^6$ cells/ml with 5%DMSO and stored in -80°C freezer overnight before being transferred into liquid nitrogen.

2.2.2 NK cell stimulation

Crosslinking assay

Purified antibody against activating NK cell receptor NK1.1 was diluted to a concentration of 10μg/ml in PBS. Tissue culture plates were coated with this crosslinking antibody. The plates were then incubated at 37^0C for 90 min before being gently rinsed with sterile PBS.

Harvesting and stimulating cultured NK cells

Cultured NK cells were harvested on day 3 or 6 and washed out of IL-15 and stimulated for 18 hours either in the NK1.1 coated wells and/or with the cytokines: IL-15 (2-10ng/ml, Peprotech), IL-2 (20ng/ml, NCI preclinical repository), IL-12 (10ng/ml, Miltenyi Biotech) and inhibitors; 2-deoxy-D-glucose (0.25-5mM, Sigma), rapamycin (20nM, Fisher), oxalate (0.5mM-2mM Sigma), oligomycin (0.5-4nM, Sigma), 25-hydroxycholesterol (1μg/ml), PF429242 (10μM), or BCH (25mM). Where indicated, cultured NK cells were stimulated in glucose-free medium containing dialysed FCS supplemented with or without the addition of glucose (Sigma). Where indicated, 3 day cultured NK cells were stained with CFSE to study NK cell proliferation on the basis of CFSE dilution.

NK cell purification

Where indicated, cultured NK cells were purified by magnetic separation using the NK Cell Isolation Kit II (Miltenyi Biotech) or EasySep™ NK cell isolation kit according to the manufacturer's guidelines for biochemical analyses.

Co-culture assay

Purified NK cells were either co-cultured with B16 melanoma cells or left unstimulated for 24 hours. CD25 expression was assessed using flow cytometry. These same cells were washed out of tumour cells and re-stimulated with either low dose IL-15 or IL-2 for 24 hours and analysed for functional outputs by flow cytometry.

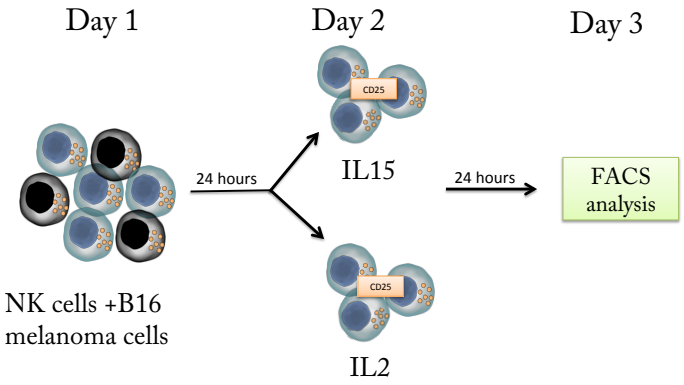


Table 2.2.1 Buffers/Media

Buffers/Media	Composition
Cell culture media	RPMI medium (+) L-Glutamine, FCS (10%), Penicillin/Streptomycin (1%), β -mercaptoethanol (50uM)
Supplemented glucose free media	10% Dialayzed FCS, 1mM Sodium Pyruvate, 1x Vitamin Cocktail, 1x Selenium/Insulin Cocktail, 50μM β-mercaptoethanol and 1% Penicillin/Streptomycin, +/- Glucose 10mM,
B16 culture medium	DMEM, Glutamax™ FCS (10%), Penicillin/Streptomycin (1%)
K562 culture medium	IMDM (+) L-Glutamine, FCS (10%), Penicillin/Streptomycin (1%)

RNA lysis Buffer	RLT buffer (Qiagen), 10% - β mercaptoethanol
FACS Buffer	Dulbecco's phosphate buffered saline, Cell culture media (10%)
Nuclear permeabilization buffer	FACS Buffer, 0.25% PFA, 0.2% Tween-20
MACS Buffer	To make up 1L: 800mls ddH_2O, 4 PBS tablets, 3.2mls EDTA (1M) 0.5% FCS
Cell-tak	For 20 wells: 13ul Cell-Tak, 4180.5ul Sodium Bicarbonate (0.1M), 6.5ul NaOH (1M)
Running Buffer	25mM Tris, 192mM Glycine, 0.1% SDS
Transfer Buffer	25mM Tris, 192mM Glycine, 10% methanol
Stripping Buffer	0.7% β-mercaptoethanol, 2% SDS, 62.5mM Tris
Protein lysis buffer	100mM Tris/HCl pH 6.7, 20% Glycerol, 4% SDS, 5% β-Mercaptoethanol, 0.1% bromophenol blue
PBST	1x PBS, 1ml Tween-20

Table 2.2.2. Cell culture stimulants

Compound	**Stock concentration**	**Final concentration**
IL-2	20μg/ml	20ng/ml
IL-15	20ug/ml	5ng/ml
IL-12	50μg/ml	10ng/ml
IL-18	100μg/ml	50ng/ml
Rapamycin	1mM	20nM
Oligomycin	2mM	2nM, 4nM
2-DG	1M	1mM, 2mM 5mM
Glucose	1M	10mM

Oxalate	200mM	0.5, 1mM, 2mM
BCH		25mM
25HC	1.8mg/ml	1µg/ml
PF429242	100mM	10µM
Anti-NK1.1	1mg/ml	10µg/ml

2.2.3 Flow cytometry analysis

Extracellular staining

Following the 18-hour incubation after the stimulations, cells were transferred to labeled FACS tubes and washed with 1ml FACS buffer per tube before being centrifuged at 1400rpm for 3 minutes. Cell pellets were dislodged, followed by the addition of Fc block (50ul per sample of 1 in 50 Fc block:FACS buffer) to block non-specific antibody binding. The cells were incubated in Fc block at 4°C for 5 minutes. Staining antibodies for NK cell surface markers were then added in a final volume of 100ul antibody mastermix per sample, at concentrations outlined in table 2.1.4.1. Cells were incubated in the dark for 20 min at 4°C. Following the incubation, cells were washed with 1ml FACS buffer and centrifuged at 1400rpm 3 minutes before acquisition by flow cytometry. Live cells were gated according to their forward scatter and side scatter. Data were acquired on a FACS Canto or FACS Fortessa (Becton Dickinson) analysed using FlowJo software (TreeStar).

Where indicated, Propidium Iodide (PI) was added to the cells, just prior to the analysis. Uptake of PI by cells indicated damaged/non viable cells.

Intracellular staining

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

Phospho-S6 ribosomal protein intracellular staining

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

c-Myc staining

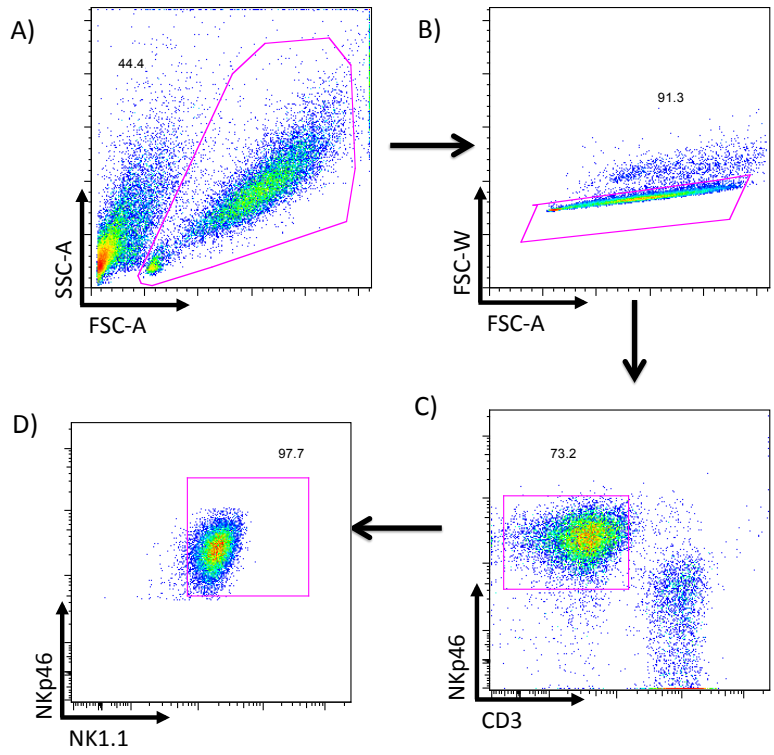
$0.2x10^6$ cultured NK cells were washed and resuspended in FACS buffer. The cells were transferred to a 96 well plate and stained with extracellular antibodies. Following the 20 min incubation, the cells were washed again and permeabilised by incubating in 200µl of FACS buffer containing 0.5% PFA and 0.2% Tween for a minimum of 2 hours at room temperature. After the permeabilization, the cells were stained with intracellular stains, including the cMyc antibody at the final concentration of 1 in 50 for 1 hour at room temperature. The cells were then washed once with FACS buffer and analysed by flow cytometry.

2-NBDG uptake assay

$0.5x10^6$ cultured NK cells were washed with glucose free and re-suspended in pre-warmed glucose-free media, supplemented with 10% dialyzed FCS (Fisher), 2mM Glutamine (Invitrogen/Biosciences), 1mM Sodium Pyruvate (Gibco), 1x concentration of MEM Vitamin Cocktail (Invitrogen/Biosciences), 1x concentration of selenium/insulin/transferrin Cocktail (Invitrogen/Biosciences), 50µM β-mercaptoethanol (Sigma) and 1% Penicillin/Streptomycin (Invitrogen/Biosciences), at a concentration of $1x10^6$cells/ml. After 15 minutes incubation at 37°C, cells were resuspended in 1 ml of supplemented glucose-free media with 35µM of the fluorescently labelled glucose analogue 2-NBDG (Life technologies), or in glucose-free media alone as a control. Cells were then incubated for a further 2 hours at 37°C after which they were washed twice in supplemented glucose-free media. Extracellular staining was carried out as outlined above before acquisition on a FACS Canto or FACS Fortessa (Becton Dickinson).

Gating Strategy

Lymphocytes were gated based on their forward scatter and side scatter area(A). Doubled cells were excluded based on forward scatter width (B). NKp46^{+}CD3^{-} cells were selected (C) to get out T cells; followed by a selection of NK cells double positive for 2 different NK cell markers NKp46^{+} and NK1.1^{+}(D). The cells in this gate were characterized for various functional outputs.



2.2.4. Metabolic Analysis

Metabolic Seahorse analysis

A XF-24 Extracellular Flux Analyzer (Seahorse Bioscience) was used for real-time analysis of the extra cellular acidification rate (ECAR), as a measure of glycolysis; and oxygen consumption rate (OCR), as an indicator of Oxidative Phosphorylation rates, of NK cells stimulated under various conditions. One day prior to analysis, the seahorse cartridge plate was hydrated using 1ml calibration buffer per well in a non-CO^2 incubator at 37^0C according to manufacturers instructions.

Wells of the 24well seahorse cell plate were coated with 200µl Cell-tak solution (BD Pharmingen) (See Table 2.2) and incubated at room temperature for 20 minutes before being washed twice with ddH_2O (500µl/well). Meanwhile, $3x10^6$ NK cells per condition were transferred to 15ml falcons and washed twice in 5ml pre-warmed seahorse media. Cells were resuspended at $7.5x10^6$ cells/ml for NK cells, or $5x10^6$ cells/ml for T cells, in seahorse media supplemented with relevant treatments. 100µl of cells were added to designate cell-tak coated wells in quadruplicate of the seahorse cell plate. The cell plate was gently centrifuged at 1200rpm for 3 minutes with the brake off before incubation in a non-CO_2 incubator for 20 minutes. Meanwhile, 70µl of inhibitors were added to designate ports of the seahorse cartridge plate prior to calibration. Inhibitor concentrations were as follows; oligomycin (2µM), fluoro-carbonyl cyanide phenylhdrazone (FCCP 0.5µM), rotenone (100nM) plus antimycin (4µM) and 2DG (30mM). Following 20 minutes incubation of cell plate, 500µl seahorse media containing relevant treatments was added to each well, and 600µl seahorse media was put in the blank wells. After calibration of the seahorse cartridge, the cell plate was put in the seahorse machine for analysis.

Glycolytic Measurements

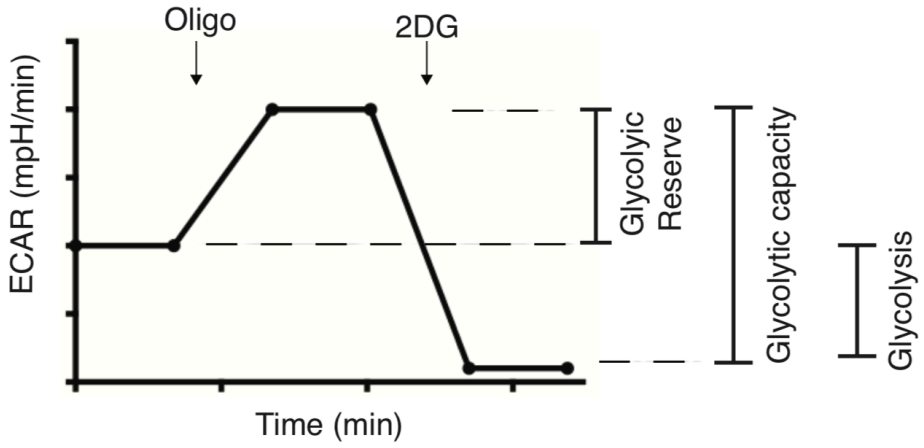


Figure 2.2.4.1. Determining Glycolytic rates using the Seahorse metabolic flux analyser

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

Oxidative Phosphorylation Measurements

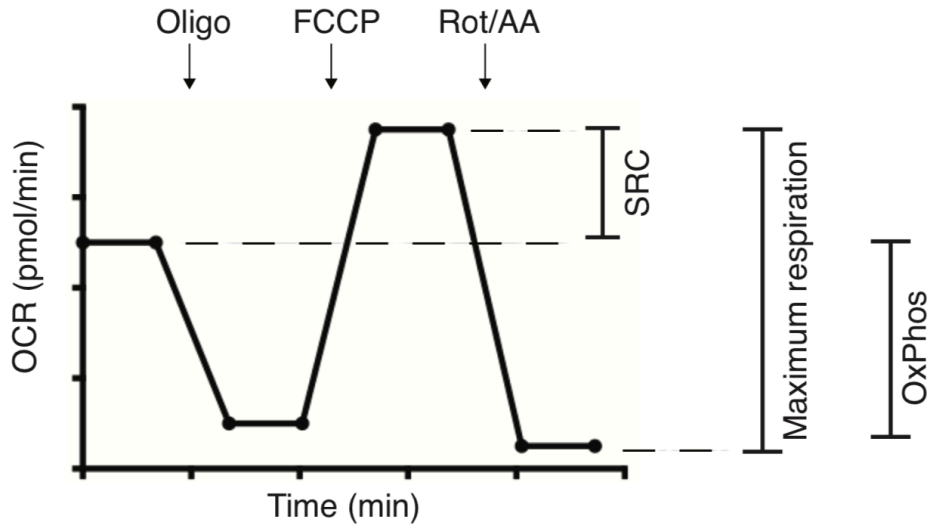


Figure 2.2.4.2 Determining OxPhos rates using the Seahorse metabolic flux analyser

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

2.2.5. RNA analysis

RNA isolation

Cultured NK cells were purified by magnetic separation and 1 x 10^6 pure NK cells were stimulated for 18 hours. After 18 hours, stimulated cells were centrifuged at 1400rpm for 3 minutes. The supernatant was discarded and cells were washed with 1ml cold PBS. Cells were re-centrifuged at 1400rpm for 3 minutes and the cell pellet was resuspended in 600µl RLT buffer containing 1% β-Mercaptoethanol. RNA lyates were stored at -80ºC. 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).

cDNA synthesis

1µg of isolated RNA was made up to 16µl with DEPC H_20 and added to 4µl of qScript cDNA Supermix (5x) (Quanta BioSciences), containing the reverse transcriptase enzyme required to carry out cDNA synthesis. The reverse transcription was carried out in Techne's ^{3}Prime Thermal Cycler at the following incubation times:

Time	Temperature
5 minutes	25ºC
30 minutes	42ºC
5 minutes	85ºC
Hold	4ºC

Primer design

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

Gene Name	Primer sequence 5'-3'
Rplp0 forward	CATGTCGCTCCGAGGGAAG
Rplp0 reverse	CAGCAGCTGGCACCTTATTG
Ldha forward	CTGGGAGAACATGGCGACTC
Ldha reverse	ATGGCCCAGGATGTGTAACC
Glut1 forward	GGAATCGTCGTTGGCATCCT
Glut1 reverse	CGAAGCTTCTTCAGCACACTC
Pfk forward	GTTTGGAAGCCTCTCCTCCTC
Pfk reverse	AGAGGTCAACACGGCGATG
Gapdh forward	CATGGCCTTCCGTGTTCCTA
Gapdh reverse	CCTGCTTCACCACCTTCTTGAT
Hex2 forward	TCGCCTGCTTATTCACGGAG
Hex2 reverse	CCATCCGGAGTTGACCTCAC
Pkm1 forward	AAACAGCCAAGGGGGACTAC
Pkm1 reverse	TTATAAGAGGCCTCCACGCTG
Pkm2 forward	GCTATTCGAGGAACTCCGCC
Pkm2 reverse	AAGGTACAGGCACTACACGC
Acat2 forward	CCAGAGCGACAAGATGAATGC
Acat2 reverse	CCACAACCTGCCGTCAAGA
Acly forward	CCCCAAGATTCAGTCCCAAGTC
Acly reverse	GATCCCCAGTGAAAGGGTAGAC

Fasn forward	GGTATAGACGACGGGCACAG
Fasn reverse	GCCATTCCAGGTAAATGGGC
Hmgcs1 forward	CATTGGGCGGCTAGAAGTTG
Hmgcs1 reverse	ACCAGAGCATATCGTCCATCC

Quantitative Real Time PCR

Quantitative Real time PCR was performed in a 96 well plate format using iQ SYBR Green based detection (VWR) in a 7900HT Fast Real-Time PCR System (Applied Biosciences) to quantify the relative amounts of steady-state mRNA expression for genes of interest against RpLp0 as an internal control. Each 10ul reaction contained: 0.5µl cDNA, 0.4µl (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 fast cycle conditions;

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

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

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

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

2.2.6. Protein manipulation

Protein Extraction

Cultured NK cells were purified by magnetic separation and 2 x 10^6/condition pure NK cells were stimulated for 18 hours. After 18 hours, stimulated cells were centrifuged at 1400rpm for 4 minutes. Cell pellets were washed with 1ml cold PBS and recentrifuged at 1400rpm for 4 minutes. Cell pellets were resuspended and lysed in 1X protein lysis sample buffer + DTT 25μM (see table 2.2.1) at a concentration of 10x10^6cells/ml. Samples were denatured at 95˚C for 15 minutes before use and stored at -20˚C.

Sodium Dodecyl-Sulphate Poly-Acrylamide Gel Electrophoresis (SDS PAGE)

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

Table 2.2.6.1 10% gel composition

Reagent	Stacking gel	Resolving gel
1M Tris	625ul (pH 6.8)	2.25ml (pH 8.8)

1% SDS	500ul	600ul
40% acrylamide	500ul	1.5ml
1.5% APS	250ul	300ul
TEMED	5ul	6ul
ddH_20	3.12ml	1.344ml

Western Blot

For the transfer of protein onto nitrocellulose membrane, SDS-PAGE gels were removed from the running plates and assembled into a gel-membrane sandwich as dictated in the manufacturer's protocol of the transfer apparatus. Nylon sponge pads, Whatmann 3-MM chromatography paper and Hybond-C extra Nitrocellulose membrane (Amersham Biosciences) were soaked in transfer buffer containing 10% methanol. Nitrocellulose membrane was activated in absolute methanol before use. The sandwich was loaded into the transfer apparatus with the nitrocellulose membrane closest to the anode. Proteins were transferred at 45V for 2 hours or 15V overnight on ice. To determine quality of transfer, membranes were stained with Ponceau S solution for 30 seconds, before gently rinsing with water. Membranes were blocked in a 5% milk/PBST solution for 40 minutes at room temperature. Membranes were washed three times in PBST for 5 minutes before incubation in primary antibody (see table 2.1.4.2.) for 4 hours at room temperature. After primary antibody incubation, membranes were washed three times for 5 minutes in PBST before incubation with secondary antibody (1/5000 in 5% milk/PBST) for 1 hour at room temperature. After secondary antibody probing, membranes were washed three times for 5 minutes in PBST. ECL (Merck Millipore) was applied to the membrane and luminescence assessed by GelDoc XR^+ system.

2.2.7 Killing assay

To perform a killing assay, K562 cell line, which is a human myleogenous leukaemic cell line was used. K562 cells lack MHC class 1 molecules, and therefore highly susceptiple to lysis by NK cells. 6-8 million K562 cells were counted and washed thoroughly out of media by cetrifugating twice at 1400rpm for 4 min. The cells were resuspended in 2 ml PBS and counted again. The cells were then spun down and resuspended at a concentration of 2 million cells/ml in PBS + 20uM Calcein AM viability dye. Calcein AM is cleaved by intra-cellular esterases to become fluorescent. Cells actively take up the dye after incubation at 37 degrees for 30 min and appear yellow-green after successful labelling. Following 30 min incubation, labelled K562 cells are thoroughly washed in wash buffer (20%FCS in PBS) 3 times. After the washed the cells were resuspended in 2 mls of RPMI and counted. The cells were resuspended at a concentration of $0.3x10^6$ cells/ml and allowed to rest for 1 hour at 37^{o} C. While the K562s were resting, untrained and trained cells were counted, washed and plated in a 96 well plates according to the ET ratios 5:1, 2:1 and 1:1 in triplicates. K562s were washed again following the 1 hour resting and resuspended at $0.3x10^6$ cells/ml in RPMI and plated in with the NK cells. K562s were also plated on their own without NK cells, to measure spontaneous release. Additionally, K562 cells alone were plated in the presence of 8ul of 10% TritonX to measure maximum release of Calcein AM.

After 4 hours of incubation, the plate was spun at 200g for 4 min, and 75ul of supernatant was aspirate into black plate used for spectramax plate reader. The killing capacity of NK cells is measured by measuring the Calcein released into the supernatant by dying cancer cells.

Spectra max settings

Mode:Fluorescent

Excitation:485nM

Autocut-off: 10nM

Emission:525nM

Plate type: Costar 96 well plate black

Flashes:6

Gain: Auto

Spontaneous release measurement

Spontaneous release should be less than 30%

(Spon release values/ Max release values)x100

Killing meausrement

Average triplicate valuesand calculate %killing as follows-

(Test - Spon release) / (Max release - Spon release) x100

2.2.8 Genotyping

Ear digestion

50µl of ear lysis buffer and 5µl of 10mg/ml proteinase K was added to each ear punch in 1.5ml eppendorfs. Eppendorfs were placed in a heating block at 55˚C for 4 hours to overnight, allowing the ears to become digested. 100µl DEPC DNAse nuclease free water was added to each sample before heating to 100˚C for 7 minutes in order to inactivate proteinase K. DNA samples were stored at -20˚C until use.

PCR and gel

2µl of DNA sample from the digested ear clips was added to 23µl of cMyc master mix, while 1µl of template was added to 24µl of tamoxifen Cre master mix as described in table 2.2.7.1. PCR products were running on a 2% agarose gel.

Primer sequences

CRE forward:	5' CGGTCGATGCAACGAGTGATGAGG 3'
CRE reverse:	5' CCAGAGACGGAAATCCATCGCTCG 3'
cMyc forward:	5'CCGCGGCACTAGGACTTGATG 3'
cMyc reverse:	5' GCTCAGATCACGACTCACCGCAG 3'

Table 2.2.8.1 Genotyping master mix components per sample

Component	Tamoxifen cre (µl)	cMyc (µl)
Buffer	5 (of 5x buffer)	2.5 (of 10x buffer)
$MgCl_2$	2.5	2
10mM dNTP	1	0.5
Forward primer	0.5 (of 10pmol/µl)	0.1 (of 100pmol/µl)
Reverse primer	0.5 (of 10pmol/µl)	0.1 (of 100pmol/µl)
Enzyme	0.1 of GoTaq	0.5 of RED Taq
DEPC H_20	14.4	14.8
1%Igepal	-	2.5

Table 2.2.8.2 cMyc PCR genotyping protocol

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

Sizes	cMyc floxed	250bp
	WT	165b

Table 2.2.8.3 Tamoxifen cre PCR genotyping protocol

Step	Temperature (°C)	Time	Cycles
0	94	Hold (hot start)	N/A
1	94	5 min	1
2	94	1 min	35
2	66	2 min	35
2	72	3 min	35
3	72	5 min	1
3	4	Hold	1

Sizes	T-cre	450bp
	WT	no band

2.2.9 Statistical Analysis

GraphPad Prism 6.00 for Macintosh (GraphPad Software) was used for statistical analysis. A one-way ANOVA test was used when comparing more than two data sets with a Tukey multiple comparison post-test. A student t-test was used when there were only 2 data sets to compare. For comparison of relative values, a one-sample t-test was used to calculate p values with the theoretical value set to 1. A p-value <0.05 was considered as statistically significant. In all figures, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Chapter 3

Receptor ligation of NK cells makes them more responsive to the adaptive cytokine IL2

3.1 Introduction

NK cells can either be activated in a by-stander response, by cytokines released from other immune cells in response to inflammation or they can be activated directly by ligands present on tumour cells or virally infected cells in a receptor mediated response. When NK cells engage with a target cell through their receptors, they are known to form an immunological synapse, and release cytotoxic granules, to directly kill the target cells. NK cell effector responses have been widely studied, but little is known about the metabolic changes associated with these responses. Our group has previously shown that murine NK cells undergo a metabolic reprogramming in response to cytokines IL2 and IL12. This study investigates the metabolic changes associated with NK cell activation in response to NK cell receptor ligation. To study NK cell response with receptor ligation, we stimulated NK cells through the NK1.1 receptor as it has been previously described elsewhere (Keppel et al., 2015; Reichlin and Yokoyama, 1998).

3.2 Receptor mediated stimulation up-regulates metabolic activity in NK cells

In order to study receptor-mediated stimulation, monoclonal antibody against the NK1.1 receptor was used to crosslink and activate this receptor to stimulate NK cells. In these experiments NK cells were directly isolated from murine spleens (hereafter termed 'splenic NK cells') or cultured with low dose IL15 (10ng/ml or 12.5ng/ml), a cytokine required for DC-mediated NK cell priming *in vivo*, for 3-6 days (hereafter termed 'cultured NK cells'). NK cells were either stimulated by anti-NK1.1 antibody crosslinking or with the cytokine combination IL2 and IL12. A lower dose of IL15 (5ng or 7.5ng/ml) was included in all conditions to promote NK cell survival without activating them. NK cells supplemented with this concentration of IL15 were used as an unstimulated control in all the experiments in this study. After the 18 hour

stimulation, $NK1.1^{+}NKp46^{+}CD3^{-}$ NK cells were analysed by Flow cytometry (*Refer to section 2.2.3 for Gating strategy).* Cultured NK cells increased in size following stimulation with the anti-NK1.1 antibody and this increase was comparable to that seen in cytokine stimulated NK cells **(Fig 3.1 A,B).** Increased cell size is a result of lymphocyte blastogenesis, which indicates increased metabolic demands for nutrients. Indeed, the expression of CD71, the transferrin receptor was found to be significantly upregulated in cultured NK cells stimulated through the NK1.1 receptor. **(Fig 3.1 C,D)** as was previously seen for IL2/12 stimulated NK cells (Donnelly et al., 2014). Additionally, there was also a significant increase in the expression of CD98, a component of the L-amino acid transporter in NK1.1 receptor stimulated splenic NK cells **(Fig 3.2)**.

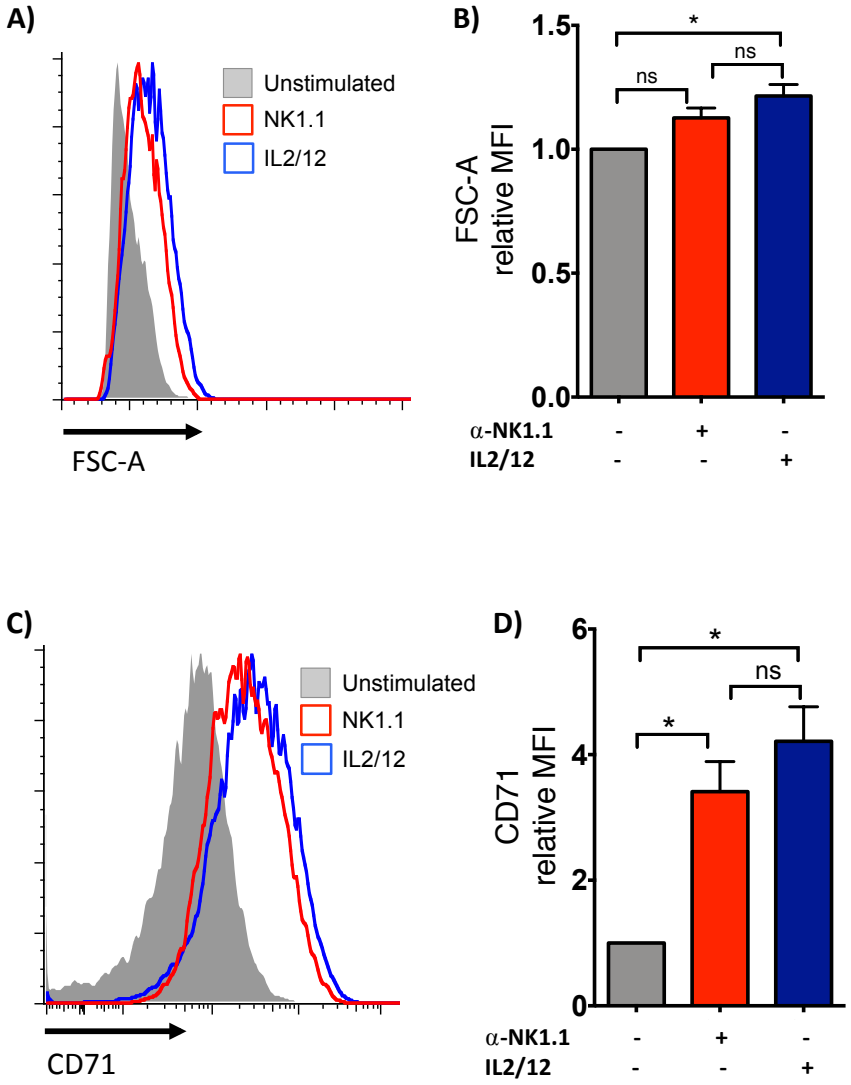


Figure 3.1 NK1.1 ligation induces cell growth and the expression of the nutrient transporter CD71 on cultured NK cells

(A-D) Cultured NK cells (3 days in IL15) were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), IL2 (20ng/ml) plus IL12 (10ng/ml) or left unstimulated (low dose IL15 at 5ng/ml only) and analysed by flow cytometry for FSC-A and CD71 expression. Pooled data is expressed relative to unstimulated NK cells (B, D). Data is representative (A, B) or mean +/- SEM (B, D) from 3 individual experiments. Data was analysed using a one-sample students t test against a theoretical value of 1. αNK1.1 and IL2/IL12 conditions were compared using a paired students t-test. (*$p < 0.05$; ns, non-significant).

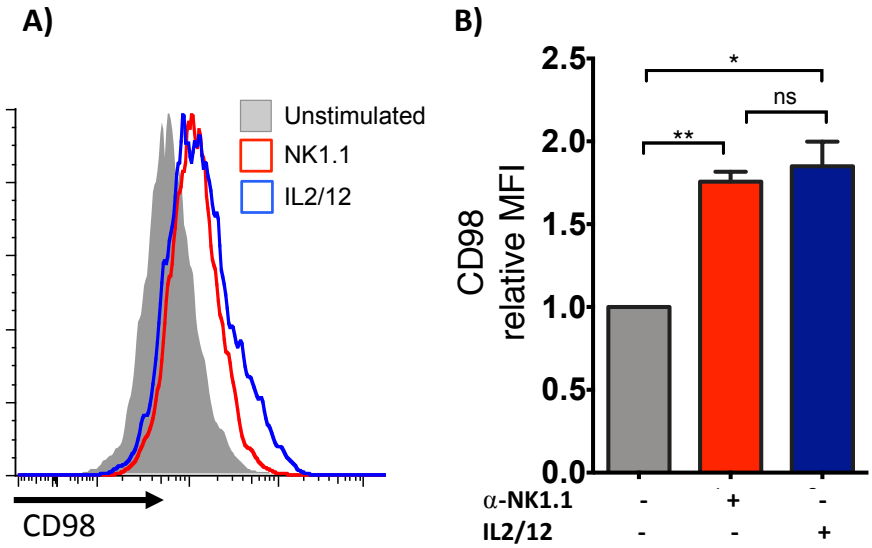


Figure 3.2 NK1.1 ligation induces increased expression of nutrient transporter subunit CD98 on splenic NK cells

(A-B) Splenic NK cells were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), IL2 (20ng/ml) plus IL12 (10ng/ml) or left unstimulated(low dose IL15 at 5ng/ml only) and analysed by flow cytometry for CD98 (A). Pooled data is expressed relative to unstimulated NK cells (B). Data is representative (A) or mean +/- SEM (B) from 3 individual experiments. Data was analysed using a one-sample student's t test against a theoretical value of 1. αNK1.1 and IL2/IL12 conditions were compared using a paired students t-test. (*$p < 0.05$; ** < 0.01, ns, non-significant)

Considering that the nutrient uptake receptors were up regulated in receptor stimulated NK cells, the next step was to measure the uptake of nutrients itself. For this, NK cells were stimulated as described above and incubated with 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose i.e. 2-NBDG, a fluorescent glucose analogue for the final 30 minutes of the 18 hour stimulation. NK1.1 receptor stimulated NK cells, both splenic and cultured NK cells, significantly up-regulated 2-NBDG uptake compared to the unstimulated NK cells and to a comparable extent to IL2/IL12 cytokine stimulated cells **(Fig 3.3).** While this assay is not an accurate indication of glucose uptake, it reflects on the NK cells' increased capacity for nutrient uptake following activation. Together these data argue that NK cells increase cellular metabolism in response to NK1.1 stimulation.

Recently, it was published that NK cells up-regulate the expression of the amino acid transporter Slc7A5, a system L transporter, which transports large amino acids including leucine, tryptophan, phenylalanine and methionine (Loftus et al., 2018). The uptake of kynurenine, which is a product of tryptophan catabolism, into lymphocytes can be used as a measure of amino acid transport through the system L transporters (Sinclair et al., 2018). Indeed, it was observed that NK cells increase their uptake of fluorescent kynurenine in response to NK1.1 receptor ligation, albeit, not to the same degree as IL2/IL12 cytokine stimulated NK cells **(Fig.3.4).**

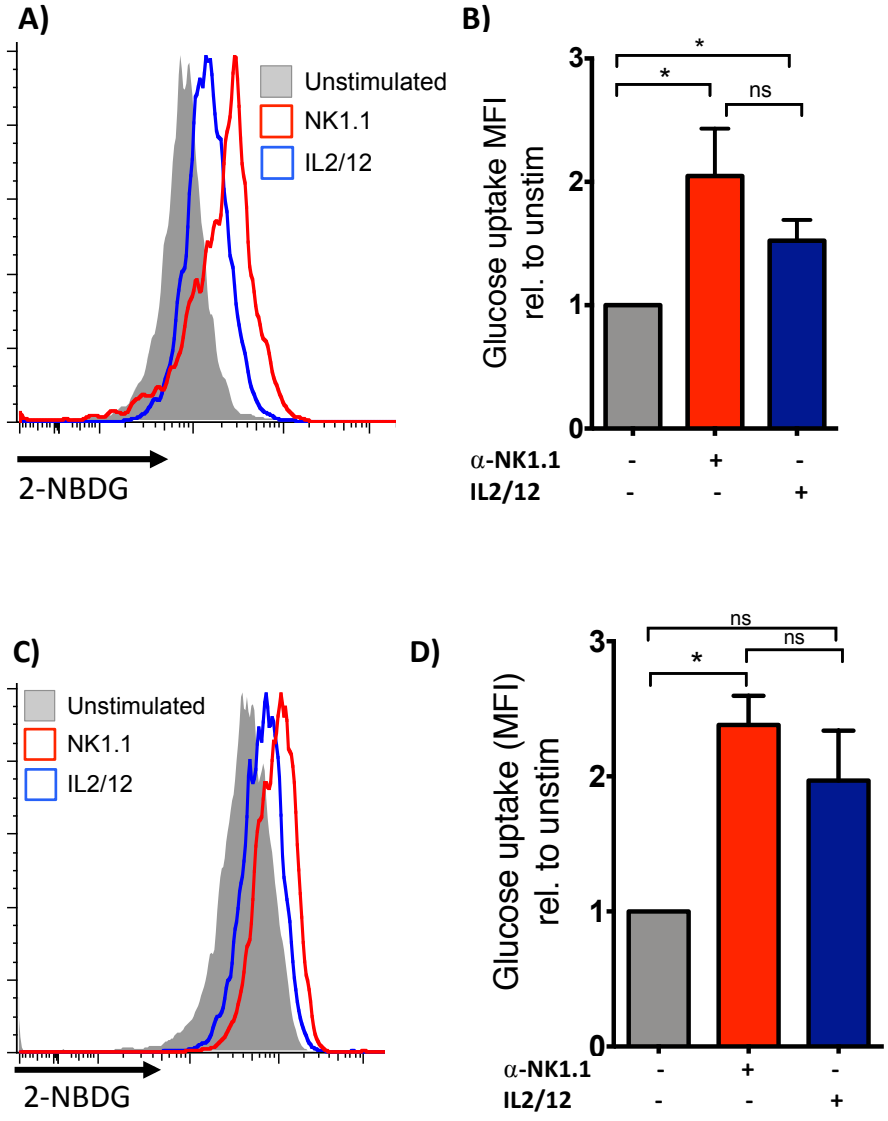


Figure 3.3 NK1.1 receptor ligation increases 2-NBDG uptake insplenic and cultured NK cells

Splenic NK cells (A,B) or cultured NK cells (6 days in low dose IL15) (C,D) were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), IL2 (20ng/ml) plus IL12 (10ng/ml) or left unstimulated (low dose IL15 at 5ng/ml only). 2-NBDG (35μM) was added to the NK cells for the final 30 minutes and 2NBDG uptake analysed by flow cytometry. Data is representative (A,C) or mean +/- SEM (B,D) of 3 independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1. αNK1.1 and IL2/IL12 conditions were compared using a paired students t-test. (*$p < 0.05$; ns, non-significant).

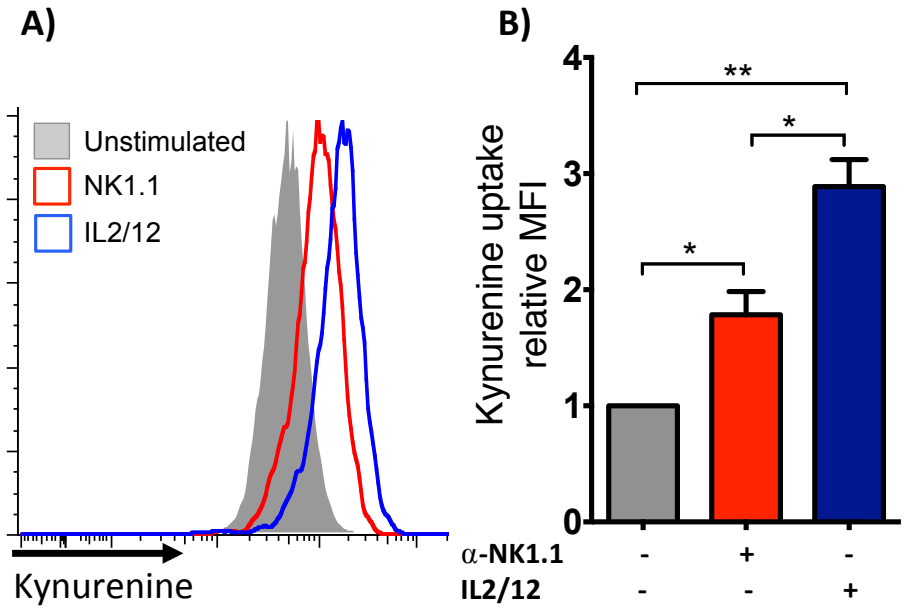


Figure 3.4 NK1.1 receptor ligation induces increased Kunurenine uptake in 6 day cultured NK cells.

(A-B)Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), IL2 (20ng/ml) plus IL12 (10ng/ml) or left unstimulated (low dose IL15 at 5ng/ml only). Kynurenine was added to the NK cells for the final 5 minutes and kynurenine uptake was analysed by flow cytometry. Data is representative (A) or mean +/- SEM (B) of 3 independent experiments. Data was analysed using a one-sample students t test against a theoretical value of 1. αNK1.1 and IL2/IL12 conditions were compared using a paired students t-test. (*$p < 0.05$; **$p < 0.01$).

Increased expression of nutrient transporters and associated nutrient uptake provided a clear indication that cellular metabolism is increased in NK cells stimulated through the NK1.1 receptor for 18 hours. To directly measure metabolic rates, NK cells were next analysed using the seahorse extracellular flux analyser. Cultured NK cells were purified by magnetic bead cell sorting and stimulated by either NK1.1 receptor cross-linking, IL2/IL12 cytokine or left unstimulated for 18 hours. Rates of glycolysis and glycolytic capacity were determined by measuring the extracellular acidification rate (ECAR) which measures lactate secretion i.e. aerobic glycolysis, following the sequential addition of oligomycin and 2-deoxyglucose (Chapter 2 Section 2.2.4). Rates of Oxidative phosphorylation (OxPhos) and maximal respiration were determined by measuring by measuring the rate of oxygen consumption (OCR) following sequential addition of oligomycin, FCCP and antimycin A plus rotenone *(Refer to section 2.2.4 for detailed description)*. NK1.1 stimulated NK cells had significantly increased rates of glycolysis compared to the unstimulated NK cells **(Fig 3.5 A,B)**. Additionally, the glycolytic capacity of NK1.1 receptor stimulated NK cells was also substantially increased **(Fig 3.5 A,C),** which suggests that the NK cells have increased the levels of glycolytic machinery, such as glycolytic enzymes and glucose transporters. However, the magnitude of these increases was small compared to IL2/IL12 cytokine stimulated NK cells **(Fig 3.5).** Similarly, NK1.1 receptor stimulated cells showed increased rates of oxygen consumption due to increased OxPhos and increased maximal respiration **(Fig 3.6).** Again, the magnitude of these increases was small compared to cytokine stimulated NK cells. Together the data show that NK1.1 receptor stimulated NK cells have modest increased in cellular metabolic pathways when compared to NK cells stimulated with IL2/IL12.

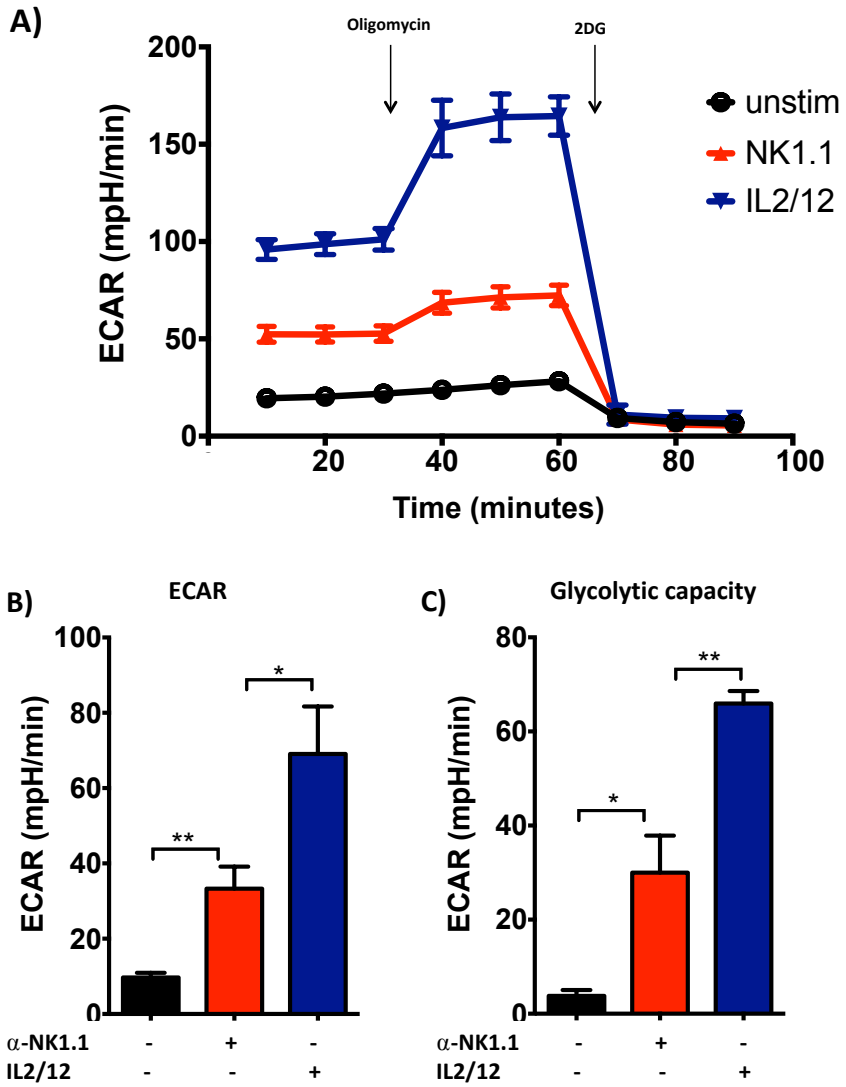


Figure 3.5 NK1.1 receptor ligation induces small increases in glycolysis in cultured NK cells

(A-C) Cultured NK cells (6 days in low dose IL15) were MACS-purified and stimulated for 18 hours through NK1.1 receptor ligation using α-NK1.1 antibody (10μg/ml) plus low dose IL15 (5ng/ml), IL2 (20ng/ml) plus IL12 (10ng/ml) or left unstimulated (low dose IL15 5ng/ml). (A) Extracellular acidification rate (ECAR) was assessed using Seahorse extracellular flux analyser with the sequential addition of inhibitors oligomycin and 2-deoxyglucose (2DG). The rates of glycolysis (B) and glycolytic capacity (C) were calculated. Data is representative (A) or mean +/- SEM (B,C) of 4 individual experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post-test. (*$p < 0.05$; **$p < 0.01$).

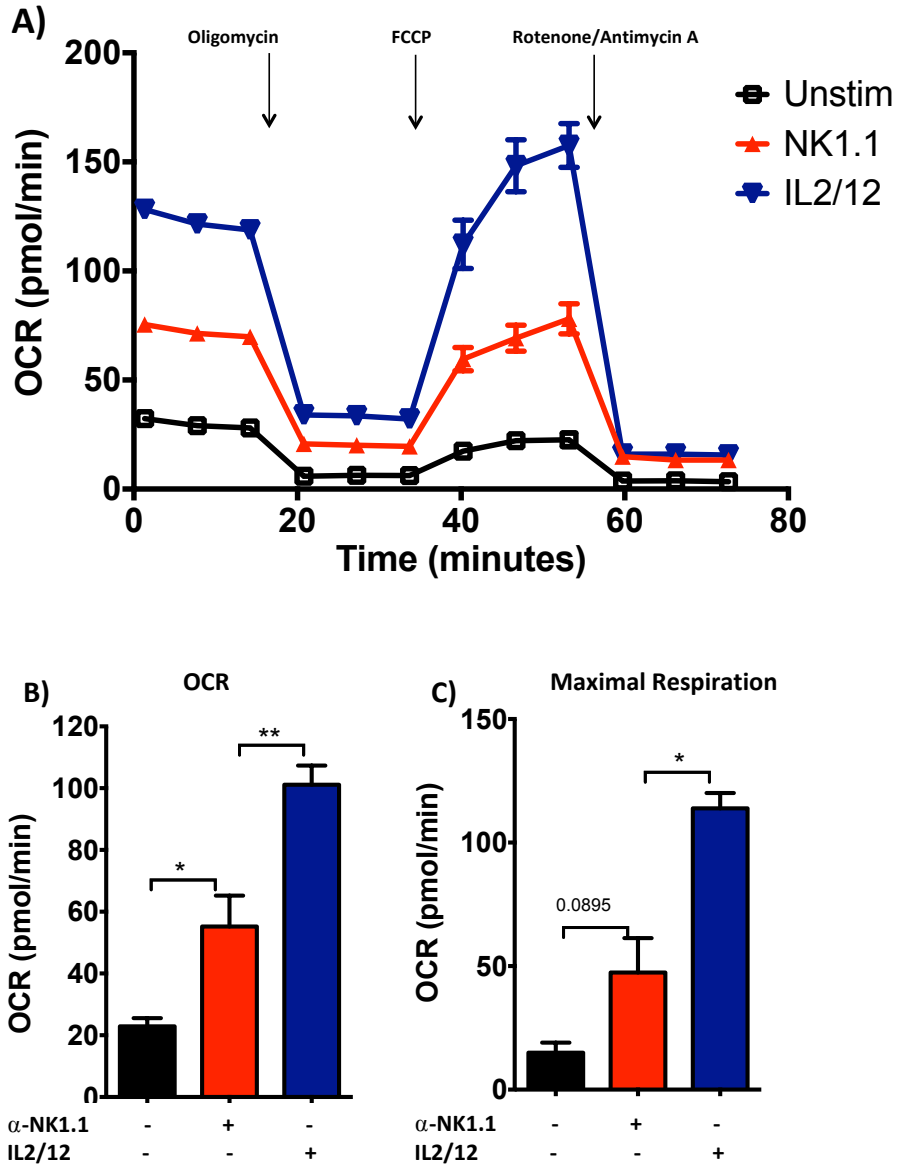


Figure 3.6 NK1.1 receptor ligation induces small increases in OxPhos in cultured NK cells

(A-C) Cultured NK cells (6 days in low dose IL15) were MACS-purified and stimulated for 18 hours through NK1.1 receptor ligation using α-NK1.1 antibody (10μg/ml) plus low dose IL15 (5ng/ml), IL2 (20ng/ml) plus IL12 (10ng/ml) or left unstimulated (low dose IL15 5ng/ml). (A) Oxygen consumption rate (OCR) was assessed using Seahorse extracellular flux analyser with the sequential addition of inhibitors oligomycin, FCCP and Antimycin A plus Rotenone. The rates of OxPhos (B) and maximal respiration (C) were calculated. Data is representative (A) or mean +/- SEM (B,C) of 4 individual experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post-test. (*$p < 0.05$; **$p < 0.01$).

3.3 Metabolic changes are accompanied by corresponding functional responses in receptor stimulated NK cells

After establishing that NK1.1 receptor stimulated NK cells moderately up-regulate their metabolic pathways, we asked whether this change in metabolism was accompanied with an increased NK cell effector response. Upon activation, NK cells respond either by releasing cytotoxic granules or by producing pro-inflammatory cytokines. Interestingly, NK1.1 receptor stimulated NK cells, that only partially up-regulate metabolism when compared to IL2/IL12 cytokine activated NK cells, significantly increased Granzyme B production **(Fig. 3.7).** However, these cells showed only a very small increase in IFN-γ production when compared with IL2/IL12 cytokine stimulated cells **(Fig 3.8).**

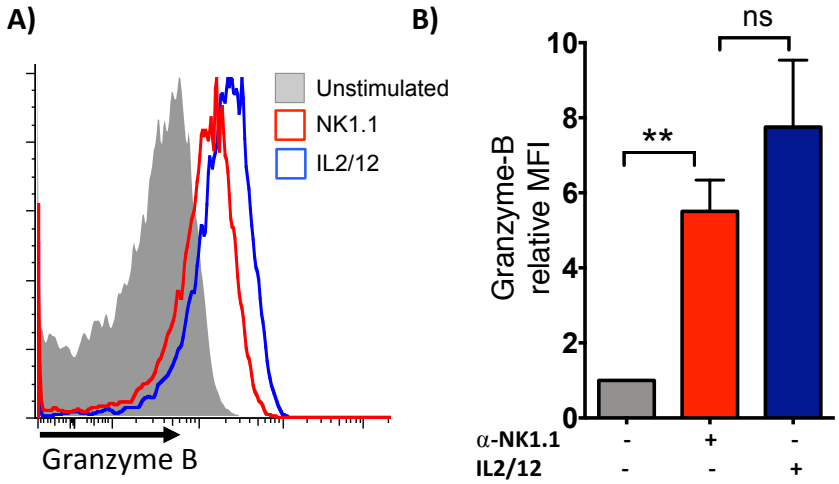


Figure 3.7 NK1.1 receptor ligation induces Granzyme B expression in cultured NK cells

(A-B) Cultured NK cells (3 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), IL2 (20ng/ml) plus IL12 (10ng/ml) or left unstimulated (low dose IL15 at 5ng/ml only). Granzyme B expression was analysed by flow cytometry. Data is expressed as relative to unstimulated (B). Data is representative (A) or mean +/- SEM (B) of 3 independent experiments. Data was analysed using a one-sample students t test against a theoretical value of 1. αNK1.1 and IL2/IL12 conditions were compared using a paired students t-test. (**$p < 0.01$; ns, non-significant).

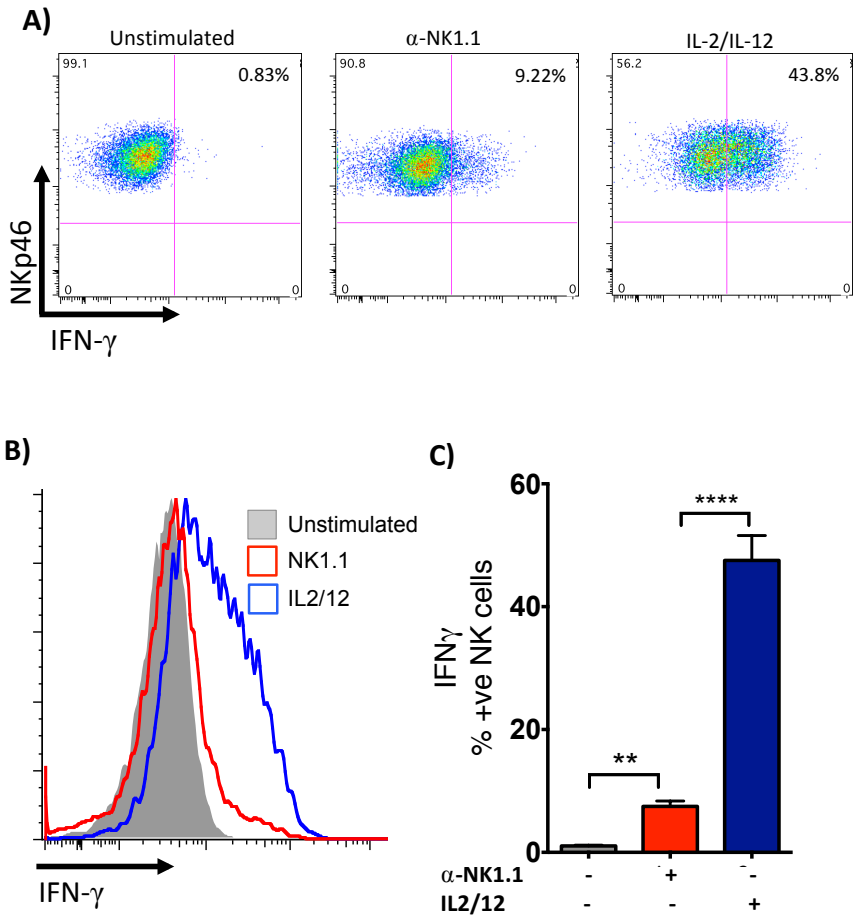


Figure 3.8 NK1.1 receptor ligation induces modest IFN-γ expression in cultured NK cells

(A-C) Cultured NK cells (3 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), IL2 (20ng/ml) plus IL12 (10ng/ml) or left unstimulated (low dose IL15 at 5ng/ml only). IFN-γ was analysed by flow cytometry. Data is representative (A, B) or mean +/- SEM (C) of 3 independent experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post-test. (**$p < 0.01$; ****$p<0.0001$)

3.4 NK1.1 ligation promotes activation induced cell death

Previous studies have shown that lymphocytes increase their metabolism in order to support the biosynthetic demands for clonal expansion. As there were significant increases in NK cell metabolism following NK1.1 receptor stimulation, changes in growth and proliferation were investigated in these cells. CFSE stained NK cells were stimulated with α-NK1.1 antibody, IL2/IL12 cytokines or left unstimulated for 24, 48 and 72 hours and their cell size (forward scatter, FSC-A), proliferation (CFSE dilution) and survival (PI staining) assessed. Growth and proliferation in NK1.1 receptor stimulated cells were compared with IL2/IL12 cytokine stimulated cells. IL2/IL12 cytokine stimulated NK cells increased in size after 24 hours and started proliferating after 48 hours **(Fig 3.9).** IL2/IL12 cytokine stimulated NK cells showed further increases in cell size and proliferation at 72 hours. In contrast, NK1.1 receptor stimulated cells did not increase in size nor engage in proliferation over the course of three days **(Fig 3.9).**

We next measured the viability of NK cells over the course of this three-day assay using propidium iodide (PI) staining. This dye is rapidly taken up by dying cells, thus cells staining positive for this dye are considered as dead or non-viable cells. The percentage of PI negative or live cells is measured as percent viability. The viability of NK1.1 receptor stimulated NK cells decreased progressively over the three-day experiment **(Fig 3.10).** In contrast, IL2/IL12 cytokine stimulated NK cells remained viable over the course of three days. Interestingly, NK1.1 receptor stimulated NK cells had reduced viability compared to unstimulated NK cells that were given a low dose of IL15. Considering that NK1.1 receptor stimulated NK cells also contained low dose IL15, these data argue that NK1.1 receptor ligation induces activation induced cell death.

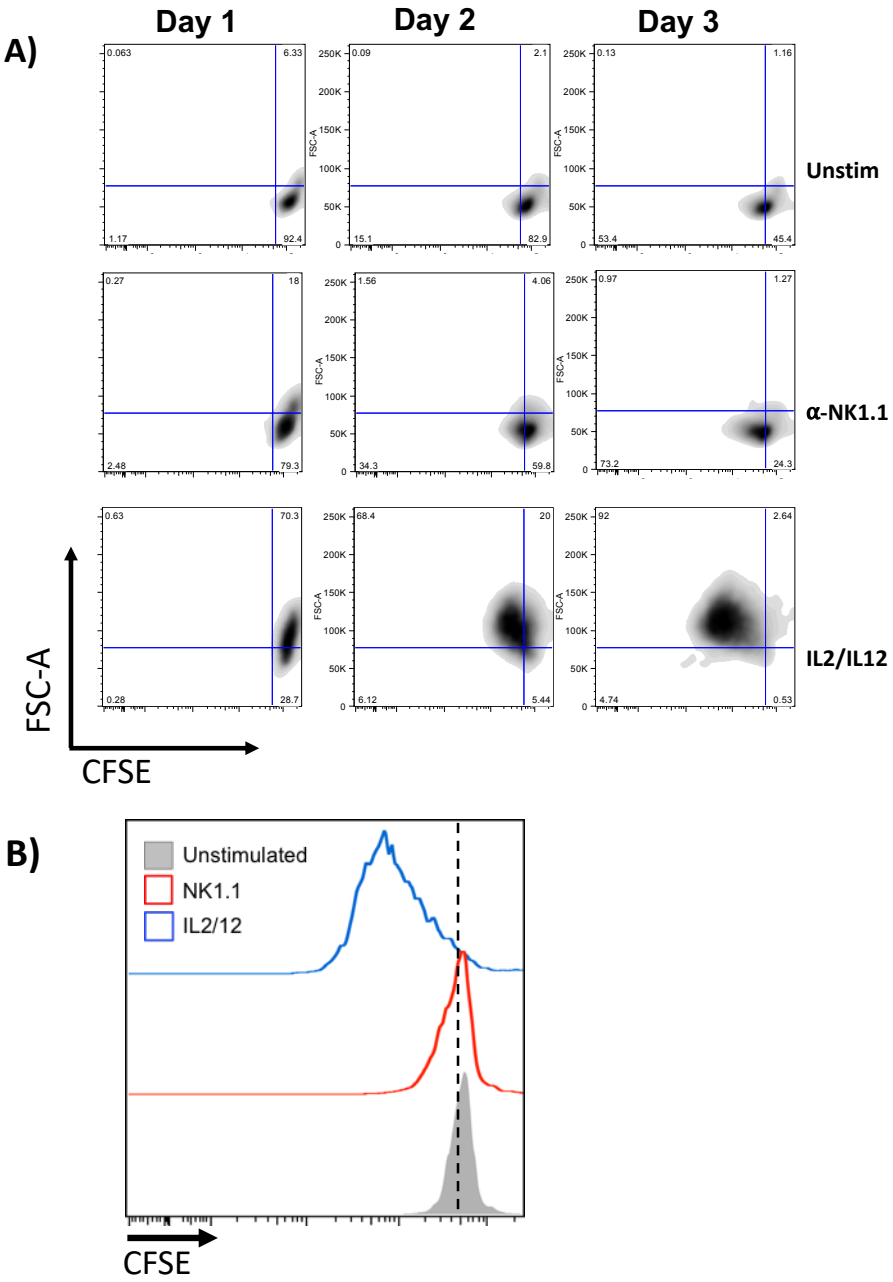


Figure 3.9 NK1.1 receptor stimulation does not induce proliferation in NK cells

(A-B)Cultured NK cells (3 days in low dose IL15) were stimulated for 24, 48 or 72 hours with α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), IL2 (20ng/ml) plus IL12 (10ng/ml) or left unstimulated (low dose IL15 at 5ng/ml only). Growth and proliferation were measured by flow cytometry using forward scatter (FSC-A) and CFSE dilution respectively. Data is representative of three individual experiments showing growth (A) and proliferation (A,B) over 3 days or at 72 hours (B).

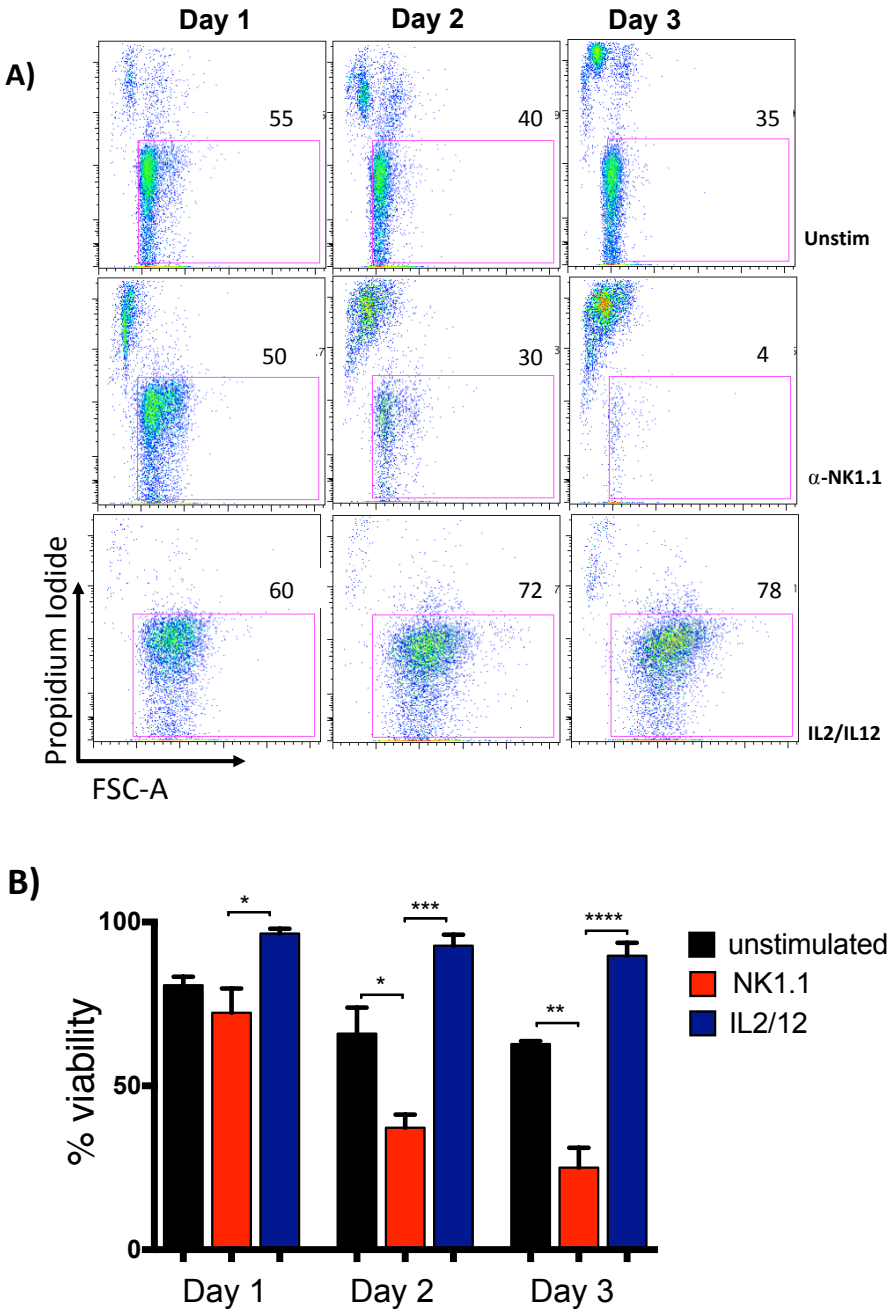


Figure 3.10 NK1.1 receptor stimulation does not support survival but causes active cell death

(A-B)Cultured NK cells (3 days in low dose IL15) were stimulated for 24, 48 or 72 hours with α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), IL2 (20ng/ml) plus IL12 (10ng/ml) or left unstimulated (low dose IL15 at 5ng/ml only). Per cent viability was measured by flow cytometry using propidium iodide (PI) staining. Data is representative (A) or mean +/- SEM (B) of 3 independent experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post-test. (*p<0.05, **p< 0.01, ***p<0.001, ****p< 0.0001).

3.5 NK1.1 receptor stimulated NK cells are more responsive to IL2 cytokine

Previous work in our lab has shown that IL12 cytokine stimulated NK cells were made more responsive to IL2 due to the induction of CD25 expression. CD25 is the high affinity IL2 receptor also known as IL2Rα. Interestingly, we found that NK1.1 receptor stimulated NK cells expressed high levels of CD25 **(Fig3.11)** much higher in fact, than previously observed in response to IL2/IL12 (Donnelly et al., 2014). Therefore, we hypothesized that NK1.1 receptor stimulation would make NK cells more responsive to IL2 cytokine. To test this hypothesis, cultured NK cells were purified by magnetic bead cell sorting and stimulated either with IL2 cytokine, α-NK1.1 antibody, α-NK1.1 antibody plus IL2 cytokine, (hereafter NK1.1+IL2) or left unstimulated in low dose IL15 for 18 hours and IL2 signalling was assessed by measuring mTORC1 activation by western blot analysis *(Refer to section 2.2.6).* mTORC1 is well established to be stimulated downstream of the IL2 receptor and mTORC1 activity can be assessed by analysing the phosphorylation of the mTORC1 substrate S6-kinase and the downstream S6K substrate S6 ribosomal protein. While NK cells stimulated with IL2 showed some activation of mTORC1, there was a much greater increase in mTORC1 signalling in response to NK1.1+IL2. In contrast, NK1.1 receptor stimulation alone did not stimulate mTORC1 signalling **(Fig3.12 A,B).** As a confirmation, phosphorylation of S6 was analysed by flow cytometry in these cells and indeed, there is an increase in the phosphorylation of S6 in NK1.1+IL2 stimulated NK cells compared to NK cells stimulated with either α-NK1.1 or IL2 alone **(Fig 3.12 C,D).** These data show that NK1.1 stimulation primes NK cells for IL2 induced signalling.

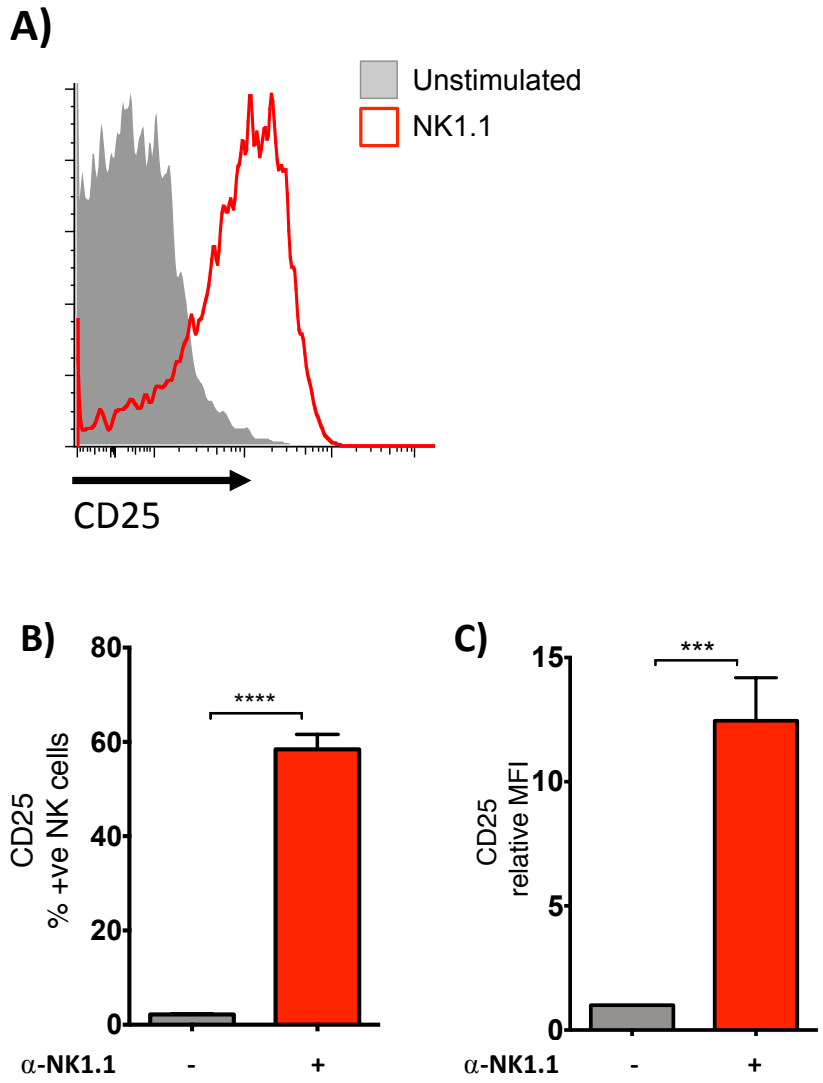


Figure 3.11 NK1.1 receptor stimulation induces high expression of CD25

(A-C) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), or left unstimulated (low dose IL15 at 5ng/ml only). CD25 expression was measured by flow cytometry. Data is representative (A) or mean +/- SEM (B,C) of 3 independent experiments. Data was analysed using a paired student's t test. (***p< 0.001, ****p< 0.0001).

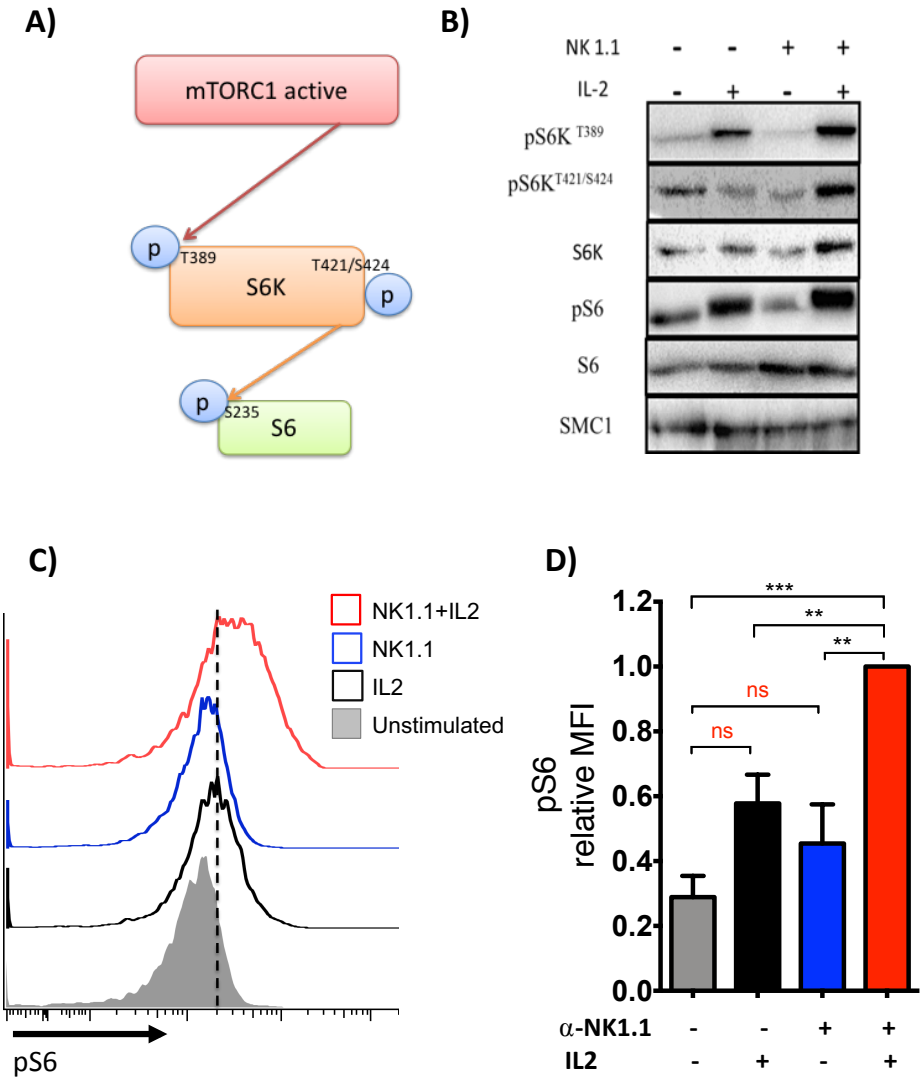


Figure 3.12 NK1.1 receptor stimulation makes NK cells more responsive to IL2 signalling

(A-D) Cultured NK cells (6 days in low dose IL15) were MACS purified and stimulated for 18 hours with IL2 cytokine (20ng/ml), α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), α-NK1.1 antibody plus IL2 or left unstimulated (low dose IL15 at 5ng/ml only). mTORC1 targets were assessed by western blot analysis. (A) Schematic representation of phosphorylation sites of downstream mTORC1 targets. Data is representative (B,C) or mean +/-SEM of three individual experiments. Data was analysed using a one-sample students t test against a theoretical value of 1. Unstimulated, αNK1.1 and IL2 conditions were compared using a one-way ANOVA followed by Tukey's multiple comparison post-test. (**$p < 0.01$; ***$p<0.001$) or (ns, non-significant).

The next step was to determine the consequences of this enhanced IL2 signalling in NK1.1 receptor stimulated cells for NK cell metabolism and function. NK1.1 receptor ligation induced blastogenesis in NK cells, but the combination of NK1.1+IL2 induced a bigger increase in cell size **(Fig 3.13).** Consistent with cellular growth, there was an increase in the metabolic demands of NK1.1+IL2 stimulated NK cells. The combination of NK1.1+IL2 showed a significant increase in CD71 expression compared to NK cells stimulated with α-NK1.1 antibody or IL2 cytokine alone **(Fig3.14).** Similarly, there was an enhanced expression of the L-amino acid transporter subunit CD98 in NK1.1+IL2 stimulated NK cells **(Fig3.15).**

However, not all metabolic readouts were enhanced by NK1.1+IL2 stimulation. NK cells up-regulated the uptake of the glucose analogue 2-NBDG to the same degree in cells that were stimulated with α-NK1.1+IL2 or α-NK1.1 alone **(Fig3.16 A,B).** Similarly, NK1.1+IL2 stimulated NK cells did not have a further significant increase in kynurenine uptake compared to cells treated with IL2 or α-NK1.1 alone **(Fig 3.16 C,D)**.

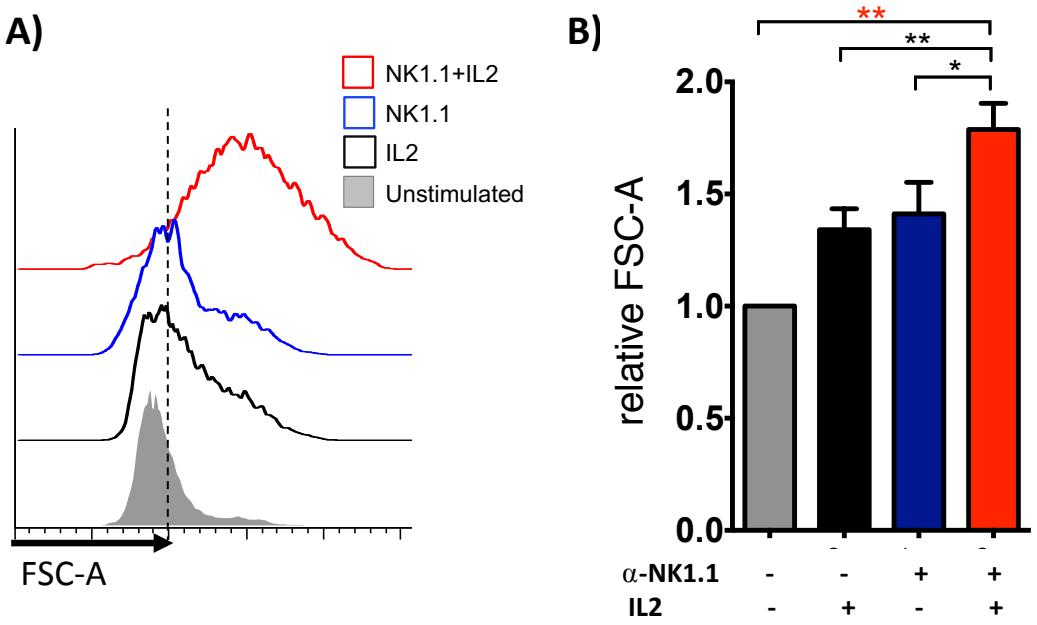


Figure 3.13 NK cells stimulated with NK1.1+IL2 undergo blastogenesis

(A-B) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with IL2 cytokine (20ng/ml), α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), α-NK1.1 plus IL2 (20ng/ml) or left unstimulated (low dose IL15 at 5ng/ml only). Cell size was analysed by flow cytometry using forward scatter-area (FSC-A). Pooled data is expressed relative to unstimulated. Data is representative (A) or mean +/- SEM (B) of four independent experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post-test; Unstim and NK1.1+IL2 were compared using one-sample student's t-test against a theoretical value of 1. (*$p < 0.05$; **$p<0.01$)

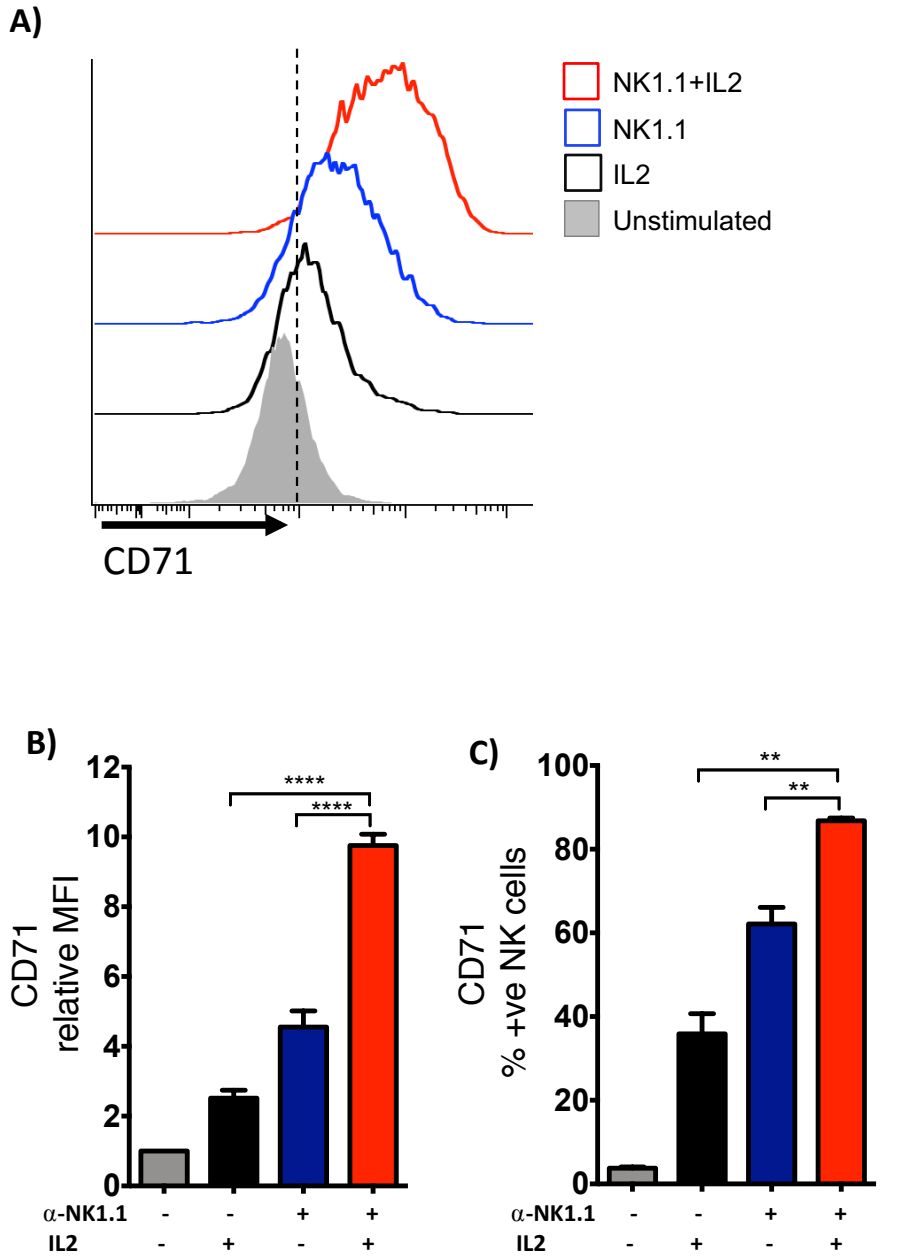


Figure 3.14 NK cells stimulated with NK1.1+IL2 increase the expression of the transferrin receptor, CD71

(A-C) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with IL2 cytokine (20ng/ml), α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), α-NK1.1 plus IL2 (20ng/ml) or left unstimulated (low dose IL15 at 5ng/ml only). CD71 expression was analysed by flow cytometry. Pooled data is expressed relative to unstimulated (B). Data is representative (A) or mean +/- SEM (B,C) of four independent experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post-test (**p < 0.01; ****p<0.0001)

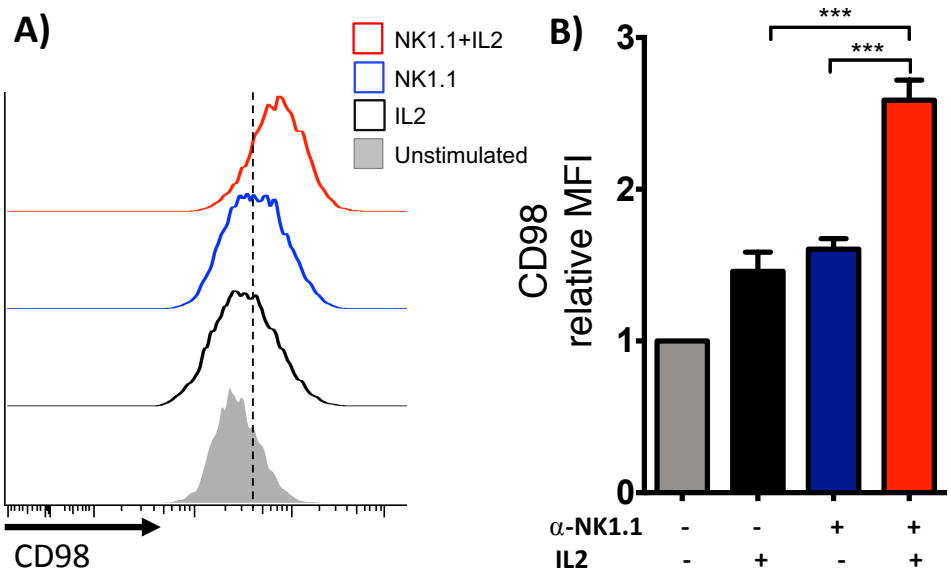


Figure 3.15 NK cells stimulated with NK1.1+IL2 increase the expression of the nutrient transporter subunit, CD98

(A-B) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with IL2 cytokine (20ng/ml), α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), α-NK1.1 plus IL2 (20ng/ml) or left unstimulated (low dose IL15 at 5ng/ml only). CD98 expression was analysed by flow cytometry. Pooled data is expressed relative to unstimulated (B). Data is representative (A) or mean +/- SEM (B) of four independent experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post-test. (***p<0.001).

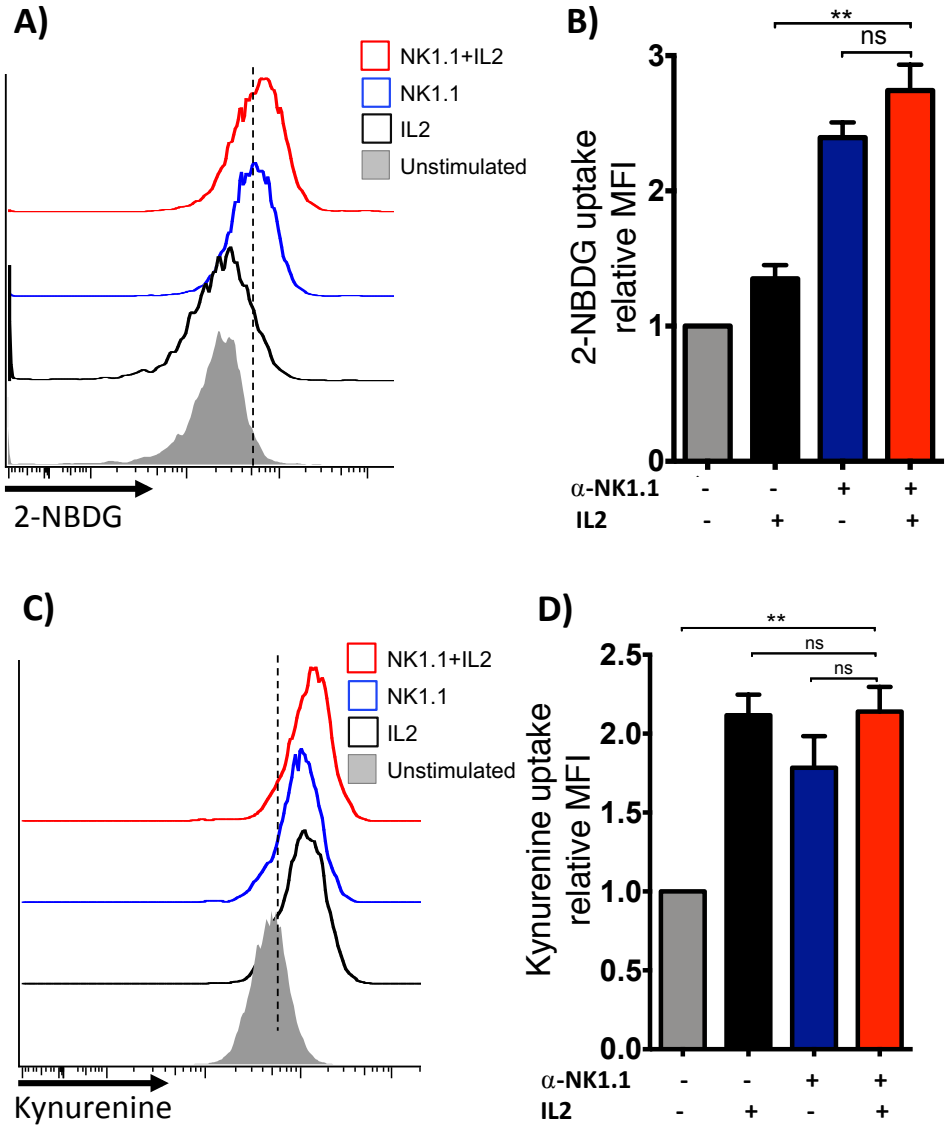


Figure 3.16 NK cells stimulation with NK1.1+IL2 induces increased nutrient uptake.

(A-D) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with IL2 cytokine (20ng/ml), α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), α-NK1.1 plus IL2 (20ng/ml) or left unstimulated (low dose IL15 at 5ng/ml only). 2-NBDG was added to the cells for the final 30 minutes (A,B); kynurenine was added for the final 5 minutes(C,D) and their uptake was measured by flow cytometry. Pooled data is expressed relative to unstimulated (B,D). Data is representative (A,C) or mean +/- SEM (B,D) of four to 6 independent experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post-test. Unstim and NK1.1+IL2 were compared using one sample t-test against a theoretical value of 1. (**$p < 0.01$; ns, non-significant)

Taken together these data indicated that NK1.1 ligation affords NK cells increased sensitivity to the adaptive cytokine IL2.

3.6 IL2 facilitates growth, proliferation and survival in NK1.1 receptor stimulated NK cells

As already seen in Figure 3.9, NK1.1 receptor stimulation did not induce robust proliferation or blastogenesis. IL2 cytokine stimulates multiple signalling pathways associated with cellular growth and proliferation. Therefore, we considered whether NK1.1 receptor stimulation primed NK cells for IL2 induced growth and proliferative responses. For this, cultured NK cells were stimulated with IL2 cytokine, α-NK1.1, α-NK1.1 plus IL2 or left unstimulated for 24, 48 and 72 hours. As explained in section 3.4, these NK cells were studied over the course of 3 days for cell growth, proliferation and viability. While IL2 alone did induce a small increase in NK cell size and some NK cell proliferation, NK1.1+IL2 stimulated cells were bigger and proliferated more over the course of three days **(Fig 3.17).** Figure 3.10 showed that NK1.1 receptor stimulation actively induced NK cell death between 24 and 48 hours post stimulation. IL2 cytokine provided a survival signal that prevented this decrease in NK cell viability **(Fig 3.18).** This data shows that increased expression of CD25 and the resultant IL2 sensitivity enables longevity in receptor ligated NK cells, which are otherwise short acting.

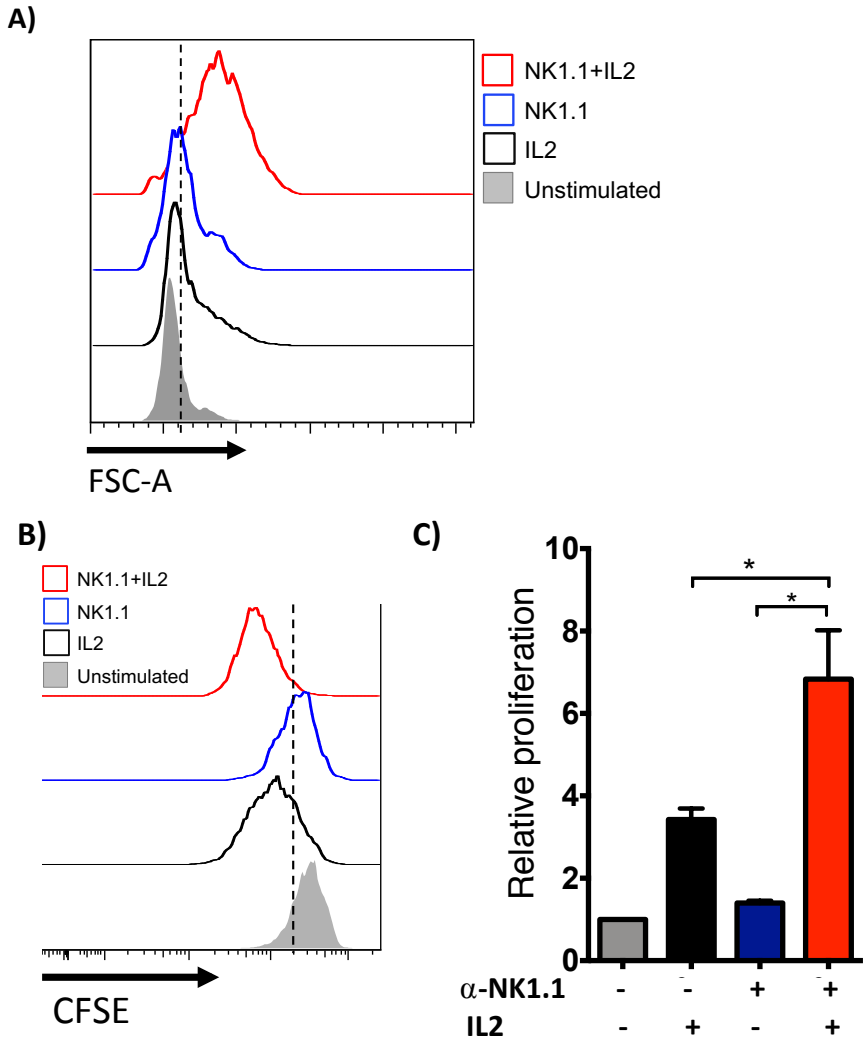


Figure 3.17 NK cells stimulation with NK1.1+IL2 induces proliferation and blastogenesis.

(A-C) Cultured NK cells (3 days in low dose IL15) were stimulated for 24, 48 or 72 hours with IL2 cytokine (20ng/ml), α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), α-NK1.1 plus IL2 (20ng/ml) or left unstimulated (low dose IL15 at 5ng/ml only). Growth and proliferation were assessed by flow cytometry using forward scatter (FSC-A) (A) or CFSE dilution respectively (A). Pooled data is expressed relative to unstimulated as fold increase in proliferation(C). Data is representative (A,B) or mean +/- SEM (C) of three independent experiments. Data was analysed using one-way ANOVA with a Tukey multiple comparison post-test. (*$p < 0.05$).

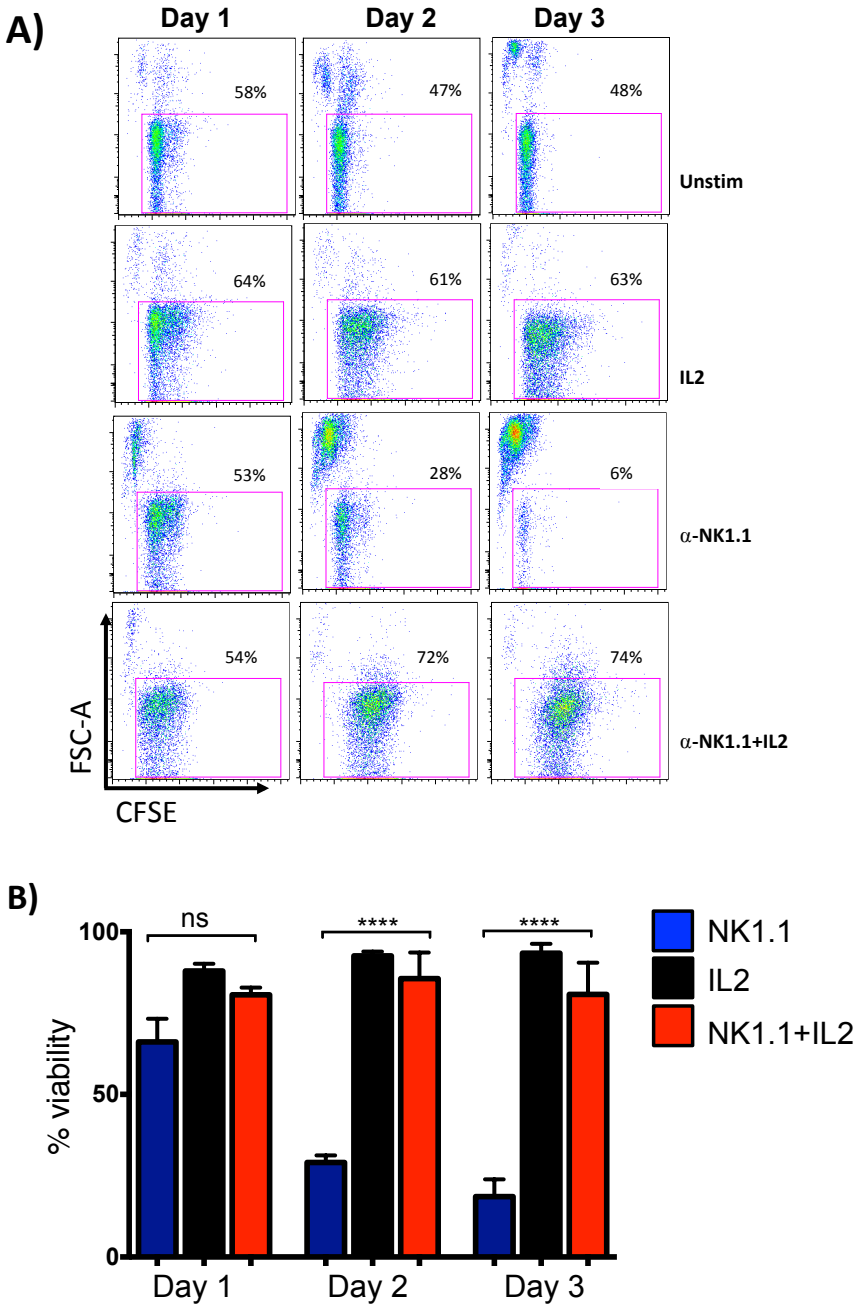


Figure 3.18 Increased IL2 sensitivity promotes cell survival in NK1.1+IL2 stimulated NK cells.

(A-B) Cultured NK cells (3 days in low dose IL15) were stimulated for 24, 48 or 72 hours with IL2 cytokine (20ng/ml), α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), α-NK1.1 plus IL2 (20ng/ml) or left unstimulated (low dose IL15 at 5ng/ml only). Per cent cell viability was assessed by flow cytometry using propidium iodide (PI) staining. Data is representative (A) or mean +/- SEM (B) of three independent experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post-test (****$p < 0.0001$; ns, non-significant).

3.7 IL2 promotes robust metabolic and functional responses in NK1.1 receptor stimulated NK cells

Elevated nutrient transporters and proliferation and blastogenesis indicate that NK1.1+IL2 stimulated NK cells are much more metabolically active than NK cells stimulated with α-NK1.1 or IL2 cytokine alone. To confirm this, NK cells were analysed using the seahorse extracellular flux analyser as explained in section 3.2. Cultured and purified NK cells were stimulated with IL2 cytokine, α-NK1.1, α-NK1.1 plus IL2 or left unstimulated for 18 hours. It was observed that IL2 cytokine and NK1.1 receptor ligation alone induced increased rates of glycolysis in NK cells but stimulation of NK cells with NK1.1+IL2 resulted in an additional increase in glycolysis. NK1.1+IL2 stimulated NK cells also showed the highest rates of glycolytic capacity **(Fig 3.19)**. Similarly, IL2 and NK1.1 stimulations alone induced a small increase in the rate of OxPhos but there was a further increase in OCR values of NK cells stimulated with NK1.1+IL2. Again, the maximal respiration in NK1.1+IL2 stimulated NK cells was also much higher compared to that seen in NK cells stimulated with either IL2 cytokine or α-NK1.1 alone **(Fig 3.20)**. Together these data confirm that NK1.1+IL2 stimulated NK cells are indeed metabolically more active than NK cells stimulated with either α-NK1.1 or IL2 alone.

A comparison of ECAR and OCR values reveals that there is an overall shift towards glycolysis in NK1.1 and NK1.1+IL2 stimulated NK cells **(Fig 3.21A).** NK1.1+IL2 stimulated cells appear to be highly energetic when the ECAR and OCR values of these NK cells are plotted on an energy map **(Fig 3.21B).** This is consistent with what is observed in IL2/IL12 cytokine stimulated NK cells **(Fig 3.21 C,D).** Increased aerobic glycolysis might indicate towards increased flux through glycolysis wherein pyruvate is predominantly converted into lactate rather than entering the TCA cycle. An increase in the glycolytic capacity in response to NK1.1+IL2 stimulation suggests that these cells have undergone

metabolic reprogramming that entails an increased expression of glycolysis associated genes. To confirm this, MACS purified NK cells were stimulated with α-NK1.1 antibody, IL2 cytokine, α-NK1.1 plus IL2 or left unstimulated for 18 hours. These NK cells were then lysed to extract RNA, and meausred by real time PCR *(Refer to section 2.2.5)*. Indeed NK1.1+IL2 stimulated NK cells expressed high levels of mRNA for glucose transporters with highly significant increases in *Glut1* and for the enzymes of the glycolytic pathway including *Hk2* and *Gapdh* compared to IL2 or NK1.1 stimulation alone **(Fig 3.22)**.

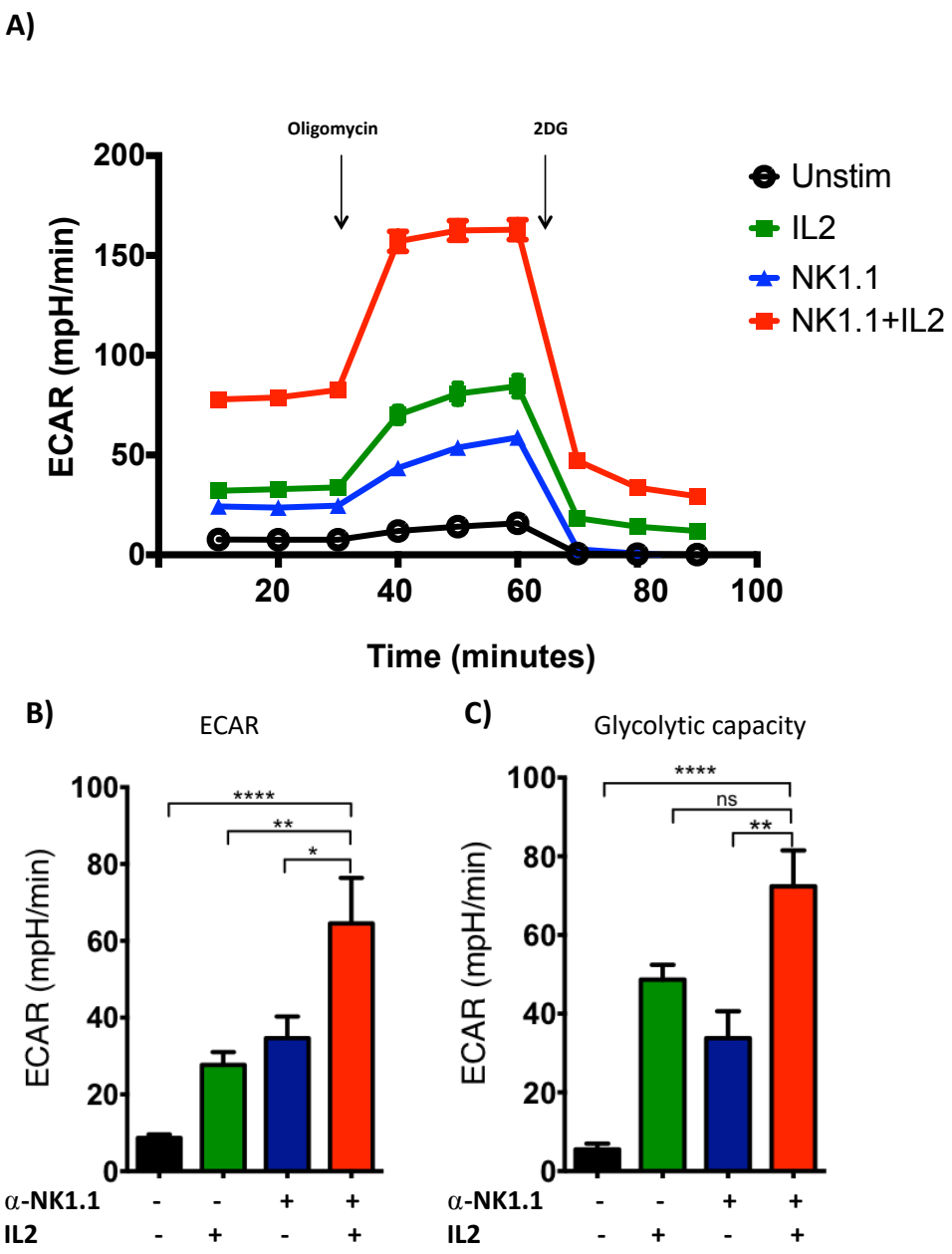


Figure 3.19 NK1.1+IL2 stimulation of NK cells results in increased glycolysis in NK cells

(A-C) Cultured NK cells (6 days in low dose IL15) were MACS-purified and stimulated for 18 hours with IL2 cytokine (20ng/ml), α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), α-NK1.1 plus IL2 (20ng/ml) or left unstimulated (low dose IL15 at 5ng/ml). (A) Extracellular acidification rates (ECAR) were assessed using Seahorse extracellular flux analyser with the sequential addition of inhibitors oligomycin and 2 deoxyglucose (2DG). The rates of glycolysis (B) and glycolytic capacity (C) were calculated. Data is representative (A) or mean +/- SEM of four independent experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post- test. (*p<0.05, **p<0.01, ****p<0.0001; ns, non-significant)

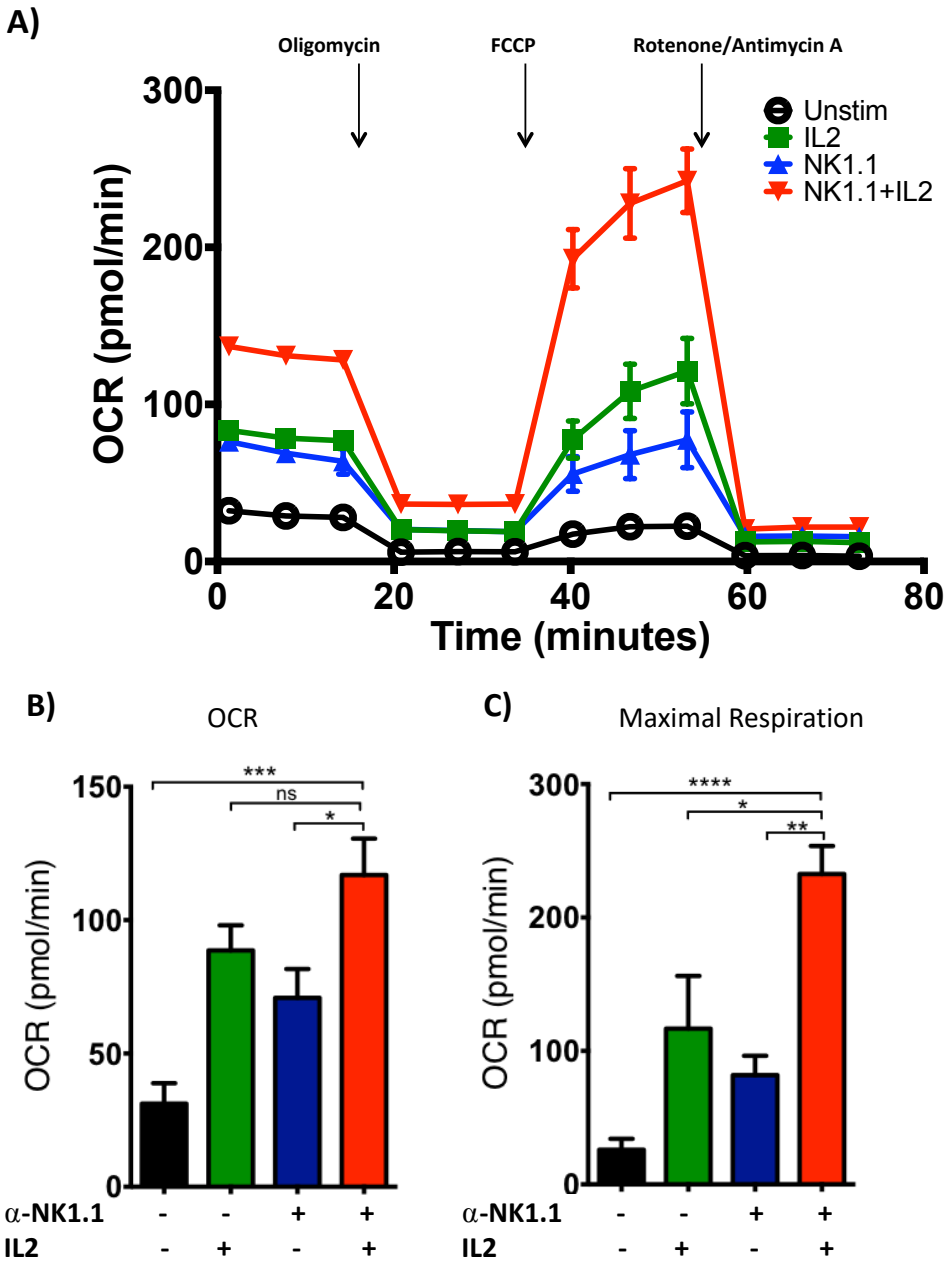


Figure 3.20 NK1.1+IL2 stimulation of NK cells results in increased OxPhos in NK cells

(A-C) Cultured NK cells (6 days in low dose IL15) were MACS-purified and stimulated for 18 hours with IL2 cytokine (20ng/ml), α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), α-NK1.1 plus IL2 (20ng/ml) or left unstimulated (low dose IL15 at 5ng/ml). (A) Oxygen consumption rates (OCR) were assessed using Seahorse extracellular flux analyser with the sequential addition of inhibitors oligomycin, FCCP and antimycin A plus rotenone. The rates of OxPhos (B) and maximal respiration (C) were calculated. Data is representative (A) or mean +/- SEM of four independent experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post- test. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; ns, non-significant)

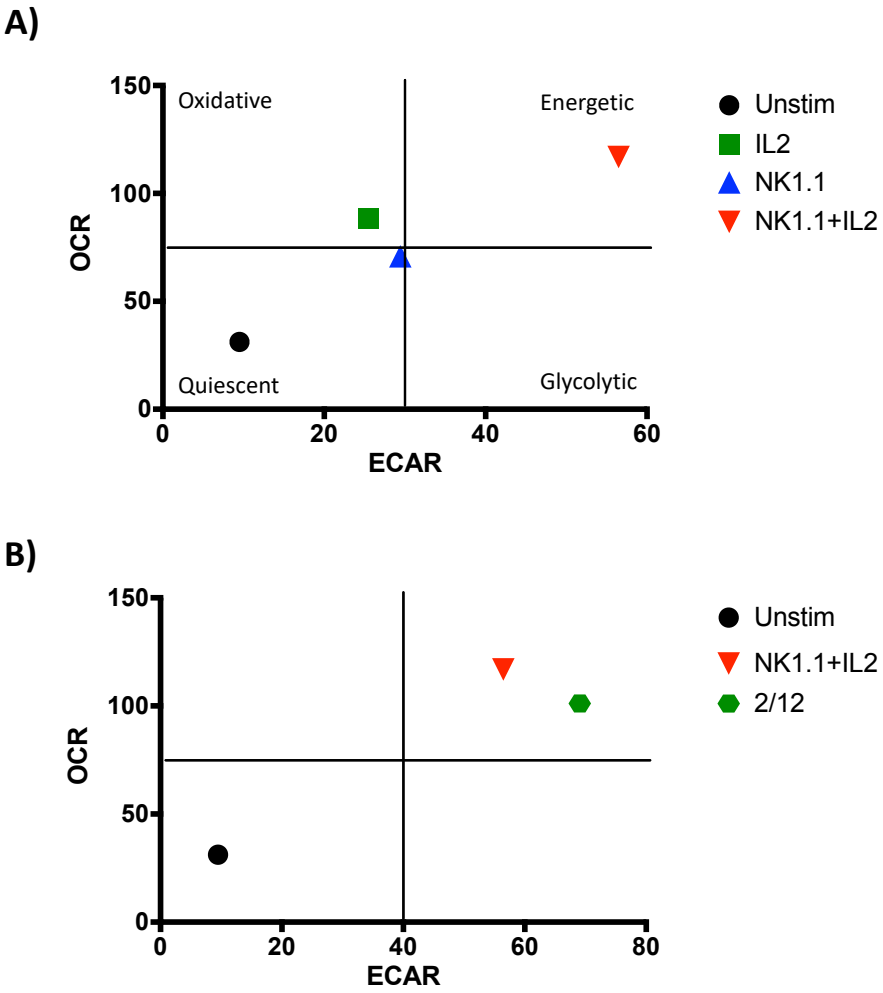


Figure 3.21 NK1.1+IL2 stimulated NK cells are highly energetic.

(A-B)Cultured NK cells (6 days in low dose IL15) were MACS-purified and stimulated for 18 hours with IL2 cytokine (20ng/ml), α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), α-NK1.1 plus IL2 (20ng/ml) or left unstimulated (low dose IL15 at 5ng/ml). (A) Extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) were assessed using Seahorse extracellular flux analyser. The rates of glycolysis were compared with the rates of OCR and plotted on an energy map (A,B) Data is representative of 4 individual experiments.

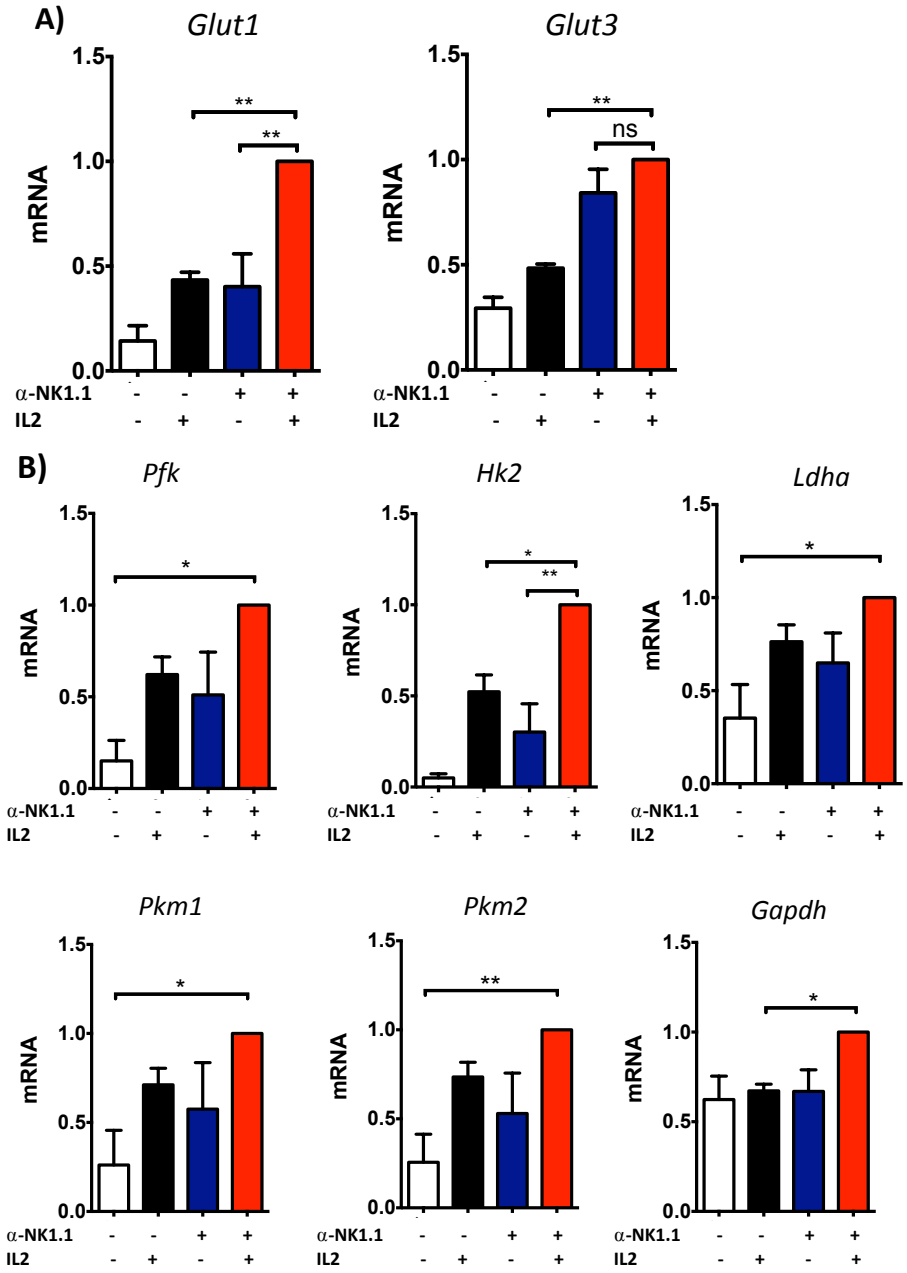


Figure 3.22 NK1.1+IL2 stimulated NK cells have increased glycolytic machinery.

Cultured NK cells (6 days in low dose IL15) were MACS-purified and stimulated for 18 hours with IL2 cytokine (20ng/ml), α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), α-NK1.1 plus IL2 (20ng/ml) or left unstimulated (low dose IL15 at 5ng/ml). Cells were lysed for RNA and mRNA transcripts were quantified by qPCR. Genes for glucose transporters (A) and enzymes of the glycolytic pathway (B) were assessed. Data is expressed relative to NK1.1+IL2. Data is mean +/- SEM of three individual experiments. Data was analysed using student's t test. (*p<0.05, **p<0.01 ns,non-significant).

Enhanced rates of cellular metabolism upon addition of IL2 cytokine to NK1.1 receptor stimulated NK cells led us to ask whether receptor stimulated NK cells were made more responsive to IL2 induced functional responses. Indeed, NK1.1 receptor stimulated NK cells significantly increased Granzyme B expression upon addition of IL2 **(Fig 3.23)**. Likewise, the expression of the pro-inflammatory cytokine IFN-γ was greatly elevated in NK1.1+IL2 stimulated NK cells **(Fig 3.24)**.

In summary, these data show that NK1.1 receptor stimulation of NK cells resulted in modest increases in NK cell metabolism and effector functions. However, while these receptor-stimulated NK cells are short lived, they are rendered more responsive to the adaptive cytokine IL2 through the induction of CD25 expression, the high affinity IL2 receptor subunit. Increased sensitivity to IL2 affords receptor-stimulated NK cells increased longevity, enhanced metabolic activity and effector responses. This implies that crosstalk between cells of the innate and the adaptive immune system is important for robust and prolonged NK cell responses.

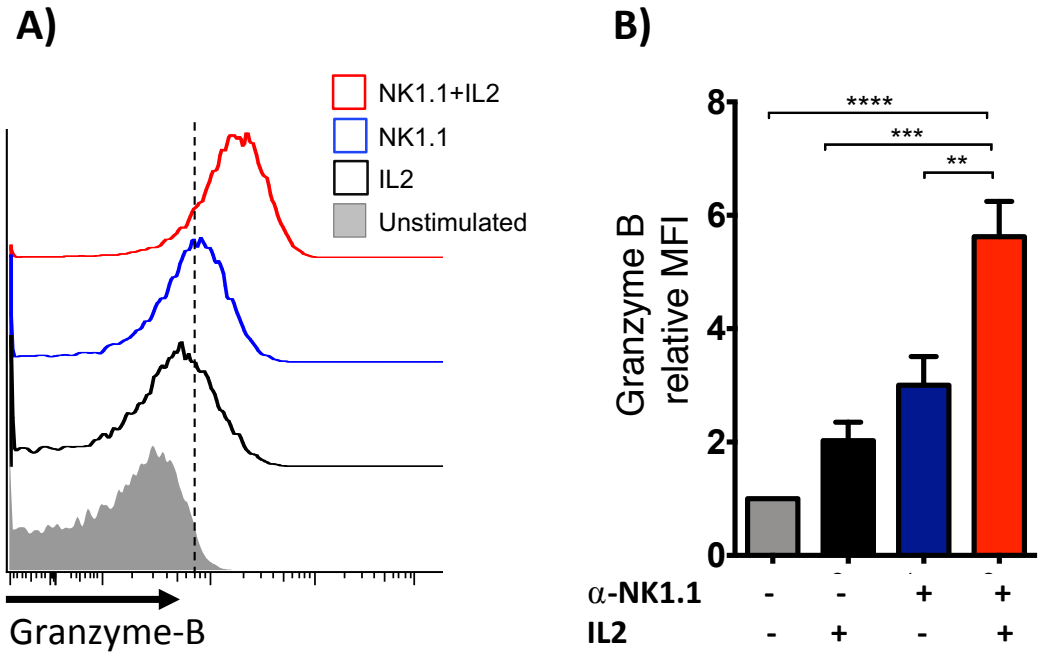


Figure 3.23 NK1.1+IL2 stimulated NK cells have increased Granzyme B expression.

(A-B) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with IL2 cytokine (20ng/ml), α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), α-NK1.1 plus IL2 (20ng/ml) or left unstimulated (low dose IL15 at 5ng/ml). Granzyme B production in NK cells was assessed by flow cytometry. Data is expressed as relative to unstimulated (B). Data is representative (A) or mean +/- SEM (B) of four independent experiments. Data was analysed using one-way ANOVA followed by a Tukey multiple comparison post test. (**p<0.01, ***p<0.001, ****p<0.0001)

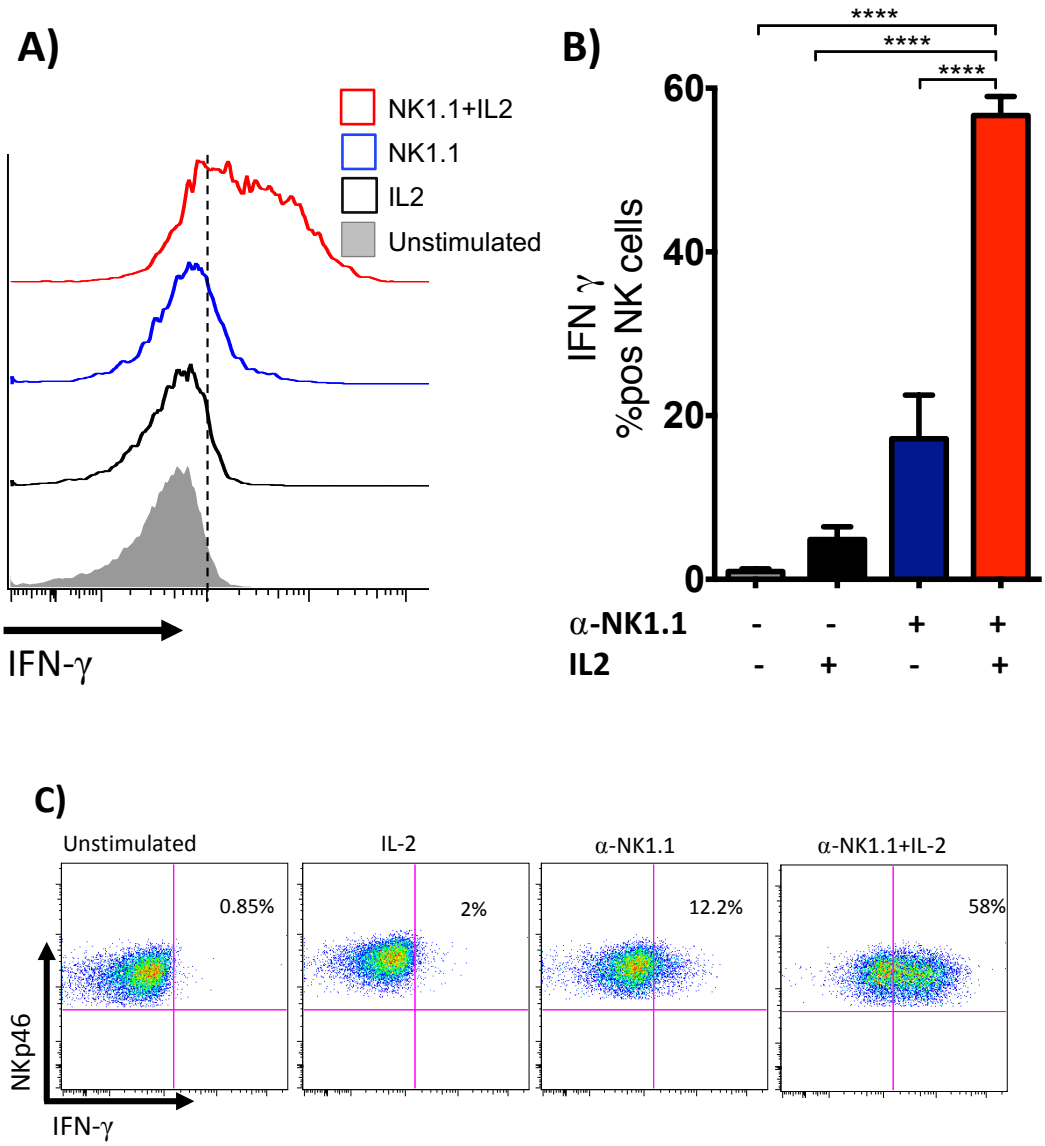


Figure 3.24 NK1.1+IL2 stimulated NK cells have increased IFN- γ expression

(A-C)Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with IL2 cytokine (20ng/ml), α-NK1.1 antibody (10μg/ml) supplemented with low dose IL15 (5ng/ml), α-NK1.1 plus IL2 (20ng/ml) or left unstimulated (low dose IL15 at 5ng/ml). IFN-γ production in NK cells was assessed by flow cytometry. Data is representative (A,C) or mean +/- SEM (B) of five independent experiments. Data was analysed using one-way ANOVA followed by a Tukey multiple comparison post-test. (****p<0.0001).

Chapter 3 Discussion

NK cells mainly function as innate immune cells and play a critical role in defence against viral infections and cancer. Unlike adaptive immune cells, that need antigen presentation to be activated, NK cells don't need prior immunization and are always ready to kill. This makes them vital for immunosurveillance. In an event of spontaneous transformation of cells, NK cells can recognize abnormal expression of self-ligands with the help of a variety of cell surface receptors they express. One NK cell can kill multiple targets but eventually undergoes anergy or apoptosis (Bhat and Watzl, 2007; Jewett and Bonavida, 1995; Jewett and Bonavida, 1996). However, NK cells are emerging to have a long-term role as well and have been shown to act alongside the adaptive immune response (Cooper et al., 2009a; Vivier et al., 2011). While cytokine induced activation has been shown to prime NK cells for a prolonged adaptive response(Donnelly et al., 2014; Lee et al., 2012; Leong et al., 2014), little is known about the role of receptor mediated NK cell activation in bridging innate and adaptive immunity.

This study shows that receptor mediated activation of NK cells through NK1.1 ligation induces metabolic pathways to support prolonged innate responses. Keppel et al showed that short-term (4-6 hours) activation of NK cells through receptor (NK1.1) ligation or cytokine stimulation did not result in significant changes in the NK cell metabolism (Keppel et al., 2015). However, when NK cells are activated for a longer duration as might be expected to happen in vivo, they need to undergo metabolic reprogramming so as to sustain prolonged activity. Indeed, it has been shown that long-term activation of NK cells by cytokines induces metabolic reprogramming in NK cells (Donnelly et al., 2014; Marçais et al., 2014a). This study was carried out on IL15 primed NK cells that were stimulated with NK1.1 for 18 hours. We demonstrate for the first time that

NK cells stimulated by receptor ligation undergo metabolic changes. A key feature of increased metabolism is increased nutrient uptake as demonstrated in T cells (Cham and Gajewski, 2005; Macintyre et al., 2014; Sinclair et al., 2018; Sinclair et al., 2013; Wang et al., 2011). Indeed, receptor ligation of NK cells show that these cells increase in size and significantly up-regulate the expression of key nutrient receptors like CD71, the transferrin receptor and CD98, which is a receptor subunit of the large neutral amino acid transporter Slc7A5. This data is supported by a recent finding reporting that CD98 expression along with its other sub-unit Slc1a5 is essential for NKG2D mediated responses in human NK cells (Jensen et al., 2017). CD71 is a target of the cMyc gene which has been shown to be essential for cytokine induced metabolic and functional responses in NK cells (Loftus et al., 2018). An increase in CD98 also indicated a role for cMyc as well as pointing towards increased uptake of nutrients. A recent paper measured the uptake of kynurenine as an indicator of increased Slc7A5 expression and increased uptake of large neutral amino acids (Sinclair et al., 2018). Indeed, NK1.1 ligated NK cells had also increased their uptake of kynurenine. Detailed metabolic analyses revealed that 18-hour receptor mediated stimulation of NK cells resulted in significant increases in the key metabolic pathways of glycolysis and OxPhos.

One study suggested that the cytotoxic effector molecules of NK cells are post-transcriptionally regulated (Fehniger et al., 2007) and that there is no increase in the mRNA levels of Granzyme B and perforin after short term activation. This study is consistent with the findings of Keppel et al, that NK cells do not require to undergo metabolic reprogramming following short term activation, as they already have the machinery to undergo cytotoxicity (Keppel et al., 2015). However, we propose that NK cells require undergoing metabolic re-programming in case of a long-term activation to facilitate sustained production of innate effector molecules.

It was noteworthy that the metabolic changes induced by receptor ligation were not as robust as seen with cytokine stimulation of NK cells. However, strikingly NK1.1 mediated activation of NK cells results in robust CD25 expression. CD25 is a subunit of the high affinity IL2 receptor (Malek, 2008). IL2 is mainly an adaptive cytokine and is secreted by activated T cells. This suggests that NK cells have mechanisms that would allow them to function alongside the adaptive immune response. CD25 is not expressed on most lymphocytes but can be up-regulated on being activated by certain cytokines (Létourneau et al., 2009). Activation of NK cells by multiple cytokines has been shown to induce CD25 expression on NK cells (Lee et al., 2012; Leong et al., 2014). Indeed, previous work in our lab has shown that IL12 induced CD25 expression is crucial to facilitate IL2 mediated metabolic and functional responses (Donnelly et al., 2014). There is also some evidence of CD25 induction upon receptor ligation. Andre et al showed that NCR ligation and soluble NKG2D ligands induced CD25 expression on human NK cells (André et al., 2004). More recently, a bispecific antibody against CD30/CD16A designed for the treatment of Hodgkin's lymphoma was shown to induce CD25 (Pahl et al., 2018) and our finding that NK1.1 ligation induces CD25 expression is consistent with these studies. Indeed, NK cell activating receptors including NK1.1, signal through ITAM motifs (Arase et al., 1997) that activates multiple signalling pathways that would be predicted to promote CD25 expression. For instance, NK1.1 signalling has been shown to induce NFκB and IκB degradation in NK cells (Gross et al., 2008). Indeed, NFκB has been shown to control CD25 expression and maintenance in T cells (Fu et al., 2013). ITAM signalling also induces the tyrosine kinases Syk that activate PLCγ through the activation of ZAP70. PLCγ has been shown to activate the transcription factor NFAT through the activation of Ca^{2+}/calcenurin signalling in T cells. (Srikanth and Gwack, 2013). An NK1.1 homologue in rats, NKR-P1, has been shown to induce the accumulation of Calcium in Rat NK cells (Ryan et al., 1991), suggesting that NFAT is induced

in NK1.1 activated NK cells. Interestingly, CD25 promoter has been shown to be a target of NFAT (Schuh et al., 1998).

As already discussed, NK1.1 ligation resulted in an up-regulation in the rates of glycolysis. The mammalian target of rapamycin complex 1 (mTORC1) has been demonstrated to be required for glycolytic reprogramming in T cell subsets (Finlay et al., 2012; Shi et al., 2011) Previous work in our lab has also shown the involvement of mTORC1 in successful glycolytic reprogramming in IL2/12 activated NK cells (Donnelly et al., 2014). Therefore, we asked the question whether mTORC1 was required for NK1.1 induced glycolytic reprogramming as well. An analysis of mTORC1 targets in response to NK1.1 revealed that NK1.1 stimulation did not induce signalling through mTORC1. NK1.1 induced signalling is comparable to TCR signalling in that both NK1.1 and TCR signal through ITAM (Arase et al., 1997; Love and Hayes, 2010). ITAM activation initiates different signalling pathways including the MEK/ERK pathway, NKκB pathway and the Ca^{2+}/NFAT signalling in both T cells and NK cells (Gross et al., 2008; Livolsi et al., 2001). Indeed, our finding that NK1.1 induced little mTORC1 activation is consistent with the study that showed that TCR ligation alone resulted in only a modest signal transduction through mTORC1 (Zheng et al., 2007).

Owing to the fact that IL2 is an activator of mTORC1 (Marzec et al., 2008), and that NK1.1 induces CD25 expression, the high affinity IL2 receptor subunit, on NK cells, we stimulated NK cells with NK1.1 and IL2 together and studied their responses. We hypothesized that NK1.1 would facilitate increased IL2 mediated mTORC1 signalling in NK cells. Indeed, NK cells stimulated with NK1.1+IL2 induced a robust increase in phosphorylation of the mTORC1 targets S6 and S6K. As it has been shown that mTORC1 is a master regulator of cellular metabolism (Laplante and Sabatini, 2009), metabolic changes in response to NK1.1+IL2 activation of NK cells were assessed. Not only was there

a substantial increase in the expression of nutrient receptors, CD71 and CD98, there was also a robust increase in the rates of glycolysis and OxPhos in NK1.1+IL2 stimulated cells compared to NK cells that were stimulated by NK1.1 or IL2 alone. Despite a clear increase in metabolism, and expression of nutrient transporters, surprisingly, there wasn't an additional increase in nutrient uptake upon addition of IL2 to the NK1.1 stimulus as measured by 2-NBDG and kynurenine uptake. This might suggest that receptor ligation alone, although does not make NK cells highly metabolically active, it is certainly preparing NK cells for a long-term response. This is consistent with the increased sensitivity of these cells to an adaptive cytokine IL2. Alternatively, there is a possibility that other amino acid transporters as well as the system L-amino acid transporter might be involved in receptor ligation mediated NK cell activation. IL2 is also known to induce pro-inflammatory cytokines and cytotoxic effectors (Janas et al., 2005; Zhou et al., 2002) in CTLs. Indeed IL2 is an important pro-inflammatory cytokine (Cooper et al., 2001). Consistent with this, NK1.1+IL2 stimulation of NK cells also induced robust increase in the expression of Granzyme B and IFN-γ in NK cells. Indeed, IL2 was shown to improve serial killing in NK cells (Bhat and Watzl, 2007).

Apart from activation of mTORC1, IL2 also results in proliferation by inducing Bcl2 through the activation of STAT5 (Lord et al., 1998; Lord et al., 2000; Moriggl et al., 1999). Bcl2 is an anti-apoptotic protein that promotes cell survival (Tsujimoto, 1998). Indeed, NK1.1+IL2 stimulated cells underwent cell growth, proliferated and survived far better as compared to NK cells stimulated with NK1.1 alone. This result is consistent with a study that demonstrated NK cell proliferation in IL2 cultured NK cells stimulated with NK1.1 ligation (Reichlin and Yokoyama, 1998). Our results revealed that NK cells that were stimulated with NK1.1 and supplemented with a low dose of IL15, survived worse between 24-48 hours after activation than the unstimulated control whereby NK cells were also supplemented with low dose of IL15. This suggests that NK1.1 ligation actively kills NK cells. This is consistent with the study done

by (Asea and Stein-Streilein, 1998) that showed that NK1.1 ligation triggered apoptosis in NK cells. These data together suggest that IL2 cytokine converts innate NK cell responses into prolonged responses that work in parallel to the adaptive immune response.

Chapter 4

mTORC1, cMyc and Srebp are essential for αNK1.1+IL2 induced metabolic and functional responses

4.1 Glycolysis and OxPhos regulate NK1.1+IL2 induced NK cell effector functions

In Chapter 3, it was established that NK cells activated through NK1.1 receptor stimulation are highly sensitive to the adaptive cytokine IL2 and that these cells have elevated metabolism and function. The next step is to determine whether this increased metabolism is required for the corresponding effector responses. To study this, NK cells stimulated with α-NK1.1 plus IL2 cytokine were treated with sub-optimal doses of various inhibitors of the glycolytic pathway and OxPhos so as to inhibit their metabolism without putting them in an energy crisis. The effects of metabolic inhibition on their functional responses were then analysed. In order to inhibit the glycolytic pathway, 2-deoxyglucose and oxalate were used which block glycolysis at distinct steps. 2-DG inhibits hexokinase, the enzyme that catalyses the first step of glycolysis, whereas oxalate inhibits pyruvate kinase isozymes, that catalyse the last step of glycolysis. Limiting the flux through glycolysis with these inhibitors resulted in a decrease in IFN-γ production **(Fig 4.1, 4.2)** and a decrease in Granzyme B expression **(Fig 4.3).**

Low doses of oligomycin (2nM), an inhibitor of ATP synthase, were used to limit OxPhos (2μM oligomycin is normally used to completely inhibit ATP synthase). This low dose of oligomycin was sufficient to inhibit NK cell effector functions, reducing the production of IFN-γ and the expression of Granzyme B **(Fig 4.4, 4.5).**

These data suggest, that the increase in cellular metabolism in response to NK1.1 plus IL2 stimulation is required for maximal NK cell effector responses.

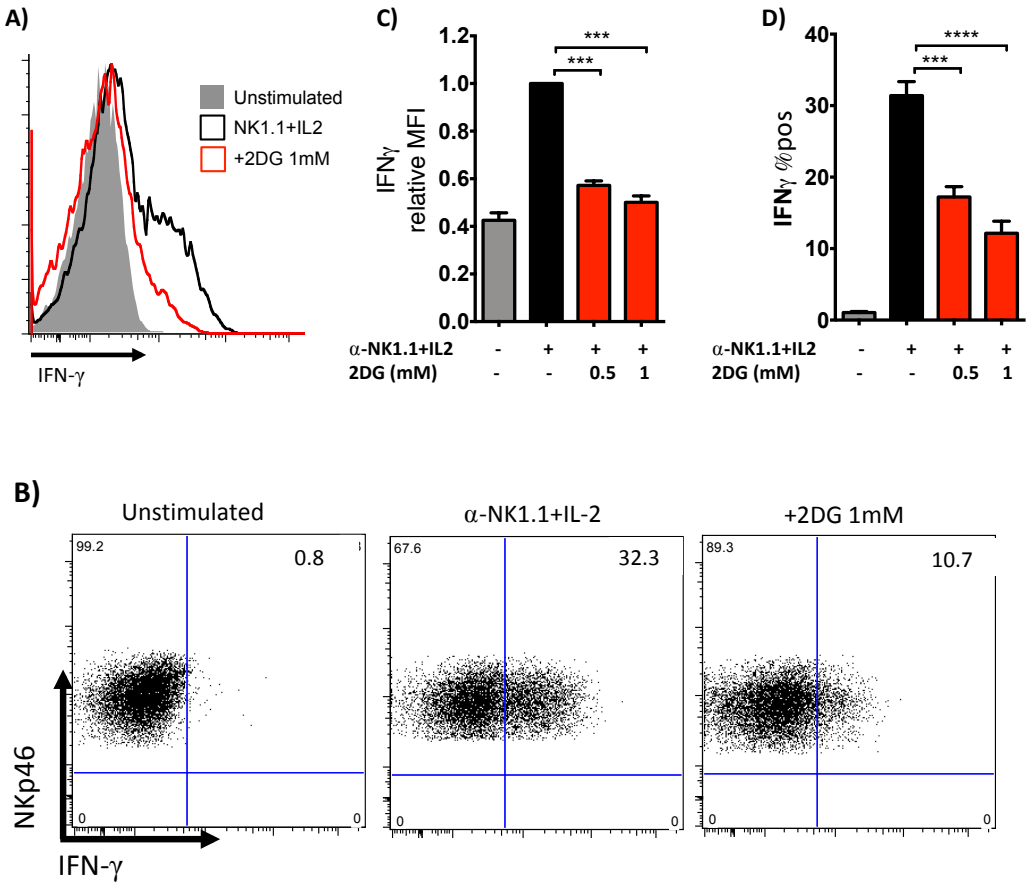


Figure 4.1 Inhibition of glycolysis using 2-deoxyglucose in NK1.1+IL2 stimulated NK cells decreases IFN-γ production

(A-D) Cultured NK cells (3 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 (20n) in the presence or absence of 2-deoxyglucose (0.5 or 1 mM) or left unstimulated (low dose IL15 at 5ng/ml only). IFN-γ was analysed by flow cytometry. Data is expressed as relative to NK1.1+IL2 (C) Data is representative (A, B) or mean +/- SEM (C,D) of 3 independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1 (C) or a one-way ANOVA with a Tukey multiple comparison test. (D). (***$p < 0.001$; ****$p<0.0001$)

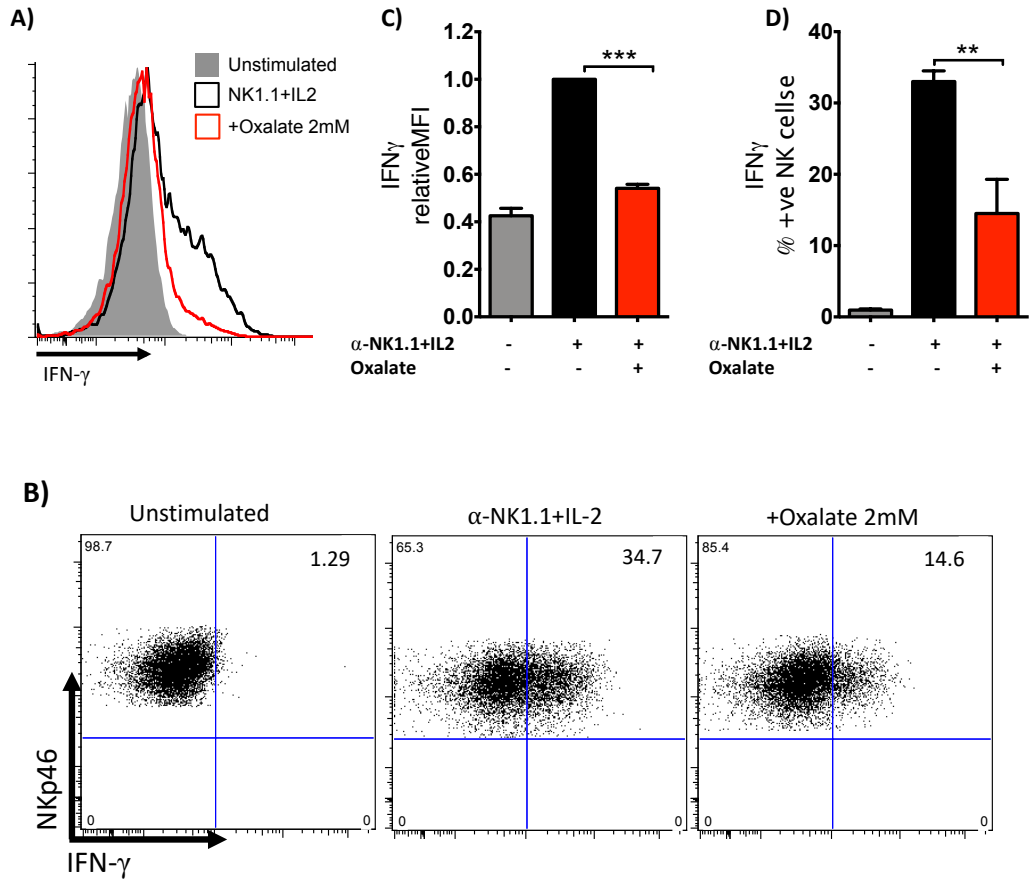


Figure 4.2 Inhibition of Glycolysis by oxalate in NK1.1+IL2 stimulated NK cells decreases IFN-γ expression

(A-D) Cultured NK cells (3 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10µg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of the glycolytic inhibitor Oxalate (2mM) or left unstimulated (in low dose IL15 at 7.5 ng/ml only). IFN-γ was analysed by flow cytometry. Data is expressed as relative to NK1.1+IL2 (C). Data is representative (A,B) or mean +/- SEM (C,D) of 3 independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1 (C), or one-way ANOVA with Tukey's multiple comparison post-test (D) (**p< 0.01, ***p<0.001).

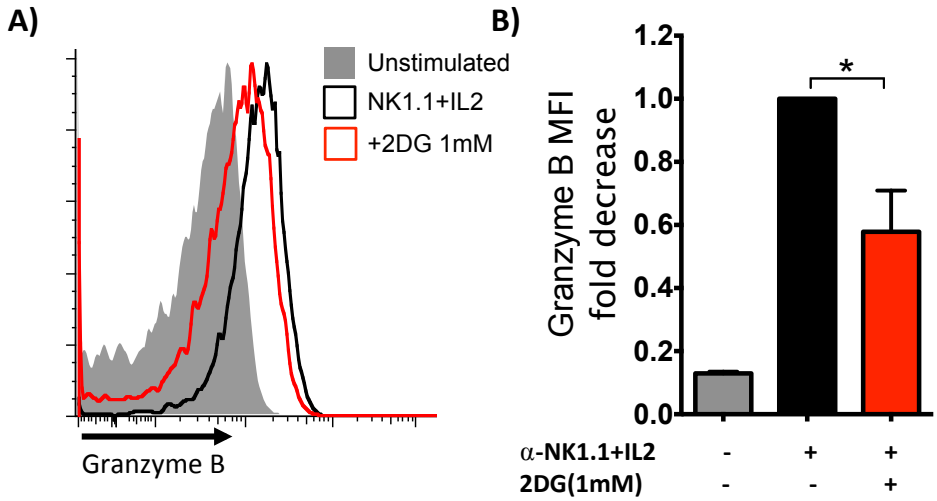


Figure 4.3 Inhibition of Glycolysis disrupts Granzyme B expression in NK1.1+IL2 stimulated NK cells

(A-B) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10µg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of the glycolytic inhibitors 2-deoxyglucose (2-DG 1mM) or left unstimulated (in low dose IL15 at 7.5 ng/ml only). Granzyme B was analysed by flow cytometry. Data is expressed as relative to NK1.1+IL2 (B). Data is representative (A) or mean +/- SEM (B) of 4 independent experiments. Data was analysed using a one sample student' t-test against a theoretical value of 1. (*p< 0.05).

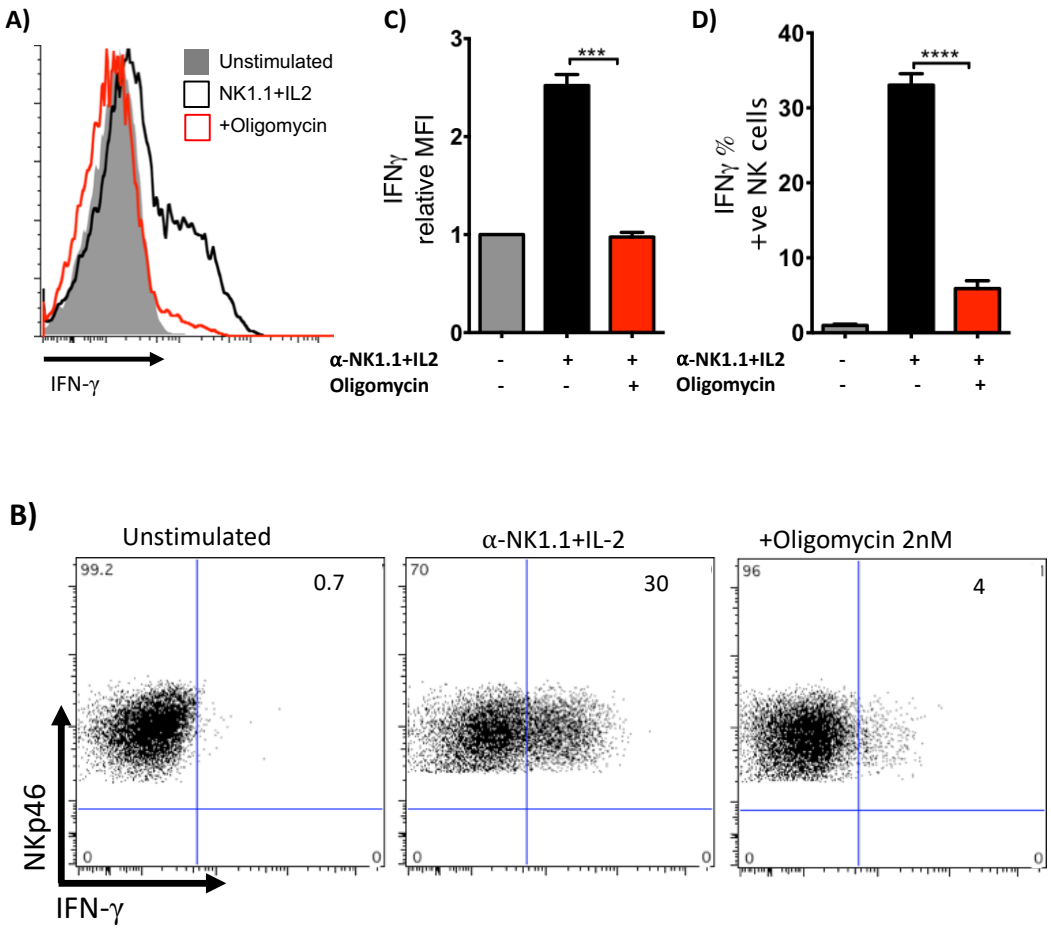


Figure 4.4 Inhibition of OxPhos in NK1.1+IL2 stimulated NK cells disrupts IFN-γ expression in NK cells

(A-D) Cultured NK cells (3 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10µg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of the ATP synthase inhibitor, oligomycin (2nM) or left unstimulated (in low dose IL15 at 7.5 ng/ml only). IFN-γ was analysed by flow cytometry. Data is expressed as relative to unstimulated (C). Data is representative (A,B) or mean +/- SEM (C,D) of 4 independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1 (C) or one-way ANOVA followed by Tukey's multiple comparison post-test (D). (***p<0.001, ****p<0.0001)

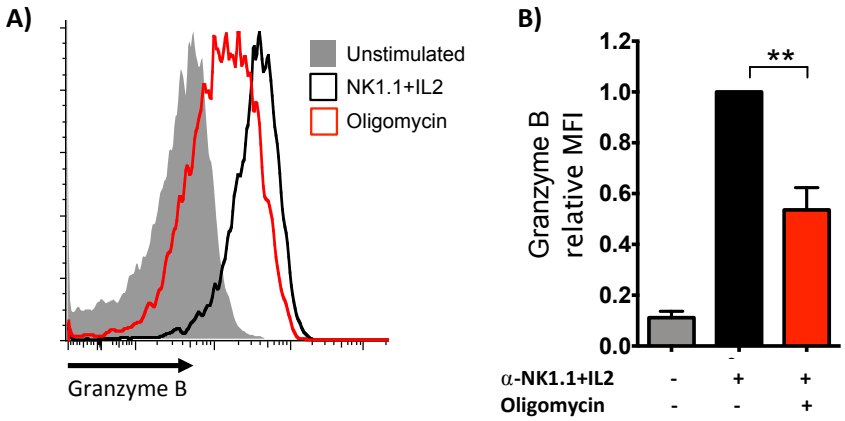


Figure 4.5 Inhibition of OxPhos disrupts Granzyme B expression in NK1.1+IL2 stimulated NK cells.

(A-B) Cultured NK cells (3 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10µg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of the ATP synthase inhibitor Oligomycin (4nM) or left unstimulated (in low dose IL15 at 7.5 ng/ only). Granzyme B was analysed by flow cytometry. Data is expressed as relative to NK1.1+IL2 (B). Data is representative (A) or mean +/- SEM (B) of 4 independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1. (**p< 0.01)

4.2 mTORC1 regulates NK1.1+IL2-induced metabolism and effector functions.

It has been previously shown in IL2/IL12 cytokine stimulated NK cells that mTORC1, an important regulator of metabolism, is essential for NK cell metabolic responses (Donnelly et al., 2014). Therefore, we asked the question whether mTORC1 was important in NK1.1+IL2 stimulated NK cells. It's already been shown in section 3.5, Fig 3.12 that NK1.1 receptor ligation makes NK cells more responsive to the IL2 signalling as demonstrated by immune blotting of the mTORC1 substrates. After establishing that mTORC1 is highly active in NK cells stimulated with α-NK1.1 plus IL2, the next step was to determine whether mTORC1 was required for the metabolic reprogramming observed in NK1.1+IL2 stimulated NK cells. Cultured NK cells were stimulated with α-NK1.1 plus IL2 cytokine in the presence or absence of the mTORC1 specific inhibitor rapamycin. Single cell flow cytometric analysis showed that rapamycin treated NK1.1+IL2 stimulated NK cells failed to increase in size, suggestive of reduced cell growth **(Fig 4.6A)**. Rapamycin treatment also resulted in decreased expression of nutrient transporters CD71 **(Fig 4.6 B,C)** and CD98 **(Fig 4.6D,E).** Additionally, the expression of glucose transporters and glycolytic enzymes were decreased in rapamycin treated NK cells, as determined by real-time q-PCR analysis **(Fig 4.7).**

The decreased expression of nutrient receptors and metabolic enzymes was associated with decreases in metabolic rates, as determined using a seahorse extracellular flux analyser *(Refer to section 2.2.4)*. Rapamycin treated NK cells failed to up-regulate their rates of glycolysis and had substantially reduced glycolytic capacity compared to untreated NK cells **(Fig 4.8)**. Similarly, NK1.1+IL2-stimulated NK cells failed to up-regulate their basal rates of OxPhos and had significantly reduced maximal respiration upon rapamycin

treatment **(Fig 4.9)**. Taken together the data shows that mTORC1 activity is required for NK1.1+IL2-induced metabolic responses.

The data in the previous section of this chapter has shown that NK cell metabolism is required for NK cell effector functions (Fig 4.1-Fig 4.5). Indeed, consistent with decreased cellular metabolism, rapamycin treated NK cells had impaired effector function. NK cells stimulated with α-NK1.1 plus IL2 cytokine in the presence of rapamycin had significantly reduced Granzyme B expression **(Fig 4.10)** and IFN-γ production **(Fig 4.11)** compared to untreated cells.

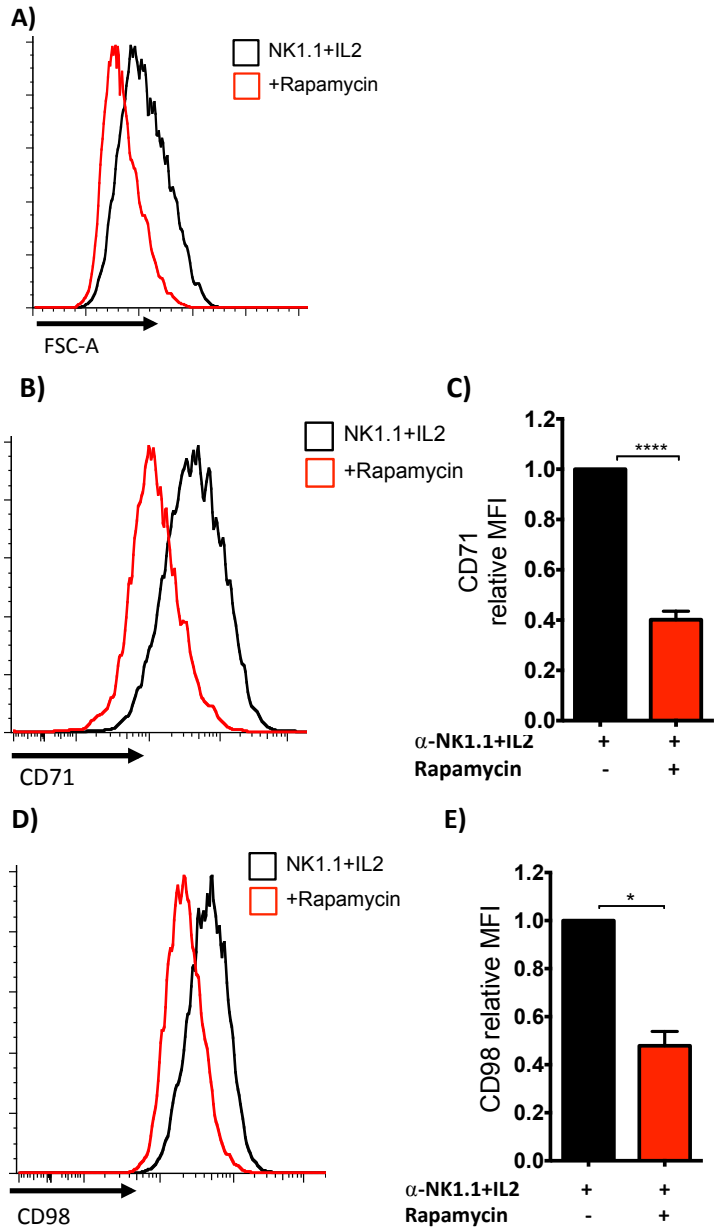


Figure 4.6 mTORC1 activity is required for NK1.1+IL2-induced growth and nutrient receptor expression.

(A-E) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10µg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of the mTORC1 inhibitor rapamycin (20nM) or left unstimulated (in low dose IL15 at 7.5ng/ml only). Cell size, CD71 and CD98 were analysed by flow cytometry. Data is expressed as relative to NK1.1+IL2 (C, E). Data is representative (A, B, D) or mean +/- SEM (C, E) of 3-5 independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1. (*p<0.05, (****p<0.0001).

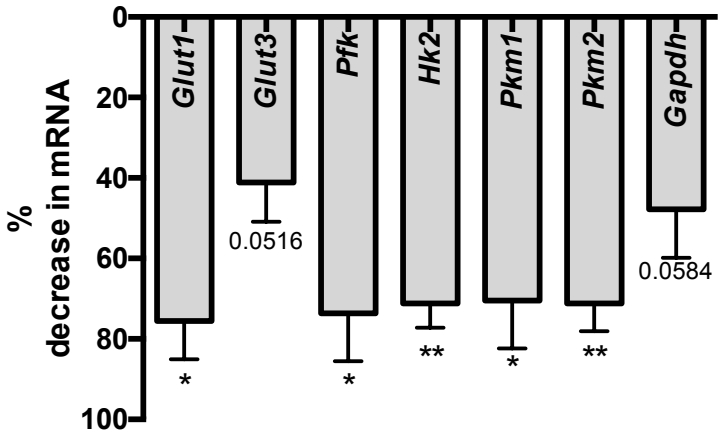


Figure 4.7 mTORC1 activity is required to maintain glycolytic gene expression in NK1.1+IL2 stimulated NK cells.

Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of the mTORC1 inhibitor rapamycin (20nM) or left unstimulated (in low dose IL15 at 7.5 ng/ml only). Cells were lysed for RNA and mRNA transcripts were quantified by qPCR. Genes for glucose transporters and enzymes of the glycolytic pathway were assessed. Data is expressed as percent reduction relative to NK1.1+IL2. Data is mean +/- SEM of three individual experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 0. (*$p<0.05$, **$p<0.01$).

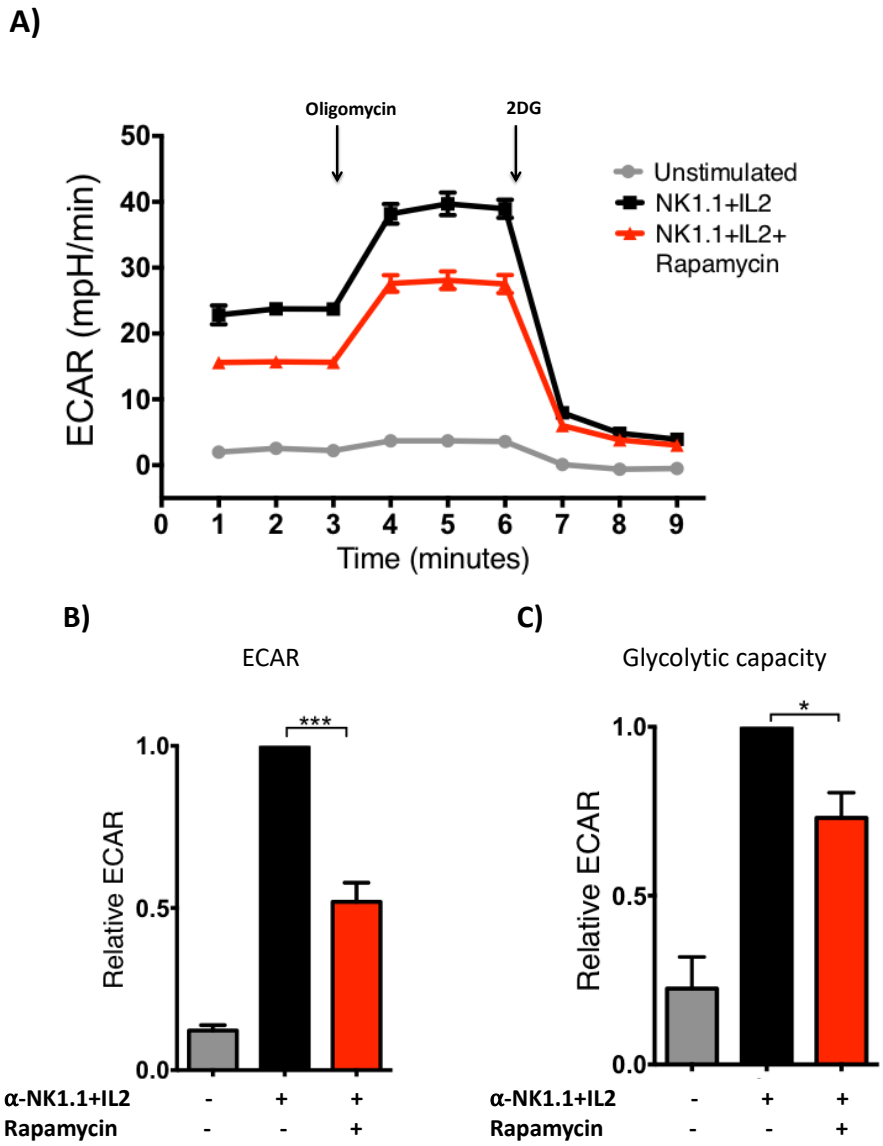


Figure 4.8 mTORC1 inhibition impairs glycolysis in NK1.1+IL2 stimulated NK cells

(A-C) Cultured NK cells (6 days in low dose IL15) were MACS-purified and stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of rapamycin (20nM) or left unstimulated (low dose IL15 at 7.5ng/ml). (A) Extracellular acidification rates (ECAR) were assessed using Seahorse extracellular flux analyser with the sequential addition of inhibitors oligomycin and 2 deoxyglucose (2DG). The rates of glycolysis (B) and glycolytic capacity (C) were calculated. Data is expressed relative to NK1.1+IL2 (B,C). Data is representative (A) or mean +/- SEM of four independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1. (*p<0.05, ***p<0.001).

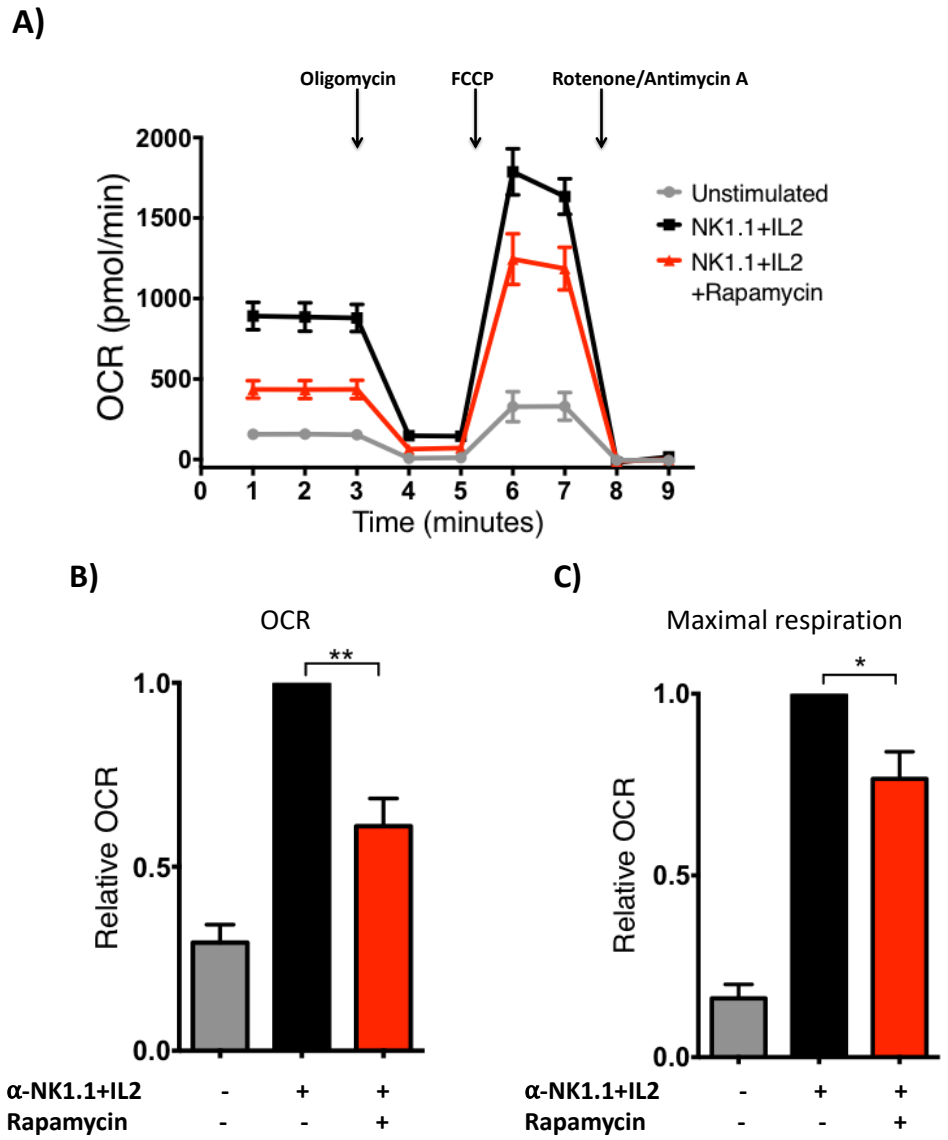


Figure 4.9 mTORC1 inhibition impairs OxPhos in NK1.1+IL2 stimulated

(A-C) NK cells Cultured NK cells (6 days in low dose IL15) were MACS-purified and stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of rapamycin (20nM) or left unstimulated (low dose IL15 at 5ng/ml). (A) Oxygen consumption rates (OCR) were assessed using Seahorse extracellular flux analyser with the sequential addition of inhibitors oligomycin, FCCP and antimycin A plus rotenone. The rates of OxPhos (B) and maximal respiration (C) were calculated. Data is expressed relative to NK1.1+IL2 (B, C). Data is representative (A) or mean +/- SEM of four independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1. (*p<0.05, **p<0.01)

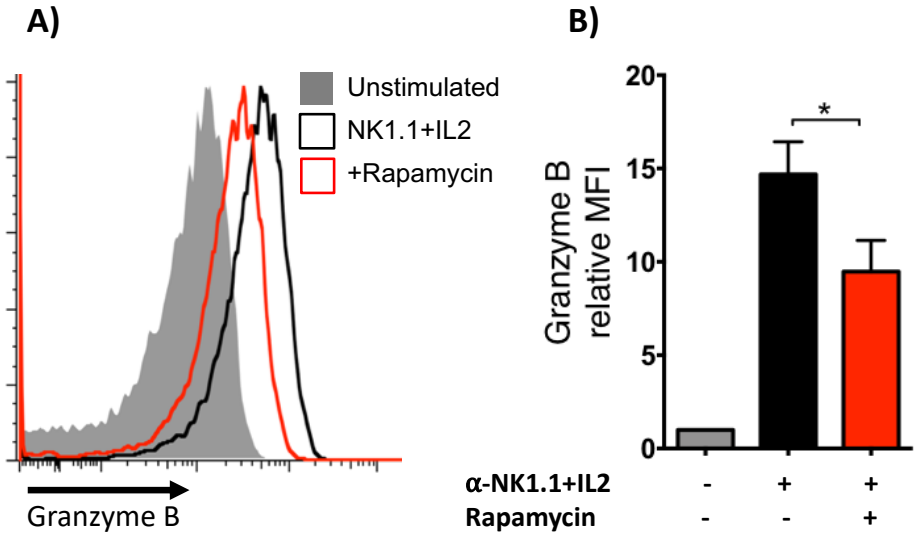


Figure 4.10 mTORC1 inhibition disrupts Granzyme B expression in NK1.1+IL2 stimulated cells

(A-B) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10µg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of rapamycin (20nM) or left unstimulated (low dose IL15 at 5ng/ml). Granzyme B production in NK cells was assessed by flow cytometry. Data is expressed as relative to unstimulated (B). Data is representative (A) or mean +/- SEM (B) of four independent experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post-test (*$p<0.05$)

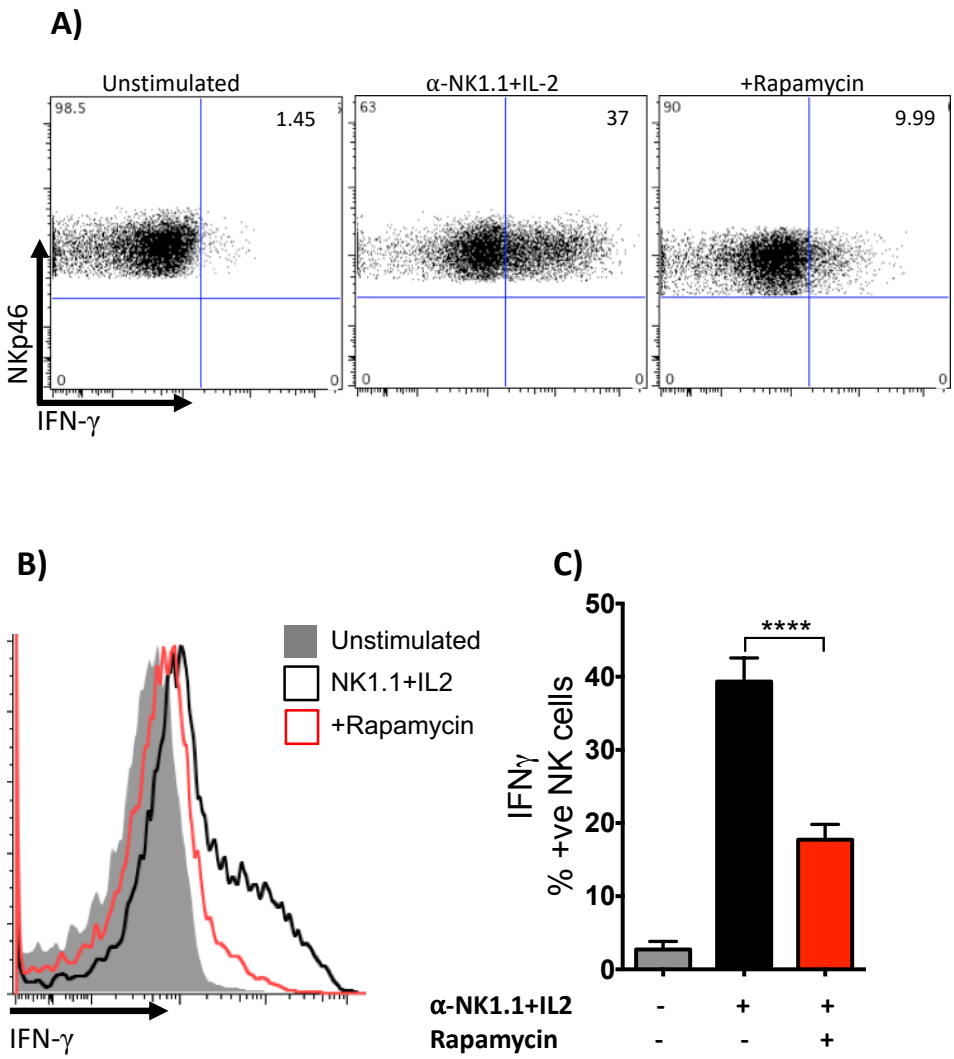


Figure 4.11 mTORC1 inhibits IFN-γ production in NK1.1+IL2 stimulated NK cells

(A-C) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of Rapamycin (20nM) or left unstimulated (low dose IL15 at 5ng/ml). IFN-γ production in NK cells was assessed by flow cytometry. Data is representative (A,B) or mean +/- SEM (C) of five independent experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post-test. (****p<0.0001).

4.3 cMyc-controls NK cell metabolism in NK1.1+IL2-stimulated NK cells

In our recent publication, we have described the role of the transcription factor cMyc in regulating cellular metabolism and function in IL2/IL12 cytokine stimulated NK cells (Loftus et al., 2018). This led us to ask whether cMyc expression was also required for metabolism and function in NK cells activated by receptor stimulation. To assess the importance of cMyc, it was important to first determine whether the expression of cMyc increases in response to receptor mediated stimulation of NK cells. Cultured NK cells were stimulated with α-NK1.1 plus IL2 cytokine for 18 hours and cMyc expression was analysed by flow cytometry. Indeed, receptor stimulated NK cells expressed increased levels of cMyc **(Fig4.12)**.

To define the role of cMyc in receptor stimulated NK cells, cMyc null NK cells were generated using cMyc$^{flox/flox}$ Tamoxifen-Cre (cMyc KO) mice, which allows for the inducible deletion of exon X of the Myc gene upon the addition of tamoxifen *(Refer to section 2.1.3)*. The control (cMyc WT) NK cells were generated from WT Tamoxifen-Cre (C57-Tcre) mice and were also treated with tamoxifen. cMyc deletion was confirmed by flow cytometric analysis of CD71 expression **(Fig 4.13)** , the gene for which is a known cMyc target (O'Donnell et al., 2006).

To study the role of cMyc for metabolic responses in NK cells, cultured cMyc KO and WT cells were purified by magnetic bead cell sorting and then stimulated with α-NK1.1 plus IL2 cytokine for 18 hours before analysis using the seahorse extracellular flux analyser *(Refer to section 2.2.4)*. cMyc null NK cells stimulated with NK1.1+IL2 had very low basal rates of glycolysis and glycolytic capacity compared to WT NK cells. Similarly, NK1.1+IL2-stimulated cMyc null

NK cells had very low rates of OxPhos and maximal respiration, compared to the high rates observed in WT NK cells.

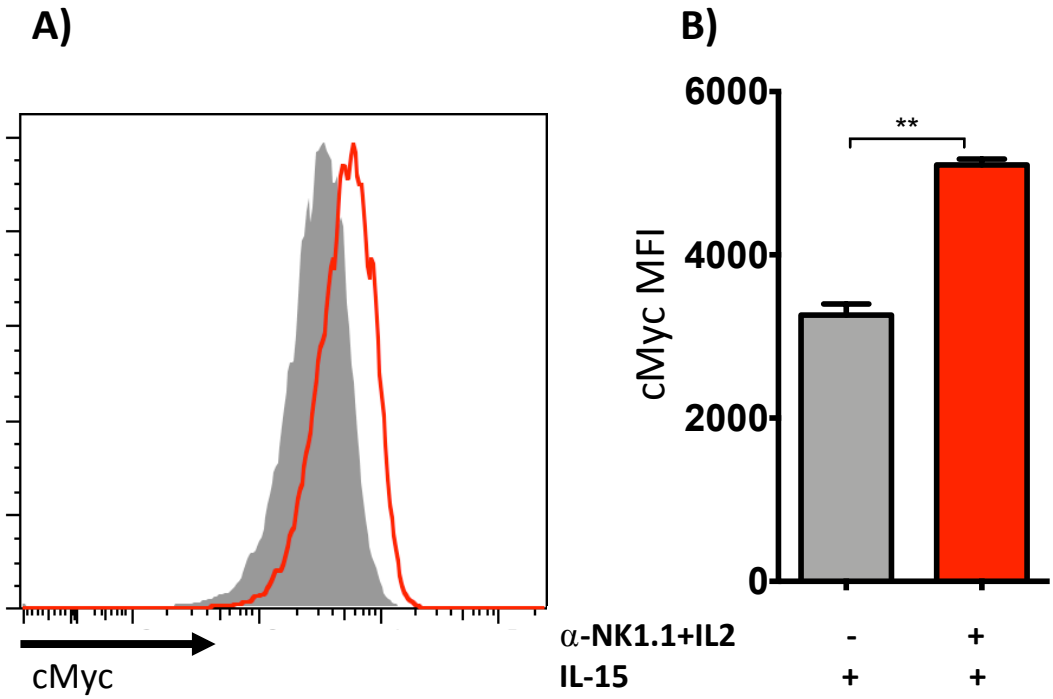


Figure 4.12 NK1.1+IL2 stimulated cells up-regulated the expression of cMyc.

(A-B) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml) or left unstimulated (low dose IL15 at 5ng/ml). cMyc expression in NK cells was assessed by flow cytometry. Data is representative (A) or mean +/- SEM (B) of three independent experiments. Data was analysed using paired student's t test. (**p<0.01).

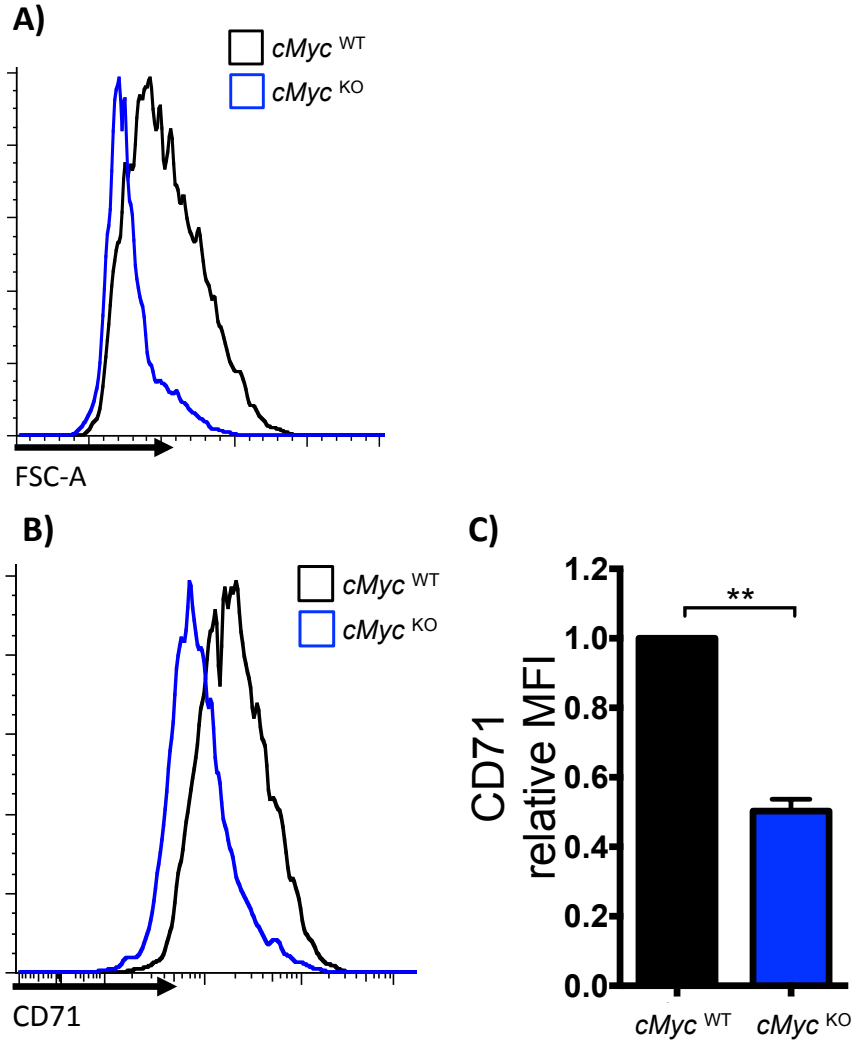


Figure 4.13 cMyc inhibits cell growth and CD71 expression in NK1.1+IL2 stimulated NK cells

(A-C) Cultured NK cells (6 days in low dose IL15 and tamoxifen) from cMyc$^{fl/fl}$ Tamox-Cre (*cMyc* ^{KO}) or C57-t-cre (*cMyc* ^{WT}) mice were MACS-purified and stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml). Cell size (A) and CD71 expression (B,C) were analysed by flow cytometry. Data is expressed relative to NK1.1+IL2 (*cMyc*^{WT})(C). Data is representative (A,B) or mean +/- SEM (C) of four independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1. (**p<0.01).

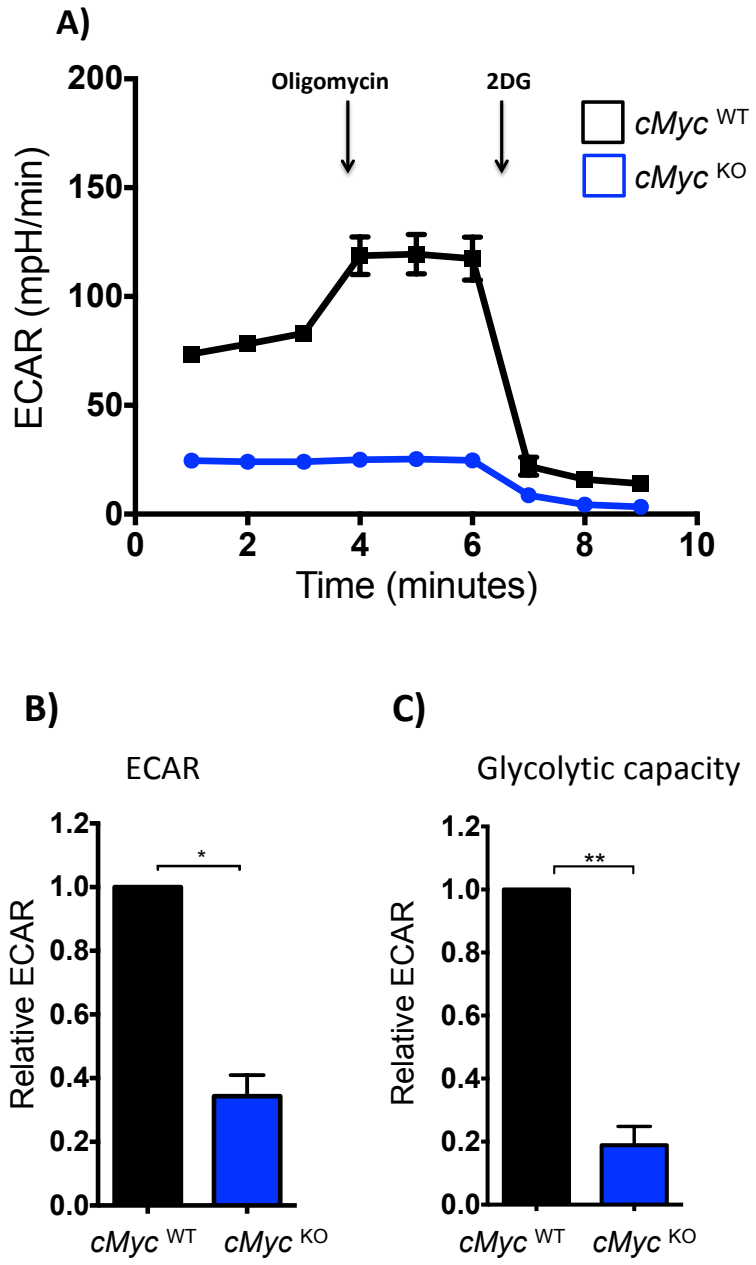


Figure 4.14 cMyc deletion impairs glycolysis in NK1.1+IL2 stimulated NK cells

(A-C) Cultured NK cells (6 days in low dose IL15 and tamoxifen) from cMyc$^{fl/fl}$ Tamox-Cre (KO) or C57-t-cre (WT) mice were MACS-purified and stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml). (A) Extracellular acidification rates (ECAR) were assessed using Seahorse extracellular flux analyser with the sequential addition of inhibitors oligomycin and 2 deoxyglucose (2DG). The rates of glycolysis (B) and glycolytic capacity (C) were calculated. Data is expressed relative to NK1.1+IL2 (B,C). Data is representative (A) or mean +/- SEM (B,C) of three independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1. (*$p<0.05$, **$p<0.01$)

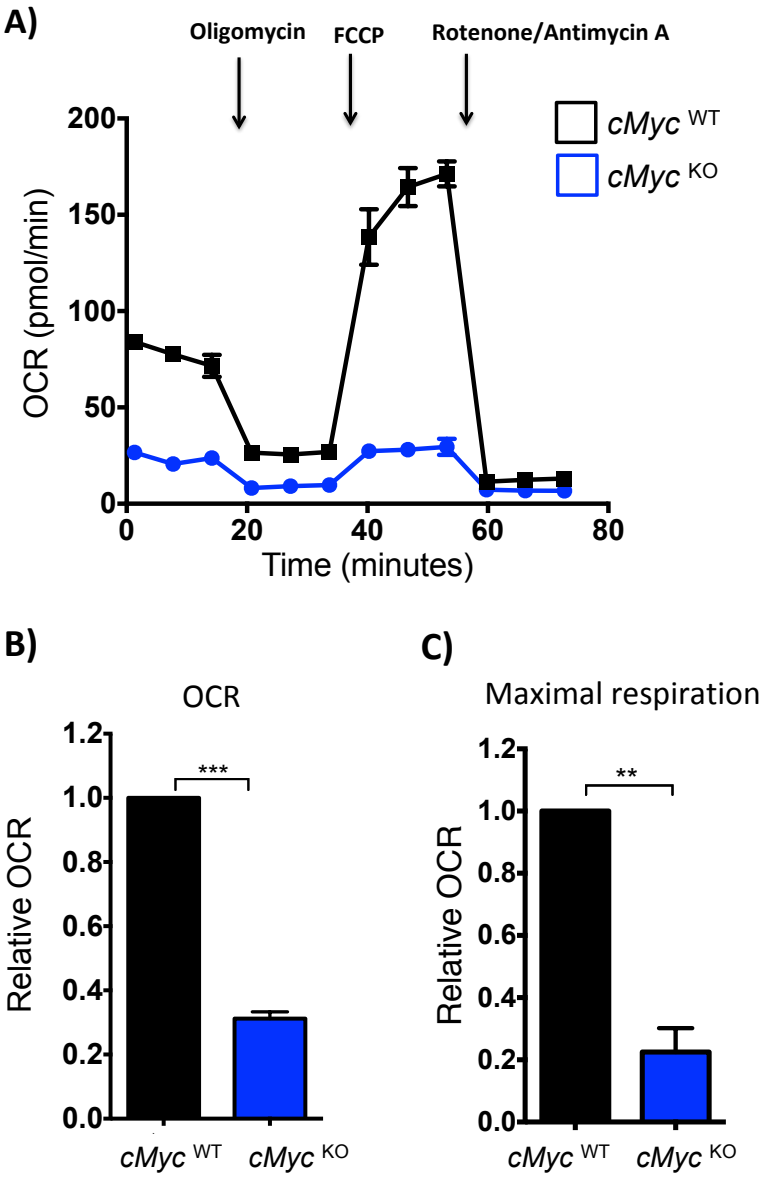


Figure 4.13 cMyc deletion impairs OXPHOS in NK1.1+IL2 stimulated NK cells

(A-C) Cultured NK cells (6 days in low dose IL15 and tamoxifen) from cMyc$^{fl/fl}$ Tamox-Cre (KO) or C57-t-cre (WT) mice were MACS-purified and stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml). (A) Oxygen consumption rates (OCR) were assessed using Seahorse extracellular flux analyser with the sequential addition of inhibitors oligomycin, FCCP and antimycin A plus rotenone. The rates of OXPHOS (B) and maximal respiration (C) were calculated. Data is expressed relative to NK1.1+IL2 (B,C). Data is representative (A) or mean +/- SEM (B,C) of three independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1. (**p<0.01, ***p<0.001)

Recent work in the Finlay lab has demonstrated that amino acid transport through the Slc7A5 amino acid transporter is essential for cMyc expression in activated NK cells. Inhibiting amino acid through the Slc7A5 transporter by treating the cells with the Slc7A5 inhibitor 2-aminobicycloheptane-2-carboxylic acid or BCH for 1 hour resulted in the loss of cMyc expression and impaired cytokine induced NK cell metabolism and function (Loftus et al., 2018).
Similarly, inhibition of amino acid transport through Slc7A5 in NK1.1+IL2 stimulated NK cells was found to inhibit NK cell effector function; BCH treated NK cells had decreased IFN-γ production and Granzyme B expression **(Fig 4.14, 4.15).**

These data confirm that cMyc is expressed upon receptor-mediated stimulation of NK cells and is required for the metabolic and functional responses following NK1.1+IL2 stimulation.

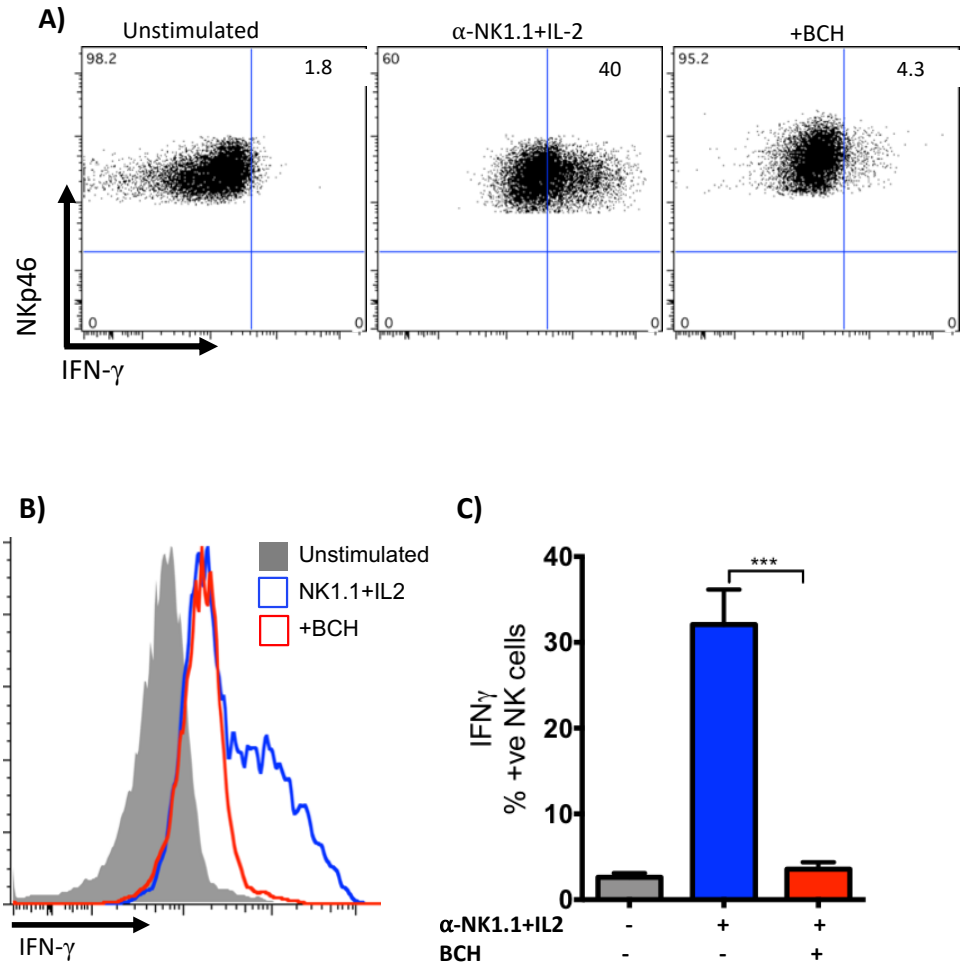


Figure 4.14 Slc7A5 inhibition with BCH inhibits IFN-γ production in NK1.1+IL2-stimulated NK cells

(A-C) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of the Slc7A5 inhibitor, 2-aminobicycloheptane-2-carboxylic acid (BCH, 25mM). IFN-γ was assessed by flow cytometry. Data is representative (A,B) or mean +/- SEM (C) of three independent experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post-test. (***p<0.001)

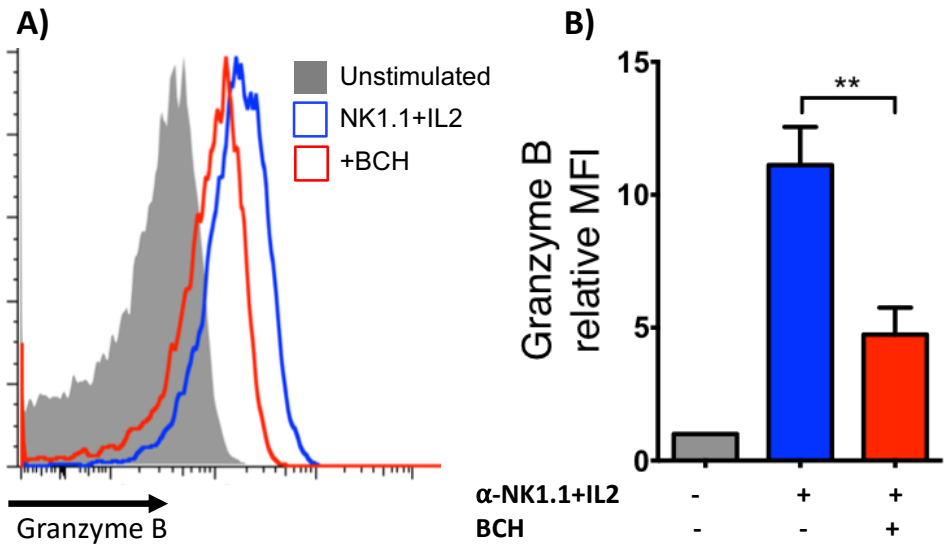


Figure 4.15 Slc7A5 inhibition with BCH inhibits Granzyme B expression in NK1.1+IL2-stimulated NK cells

(A-B) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of the Slc7A5 inhibitor, 2-aminobicycloheptane-2-carboxylic acid (BCH, 25mM). Granzyme B was assessed by flow cytometry. Data is representative (A) or mean +/- SEM (B) of three independent experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post-test. (**p<0.01)

4.4 Srebp activity is required for NK1.1+IL2-induced metabolic and functional responses.

Srebp is a transcription factor that is considered the master regulator of *de novo* lipid synthesis (Horton et al., 2002)) Recent work in our lab led to the surprising discovery that Srebp is essential for cytokine-induced NK cell metabolic reprogramming towards elevated glycolysis and OxPhos. Srebp was shown to control glucose metabolism in cytokine stimulated NK cells via its control of a novel metabolic pathway, the citrate malate shuttle, that is active in cytokine-stimulated NK cells (Assmann et al., 2017). Srebp was shown to act downstream of mTORC1 (Assmann et al., 2017; Porstmann et al., 2008), the role for which in NK1.1+IL2-induced metabolic responses has already been highlighted in the previous section. Therefore, we predicted that Srebp would be required for NK1.1+IL2-induced metabolic responses and effector functions.

Before looking at Srebp's requirement in metabolism it was necessary to determine its level of expression in NK1.1+IL2 stimulated NK cells and whether it was regulated by mTORC1 in these cells. To determine Srebp activity, cultured NK cells were purified by magnetic bead cell sorting and stimulated for 18 hours with αNK1.1 plus IL2 cytokine in the presence or absence of mTORC1 specific inhibitor rapamycin or left unstimulated. The cells were lysed for RNA following the 18-hour stimulation and established Srebp associated genes quantified by qPCR; steroyl CoA desaturase 1 (*Scd1*), ATP citrate lyase (*Acly*), acetyl CoA acetyltransferase 2 (*Acat2*), 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*Hmgcs1*) fatty acid synthase (*Fasn*). NK1.1+IL2 stimulation of NK cells resulted in a robust increase in all the above genes **(Fig4.16).** The gene expression of all the Srebp target genes significantly decreased in rapamycin treated NK cells, implying that it is activated downstream of mTORC1 **(Fig4.16).**

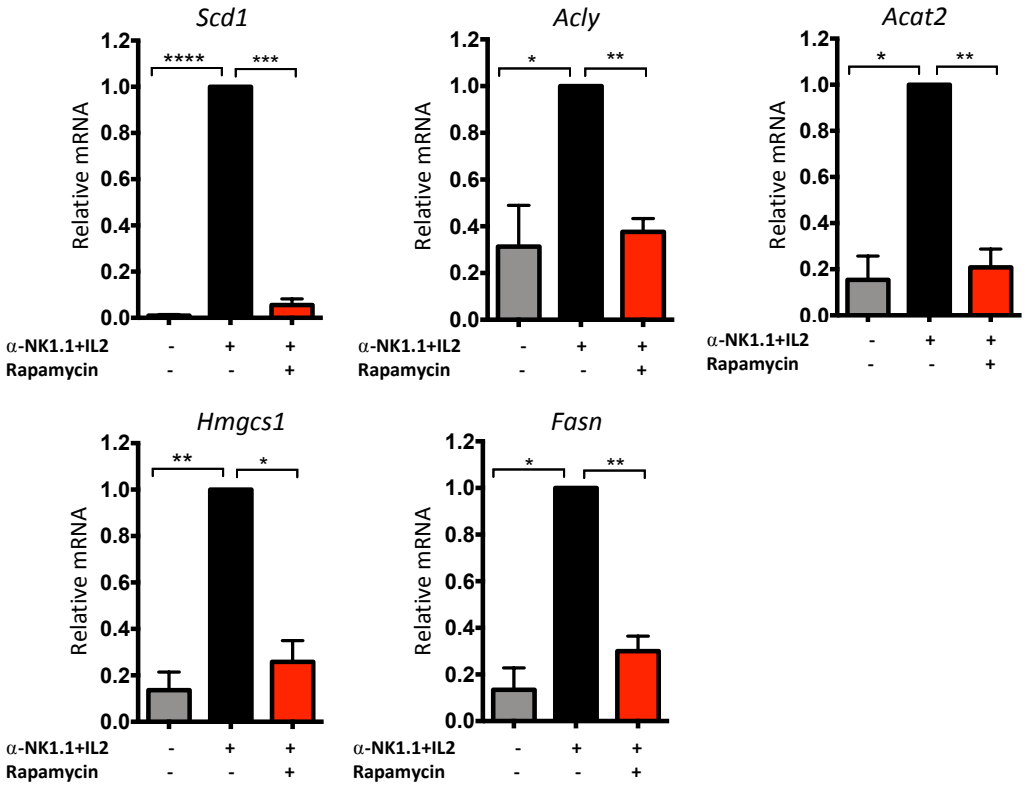


Figure 4.16 The transcription factor Srebp is highly expressed in NK1.1+IL2 stimulated cells NK cells and is controlled by mTORC1

Cultured NK cells (6 days in low dose IL15) were MACS-purified and stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of rapamycin (20nM). Cells were lysed for RNA and mRNA transcripts for Srebp target genes were quantified by qPCR. Data is expressed relative to NK1.1+IL2. Data is mean +/- SEM of three independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1. (*p<0.05, **p<0.01, ***p<0.001).

To establish the role for Srebp in receptor stimulation associated metabolic and functional responses, two different pharmacological inhibitors that inhibited Srebp activity through independent mechanisms were used; 25-hydroxycholesterol (25HC) and PF429242 (PF). 25HC prevents the translocation of Srebp into the Golgi apparatus where it is processed to an active form, while PF blocks the enzyme site-1-protease (S1P) that cleaves Srebp into an active form in the Golgi apparatus.

To determine the importance of the Srebp transcription factor in NK1.1+IL2 mediated metabolic reprogramming in NK cells, cultured NK cells were purified by magnetic bead cell sorting, and stimulated with αNK1.1 plus IL2 cytokine in the presence or absence of 25HC or PF for 18 hours. Detailed metabolic analyses were performed using the Seahorse extracellular flux analyser *(Refer to section 2.2.4)*. Both 25HC and PF had profound effects on basal glycolytic rates in NK1.1+IL2 stimulated NK cells **(Fig 4.17 A,B)**. Srebp inhibition also severely compromised glycolytic capacity **(Fig 4.17 A,C)** in these cells indicating its control of NK cell metabolic reprogramming.
Similarly, inhibition of Srebp by either 25HC or PF in NK1.1+IL2 stimulated NK cells, had a drastic impact on rates of OxPhos in these cells **(Fig 4.18 A,B)**. 25HC or PF treated NK cells also had a robust impairment in the rates of maximal respiration **(Fig 4.18 A,C)**.

On the basis of its requirement for NK1.1+IL2 associated metabolic reprogramming, it was predicted the Srebp inhibition would affect NK1.1+IL2 mediated effector functions. Cultured NK cells were purified by magnetic bead cell sorting, and stimulated with αNK1.1 plus IL2 cytokine in the presence or absence of 25HC or PF for 18 hours. Following the 18-hour stimulation, NK cell functional output was measured by analysing IFN-γ and Granzyme B production. While 25HC mediated inhibition of Srebp resulted in a significant decrease in IFN-γ production in NK1.1+IL2 stimulated NK cells, inhibiting

Srebp by PF showed a non-significant decrease in IFN-γ **(Fig 4.19)**. Srebp inhibition by either 25HC or PF substantially reduced Granzyme B production by NK1.1+IL2 stimulated NK cells **(Fig 4.20).**

Taken together these data confirm a role for the transcription factor Srebp in NK1.1+IL2 mediated metabolism and function.

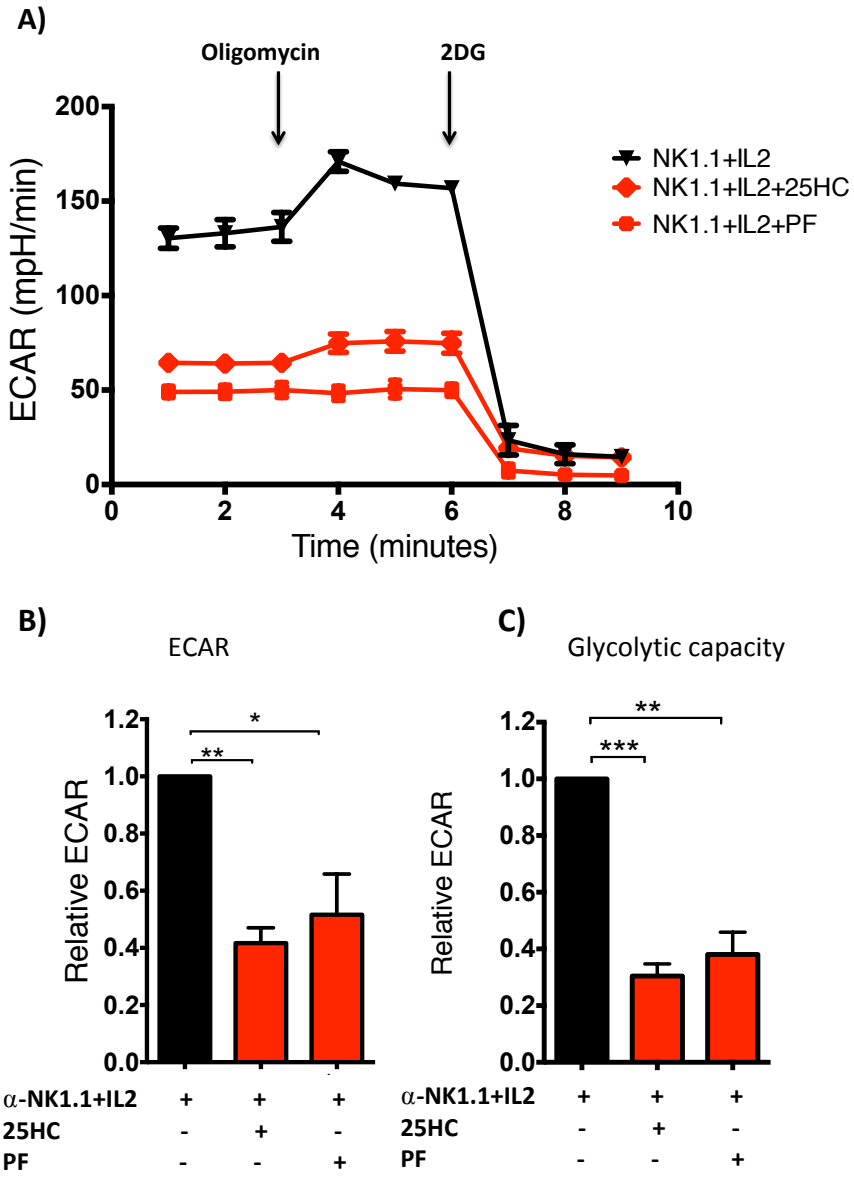


Figure 4.17 Srebp inhibition impairs glycolysis in NK1.1+IL2 stimulated NK cells

(A-C) Cultured NK cells (6 days in low dose IL15) were MACS-purified and stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of 2 separate Srebp inhibitors 25-hydroxycholesterol or 25HC (1μg/ml) and PF429242 or PF (10μM). (A) Extracellular acidification rates (ECAR) were assessed using Seahorse extracellular flux analyser with the sequential addition of inhibitors oligomycin and 2 deoxyglucose (2DG). The rates of glycolysis (B) and glycolytic capacity (C) were calculated. Data is expressed relative to NK1.1+IL2 (B,C). Data is representative (A) or mean +/- SEM (B,C) of three independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1. (*p<0.05, **p<0.01, ***p<0.001).

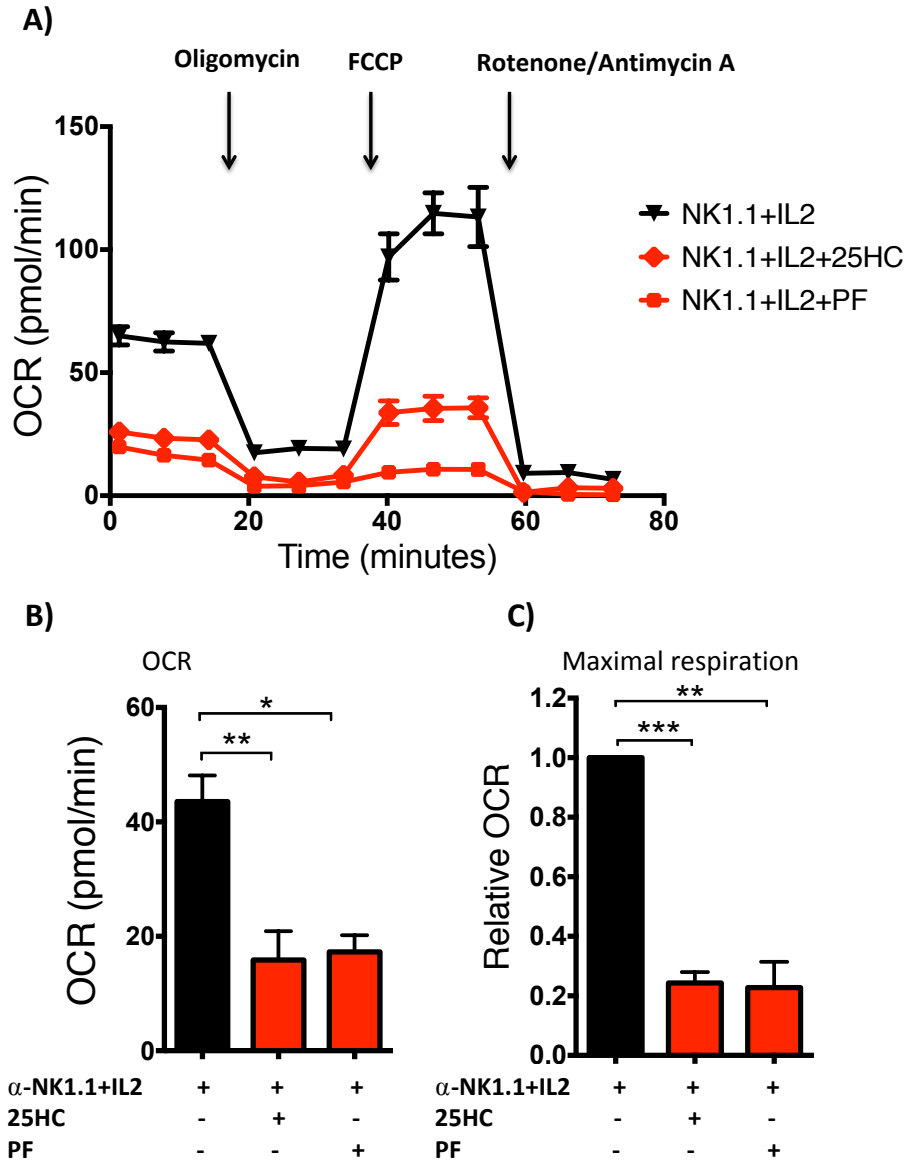


Figure 4.18 Srebp inhibition impairs OxPhos in NK1.1+IL2 stimulated NK cells

(A-C) Cultured NK cells (6 days in low dose IL15) were MACS-purified and stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of 2 separate srebp inhibitors 25-hydroxycholesterol or 25HC (1μg/ml) and PF429242 or PF (10μM). (A) Oxygen consumption rates (OCR) were assessed using Seahorse extracellular flux analyser with the sequential addition of inhibitors oligomycin, FCCP and antimycin A plus rotenone. The rates of OxPhos (B) and maximal respiration (C) were calculated. Data is expressed relative to NK1.1+IL2 (B,C). Data is representative (A) or mean +/- SEM (B,C) of three independent experiments. Data was analysed using one way ANOVA followed by Tukey's multiple comparison post test (B) or one-sample student's t-test against a theoretical value of 1(C) (*p<0.05, **p<0.01, ***p<0.001).

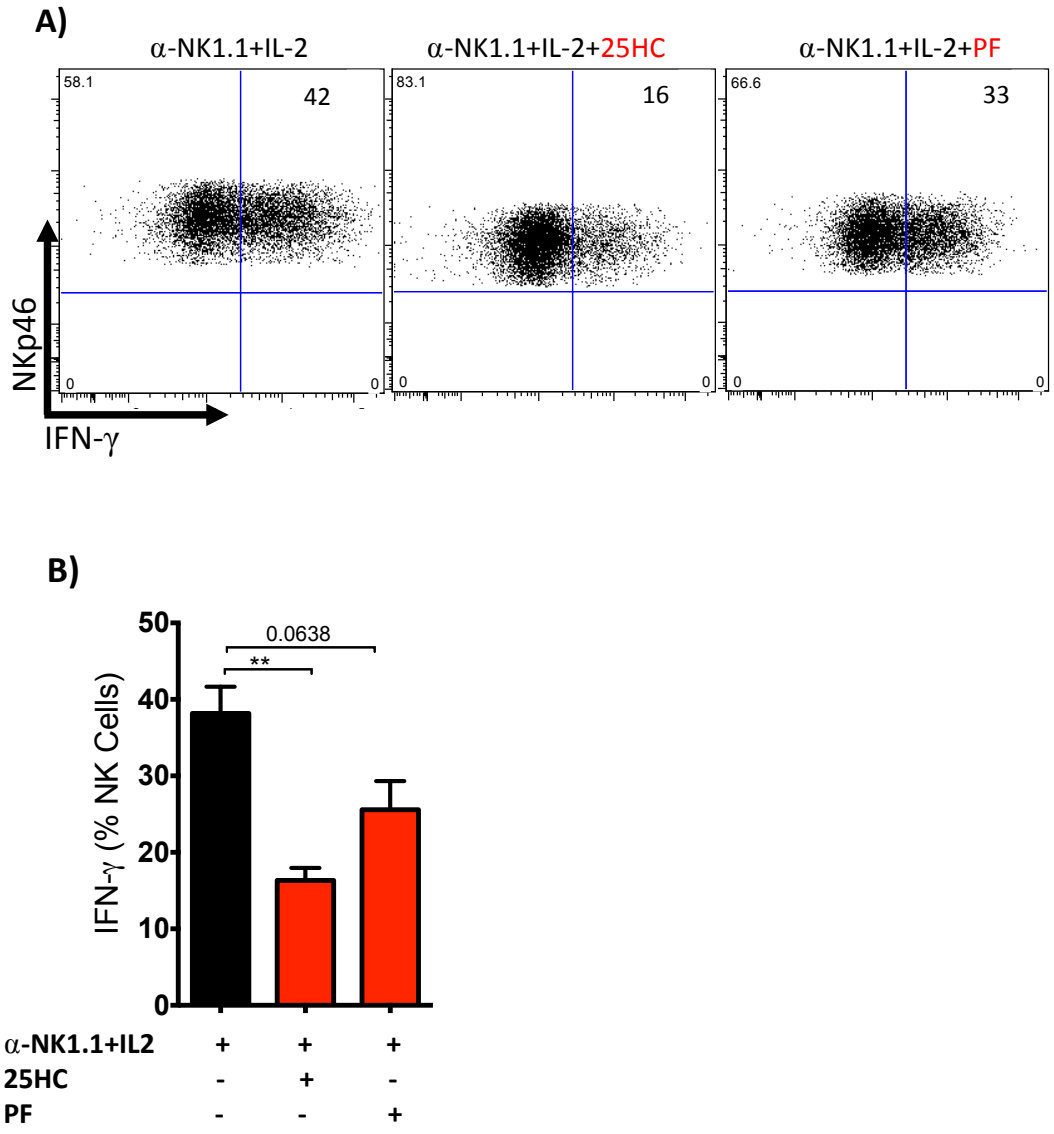


Figure 4.19 Srebp inhibition decreases IFN-γ NK1.1+IL2 stimulated NK cells

(A-B) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of the Srebp inhibitors, 25-hydroxycholesterol or 25HC (1μg/ml) and PF429242 or PF (10μM). IFN-γ was assessed by flow cytometry. Data is representative (A) or mean +/- SEM (B) of three independent experiments. Data was analysed using one-way ANOVA with a Tukey multiple comparison post-test (**p<0.01)

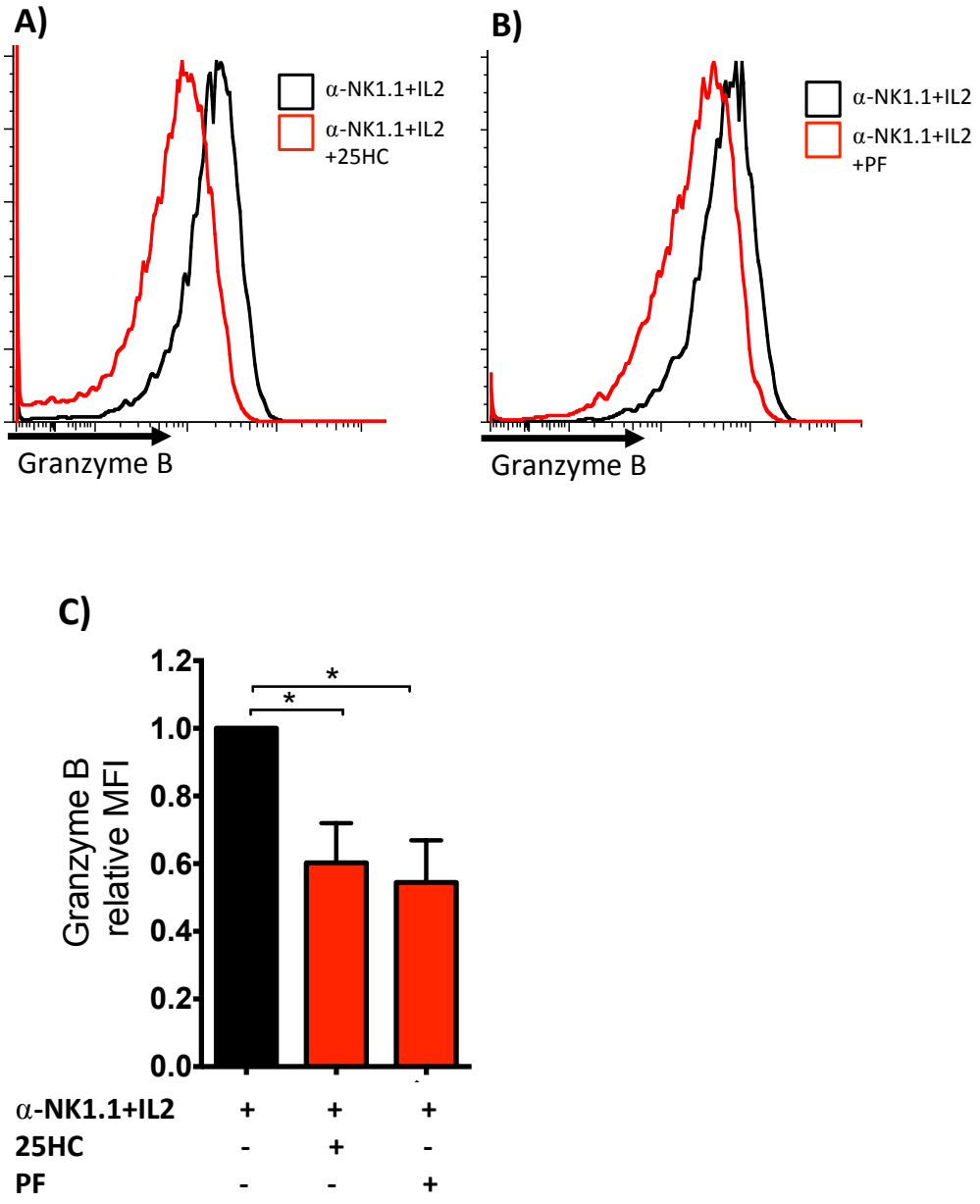


Figure 4.20 Srebp inhibition decreases Granzyme B NK1.1+IL2 stimulated NK cells

(A-C) Cultured NK cells (6 days in low dose IL15) were stimulated for 18 hours with α-NK1.1 antibody (10μg/ml) plus IL2 cytokine (20ng/ml) in the presence or absence of the Srebp inhibitors, 25-hydroxycholesterol or 25HC (1μg/ml) and PF429242 or PF (10μM). Granzyme B was assessed by flow cytometry. Data is expressed as relative to NK1.1+IL2 (C). Data is representative (A,B) or mean +/- SEM (C) of three independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1. (*$p<0.05$)

The data presented in this chapter together establish that the metabolic reprogramming in αNK1.1+IL2 stimulated NK cells is regulated by mTORC1, cMyc and Srebp. Inhibiting the metabolic pathways directly, for instance by blocking the glycolytic pathway of by the inhibition of OxPhos results in an impairment of NK1.1+IL2 mediated NK cell effector functions. Inhibition of any of the key metabolic regulators mTORC1, cMyc or Srebp also negatively impacts upon NK1.1+IL2 mediated NK cell effector responses. Taken together, the data proves that cellular metabolism controls NK cell effector responses.

Chapter 4 Discussion

There is increasing evidence suggesting that cellular metabolism controls effector function in immune cells (Chang et al., 2013; Donnelly et al., 2014; O'Neill and Pearce, 2016; Pearce and Pearce, 2013). Previous work from our lab has demonstrated that limiting the rates of glycolysis either by 2-DG or replacing glucose with galactose, significantly affected cytokine mediated NK cell effector functions (Donnelly et al., 2014). To determine the relationship between cellular metabolism and receptor mediated NK cell effector functions, we limited the rates of glycolysis and OxPhos using different inhibitors. Limiting the rates of glycolysis in NK1.1+IL2 stimulated NK cells greatly impacted IFN-γ production in these cells, indicating that glycolytic reprograming is essential for cytokine production in NK1.1+IL2 stimulated cells. This is consistent with our published work studying the relationship between cellular metabolism and function in IL2/12 stimulated NK cells (Donnelly et al., 2014). Indeed, GAPDH, a glycolytic enzyme has been shown to control IFN-γ expression in T cells, by binding to its mRNA, when it is not engaged in glycolysis (Chang et al., 2013).
While glycolytic limitation severely affected IFN-γ production in NK1.1+IL2 stimulated cells, it had a smaller but significant effect on Granzyme B expression in these cells, this is in line with the published work from our lab which shows that glycolytic inhibition by 2-DG significantly affects Granzyme B levels in IL2/12 stimulated cells (Donnelly et al., 2014). Moreover, recently published work in our lab shows that glycolysis and OxPhos in IL2/12 stimulated cells are parts of the same metabolic pathway as glucose in the principal energy source that fuels OxPhos in these cells. Metabolism of other fuels such as glutamine does not control the metabolic pathways in IL2/12 stimulated NK cells (Assmann et al., 2017; Loftus et al., 2018).

There is also supporting data in other lymphocytes wherein, inhibition of glycolysis resulted in decreased effector responses. For instance, in $CD8^+$ T cells,

glycolytic inhibition led to an increase in memory T cells and a depletion of effector T cells (Sukumar et al., 2013). In $CD4^+$ T cells, it was demonstrated that increased glucose uptake and Glut1 expression results in enrichment of T_{eff} (T effector) cells and that glucose deprivation from the culture media or Glut1 deficiency during $CD4^+$ T cell differentiation failed to yield T effector cells, while having no effect on the numbers of Treg cells (Macintyre et al., 2014; Michalek et al., 2011). In B cells, Glut1 deletion and reduced glucose uptake resulted in reduced B cell numbers and impaired antibody secretion *in vivo* (Caro-Maldonado et al., 2014).

Three key metabolic regulators have been described to control metabolism and function in IL2/12 stimulated NK cells (Assmann et al., 2017; Donnelly et al., 2014; Loftus et al., 2018) - mTORC1, Srebp and cMyc. mTORC1 is well established as a key molecule for the control of T cell metabolism and function (Waickman and Powell, 2012). However, the first report revealing the importance of mTORC1 for NK cells came as late as 2014 when it was shown that mTORC1 is essential for IL15 mediated development and activation of NK cells (Marçais et al., 2014a). Since then, work from our lab has highlighted the importance of mTORC1 in the control of IL2/IL12 mediated NK cell metabolism in mice and IL2 dependent metabolic reprograming in humans (Donnelly et al., 2014; Keating et al., 2016). This data is supported by evidence in other lymphocytes like $CD4^+$ T cells, where mTORC1 inhibition led to generation of Tregs over T effector cells like TH1 and TH17 (Delgoffe et al., 2009; Delgoffe et al., 2011). Similarly in $CD8^+$ cytotoxic T cells, mTORC1 was shown to be important for increased glucose uptake and glycolysis during CTL differentiation (Finlay et al., 2012). Indeed, mTORC1 inhibition by rapamycin treatment in $CD8^+$ cytotoxic T cells led to generation of memory $CD8^+$ T cells over effector CTLs (Araki et al., 2009). Corroborating this data, our report shows that disrupting mTORC1 activity by rapamycin affected both NK1.1+IL2 induced glycolysis and OxPhos in NK cells. Interestingly, this is in contrast to

what was seen in IL2/IL12 cytokine activated NK cells where only glycolysis was sensitive to mTORC1 inhibition. However recent research in the lab on cultured and splenic NK cell suggests that maximal induction of IL2/IL12 dependent OxPhos does require mTORC1. This is in line with the fact that mTORC1 is required for Srebp activation which is a key regulator of IL2/IL12 induced OxPhos (Assmann et al., 2017; Donnelly et al., 2014; Porstmann et al., 2008). Rapamycin treatment also resulted in the downregulation of several glycolytic genes including those encoding for the enzymes involved in the glycolytic pathway and those for glucose transporters indicating that mTORC1 regulates the metabolic machinery in NK1.1+IL2 stimulated NK cells.

Owing to the fact that glycolytic inhibition led to impairment of functional responses in NK1.1+IL2 stimulated NK cells, and having established that mTORC1 activity controlled metabolism in these cells, we hypothesized that mTORC1 activity was also required for NK cell effector functions. Indeed it has been previously shown in IL15 activated NK cells as well as IL2/IL12 activated NK cells that disruption of mTORC1 activity by rapamycin resulted in the loss of NK cell functional responses (Donnelly et al., 2014; Marçais et al., 2014a; Nandagopal et al., 2014). Likewise, mTORC1 driven metabolism was found to be required for CD56 bright NK cell function in IL2 activated human NK cells (Keating et al., 2016). In this study, we were able to show a similar requirement for mTORC1 for NK1.1+IL2 driven effector functions, including IFNγ production and Granzyme B expression.

cMyc is an important transcription factor, which, apart from being implicated in various cancers has also be shown to be important for lymphocyte responses. There is data showing that cMyc expression can be induced in an mTORC1 dependent manner (Csibi et al., 2014), but cMyc expression can also be regulated in an mTORC1 independent manner in both NK cells and T cells. For

instance, mTORC1 inhibition with rapamycin had no effect on cMyc expression in 18 hour cytokine stimulated NK cells (Loftus et al., 2018).

It is known that IL2 cytokine can induce robust expression of cMyc (Lord et al., 2000). cMyc levels in activated T cells have also been shown to be highly correlated with CD25 which is up-regulated during T cell priming (Preston et al., 2015). In fact, upon removal of IL2 or treatment with the Janus kinase inhibitor, tofacitinib, that blocks the signal transduction activated by IL-2R ligation, cMyc was rapidly lost (Preston et al., 2015). Moreover, CD71, a cMyc target gene, was shown to be highly up regulated in response to NK1.1+IL2 stimulation of NK cells in this study. Therefore, we hypothesized that cMyc would also be induced by NK1.1+IL2 activation of NK cells. Indeed, in NK1.1+IL2 stimulated NK cells, there was a significant increase in the levels of cMyc protein.

In T cells cMyc has been shown to control the metabolic response upon activation (Chou et al., 2014; Hsu et al., 2016; Wang et al., 2011). Recent publication from our group has proved that cMyc is essential for cytokine mediated NK cell blastogenesis and metabolism and is crucial in control of glycolytic machinery (Loftus et al., 2018). Therefore, we hypothesized that cMyc also controls metabolic responses in receptor stimulated NK cells. cMyc deletion using Tamoxifen inducible Myc KO mice had profound defects in NK1.1+IL2 induced NK cell metabolism. This was accompanied by an expected loss of CD71 expression and an impediment in cellular growth.

cMyc was implicated in the initiation of $CD8^+$ T cell mediated host protection against microbial infections (Chou et al., 2014). Slc7A5, the large neutral amino acid transporter, was shown to control the expression of cMyc in T cells (Sinclair et al., 2013) and cytokine activated NK cells (Loftus et al., 2018). It was demonstrated by the same groups that Slc7A5 controlled metabolic reprograming in T cells activated by receptor ligation and cytokine activated NK cells through its control of cMyc. Loss of Slc7A5 had drastic consequences for T

cell differentiation into effector subtypes (Sinclair et al., 2013). Pharmacological inhibition of Slc7A5 results in impairment of cytokine stimulated NK cell function (Loftus et al., 2018). Similar effects of Slc7A5 inhibition were seen in receptor ligation mediated NK cell function in this study. Thus, in NK1.1+IL2 stimulated NK cells, cMyc expression is induced by signalling through NK1.1 ligation and IL2R and maintained by the uptake of amino acids through the Slc7A5 transporter. Increased levels of cMyc are essential for NK1.1+IL2 mediated metabolic and functional responses.

Srebp proteins were originally described as important transcription factors for *de novo* lipid synthesis as they control the transcription of genes involved in cholesterol synthesis and unsaturated fatty acid synthesis (Horton et al., 2002). However, recent research by our group and by one other group has described a previously unknown and unexpected involvement of Srebp in the control of metabolism in cytokine activated NK cells (Assmann et al., 2017) and $CD8^+$ T cells (Kidani et al., 2013). In response to IL2/IL12 cytokine mediated activation of NK cells, there was a robust induction of all the Srebp target genes involved in de novo fatty synthesis and cholesterol synthesis. Likewise, there was also a robust induction in these genes in response to NK1.1+IL2 receptor mediated stimulation of NK cells. The expression of the Srebp target genes was found to be acutely sensitive to rapamycin which is supported by earlier reports that Srebp works downstream of mTORC1 and that Srebp inhibition has no effect on mTORC1 activity (Assmann et al., 2017; Porstmann et al., 2008).

Work by our group also revealed that cytokine activated NK cells do not engage in the TCA cycle to fuel OxPhos. Instead, they adopt a novel metabolic pathway called the citrate malate shuttle (CMS) whereby citrate in the mitochondria is shuttled into the cytosol with a concomitant import of malate through the antiporter Slc25a1. In the cytosol, citrate is broken down into oxaloacetate and acetyl Co-A, with the help of the enzyme ATP citrate lyase (ACLY) (Assmann

et al., 2017). Assmann et al uncovered an important link between Srebp and the CMS by demonstrating that genetic deletion of the Srebp chaperone protein SCAP, or pharmacologically inhibiting Srebp by 25-hydroxycholesterol (25HC) or PF429242 (PF) resulted in a failure to up-regulate genes encoding Slc25a1 and ACLY. Interestingly, there was a robust increase in the mRNA transcripts for *Acly* in NK1.1+IL2 stimulated NK cells.

Rigorous metabolomics analysis revealed that glucose taken up by cytokine activated NK cells was being converted into cytosolic citrate. This finding definitively linked Srebp to NK cell metabolism. Indeed, Srebp inhibition by 25HC or PF resulted in impairment of IL2/IL12 mediated metabolic response (Assmann et al., 2017). Therefore we looked at the effect of Srebp inhibition on NK1.1+IL2 induced metabolism. 25HC or PF dependent inhibition of Srebp resulted in a sharp decrease in NK1.1+IL2 induced metabolic responses in NK cells. Similar effects of Srebp inhibition seen in activated T cell metabolism support the data in this report (Kidani et al., 2013).

It has been demonstrated in both T cells and NK cells that Srebp controls effector responses mediated by these cells. Blockade of Srebp activity resulted in decreased clonal expansion, decreased IFNγ and TNFα in activated SCAP deficient CD8+ve T (Kidani et al., 2013). Similarly, the importance of Srebp for cytokine mediated NK cell effector functions was demonstrated by decreased IFNγ, Granzyme B production and impaired specific lysis of various cancer cell lines like YAC-1 and K562 seen in cytokine activated NK cells treated with Srebp inhibitors (Assmann et al., 2017). Likewise, Srebp inhibition by either 25HC or PF resulted in significant decrease in Nk1.1+IL2 mediated effector functions including IFNγ production and Granzyme B expression.

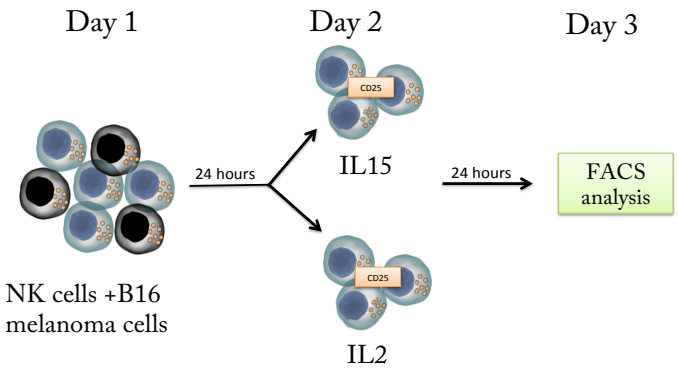
Together these data show that NK1.1+IL2 mediated metabolic responses are critically important for NK cell effector functions. The three key regulators that have also been implicated in IL2/IL12 induced metabolic responses control these metabolic pathways. Both IL12 and NK1.1 drive the expression of the high affinity IL2 receptor, CD25. Thus, it can be said that IL2 is the central driver of metabolism in activated NK cells.

Chapter 5

Natural Killer (NK) cells integrate signals received from tumour interactions and IL2 to induce robust metabolic and functional responses

5.1 Tumour interactions induce CD25 expression on NK cells to facilitate IL2 responses

In chapters 3 and 4, metabolic and functional responses induced by NK cell receptor ligation were studied in detail using a reductionist approach. An NK1.1 receptor specific antibody was used to stimulate NK cells. This led us to predict that when NK cells come in direct contact with tumour cells and their activating receptors engaged, similar signalling pathways would come into play. Using this rationale, we hypothesized that NK cells interacting with tumour cells would induce CD25 expression and thus make them more responsive to the adaptive cytokine IL2. In order to achieve this, NK cells were co-cultured with B16 murine melanoma cells in a 2:1 Effector:Target (E:T) ratio for 18 hours and then in the presence or absence of IL2 for a further 24 hours (Experimental Approach 5.1).



Experimental Approach 5.1

On Day 1, cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells overnight, washed and supplemented with low dose IL15 (7.5ng/ml) and were either left un-stimulated or stimulated with IL2 (20ng/ml) on Day 2 for a further 24 hours. On Day 3, various functional outputs were measured by FACS analysis.

A subset of NK cells co-cultured with B16 melanoma cells up-regulated the expression of CD25 **(Fig 5.1)**. It was predicted that these CD25 expressing NK

cells would be highly responsive to IL2. Indeed, when IL2 was provided (B16 +IL2) the CD25 positive NK cells produced large amounts of IFN-γ cytokine **(Fig 5.2 A,B).**

To confirm the importance of CD25 expression for IL2-induced responses we compared NK cells with high CD25 expression ($CD25^{high}$) and low CD25 expression ($CD25^{low}$) within the B16 + IL2 co-culture **(Fig 5.3A).** $CD25^{high}$ NK cells had greatly increased effector functions with elevated IFN-γ production and Granzyme B expression **(Fig 5.3 B,C, Fig 5.4).** Additionally, $CD25^{high}$ NK cells showed evidence of increased cellular metabolism; $CD25^{high}$ NK cells were larger than $CD25^{low}$ NK cells (**Fig 5.5 A**) and had increased expression of the nutrient transporter CD71 **(Fig 5.5 B,C)**.

In chapter 3, it was shown that NK1.1 stimulation faciliated increased IL2-dependent signal transduction through the mTORC1 pathway. Similarly, in B16+IL2 co-cultures, $CD25^{high}$ NK cells showed evidence of elevated IL2 signalling compared to the $CD25^{low}$ NK cells in the same culture. $CD25^{high}$ NK cells had increased levels of mTORC1 signalling, as determined by the levels of phosphorylated S6 ribosomal protein **(Fig5.6)**, and increased expression of the transcription factor cMyc **(Fig5.7)**.

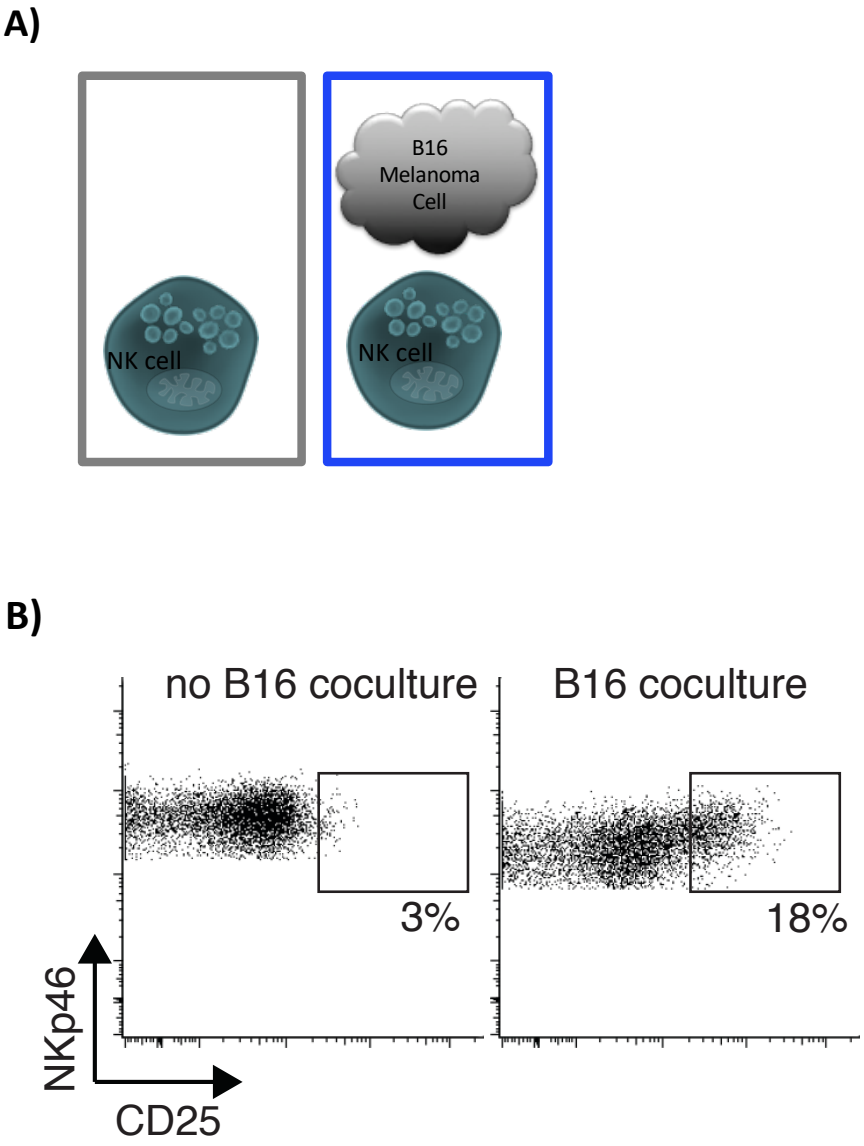


Figure 5.1 NK cells co-cultured with B16 exhibit elevated CD25

(A-B) Cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells for 18 hours, washed and supplemented with low dose IL15 (7.5ng/ml). CD25 expression was measured by flow cytometry. (A) Schematic representation of the experimental approach, (B) Representative dot plots showing CD25 expression.

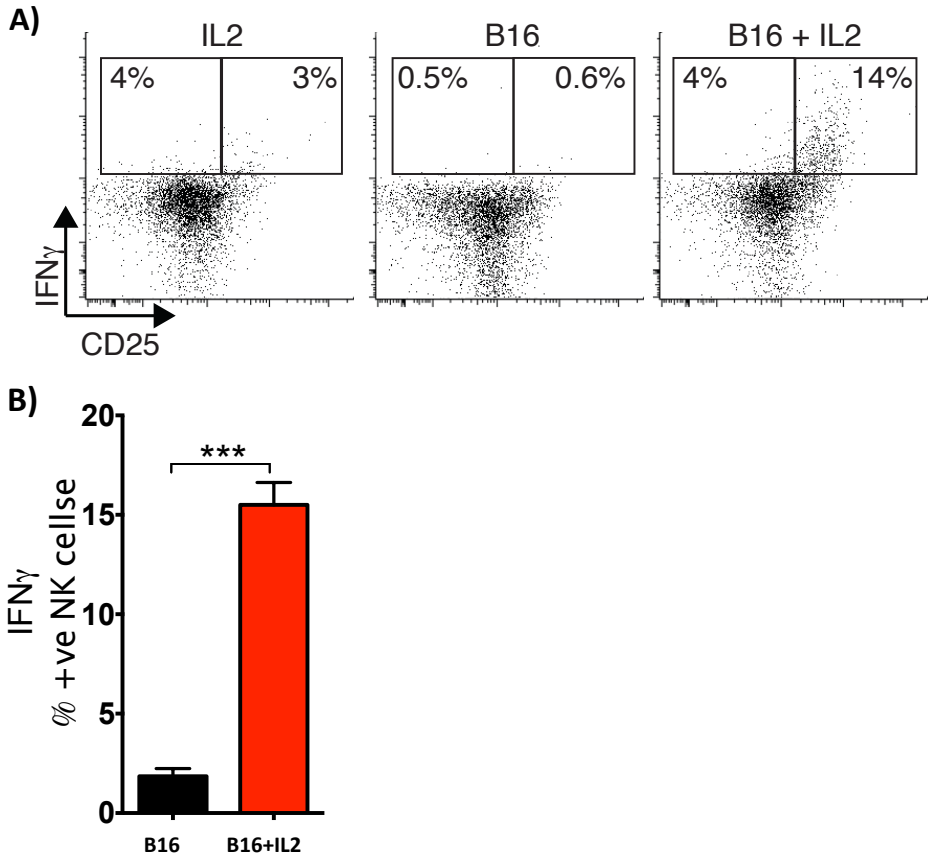


Figure 5.2 B16 co-cultured NK cells are more responsive to IL2 cytokine
(A-C) Cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells for 18 hours; washed and supplemented with low dose IL15 (7.5ng) and were either left un-stimulated or stimulated with IL2 (20ng/ml) for a further 24 hours. IFN-γ expression in $CD25^{high}$ NK cells was measured by flow cytometry. Data is representative (A) or mean +/- SEM (B) of 5 independent experiments. Data was analysed using student's t-test. (***$p < 0.001$).

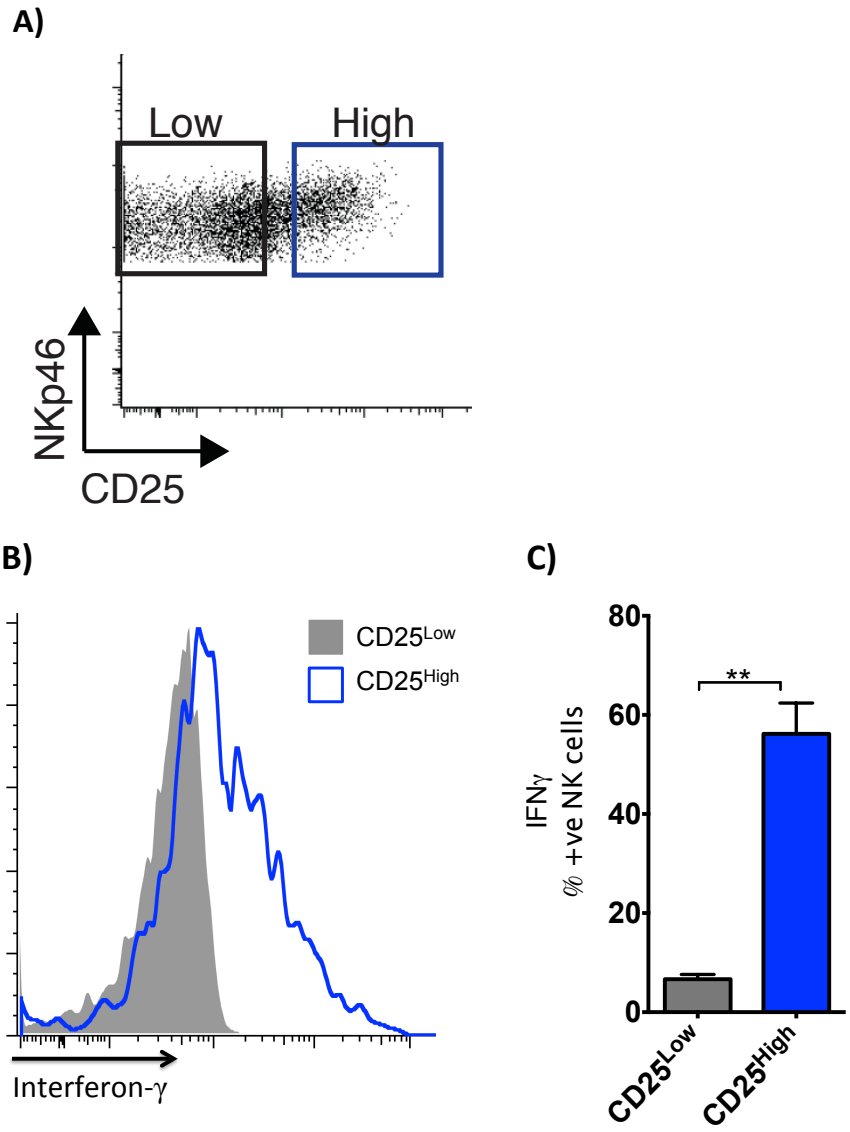


Figure 5.3 CD25 ^{high} NK cells express higher levels of IFN-γ

(A-C) Cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells for 18 hours, washed and stimulated with IL2 (20ng/ml) for a further 24 hours. CD25^{high} cells and CD25^{low} cells were determined by analysing CD25 expression by flow cytometry (A). CD25^{high} cells were compared to CD25^{low} cells. IFN-γ expression was measured by flow cytometry. Data is representative (A,B) or mean +/- SEM (C) of 5 independent experiments. Data was analysed using student's t-test. (**p < 0.01).

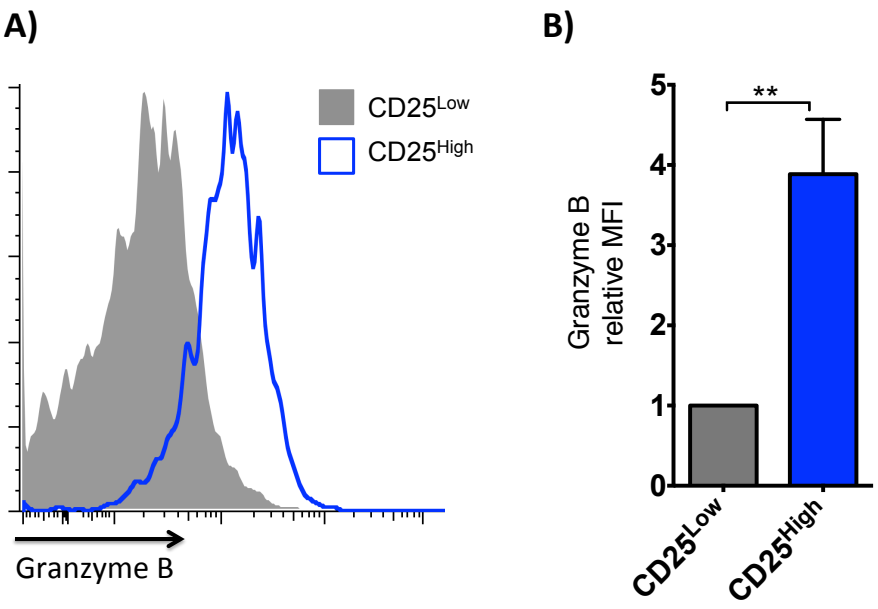


Figure 5.4 CD25 ^{high} NK cells express higher levels of Granzyme B

(A-B) Cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells for 18 hours, washed and stimulated with IL2 (20ng/ml) for a further 24 hours. CD25^{high} cells were compared to CD25^{low} cells by flow cytometry. Granzyme B expression was measured by flow cytometry. Data is expressed as relative to CD25^{high} cells (B). Data is representative (A) or mean +/- SEM (B) of 5 independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1. (**$p < 0.01$)

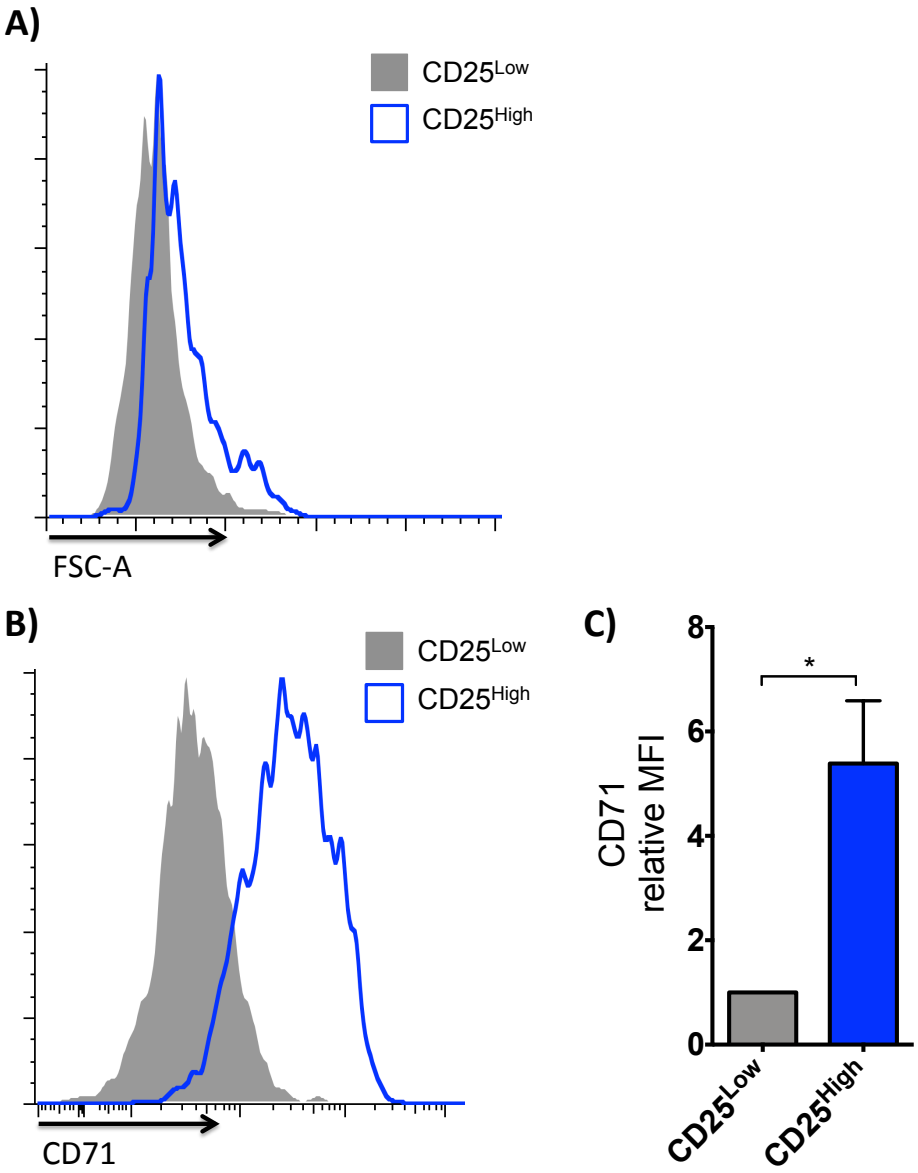


Figure 5.5 CD25^{high} NK cells are bigger in size and express increased levels of CD71

(A-C) Cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells for 18 hours, washed and stimulated with IL2 (20ng/ml) for a further 24 hours. CD25^{high} cells were compared to CD25^{low} cells by flow cytometry. Cell size (FSC-A) and CD71 expression was measured by flow cytometry. Data is representative (A,B) or mean +/- SEM (C) of 4 independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1. (*$p < 0.05$)

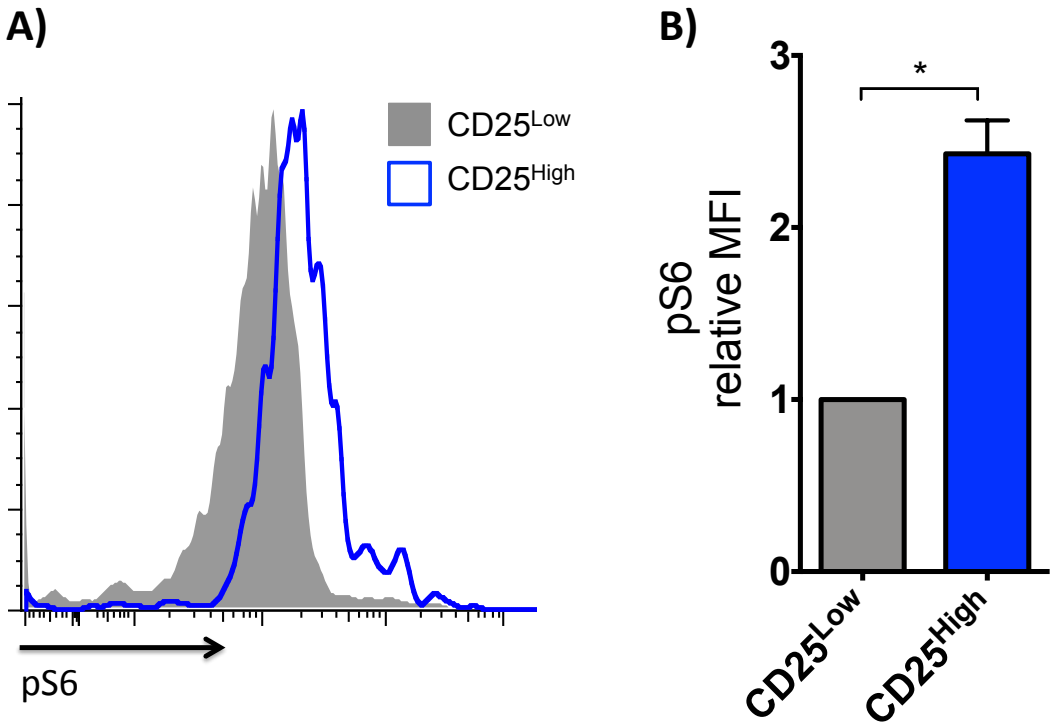


Figure 5.6 CD25 ^{high} NK cells have increased mTORC1 activity

(A-B) Cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells for 18 hours, washed and stimulated with IL2 (20ng/ml) for a further 24 hours. $CD25^{high}$ cells were compared to $CD25^{low}$ cells by flow cytometry. Phosphorylation of S6 was measured by flow cytometry. Data is representative (A) or mean +/- SEM (B) of 3 independent experiments. Data was analysed using a one-sample student's t-test against a theoretical value of 1. (*$p < 0.05$)

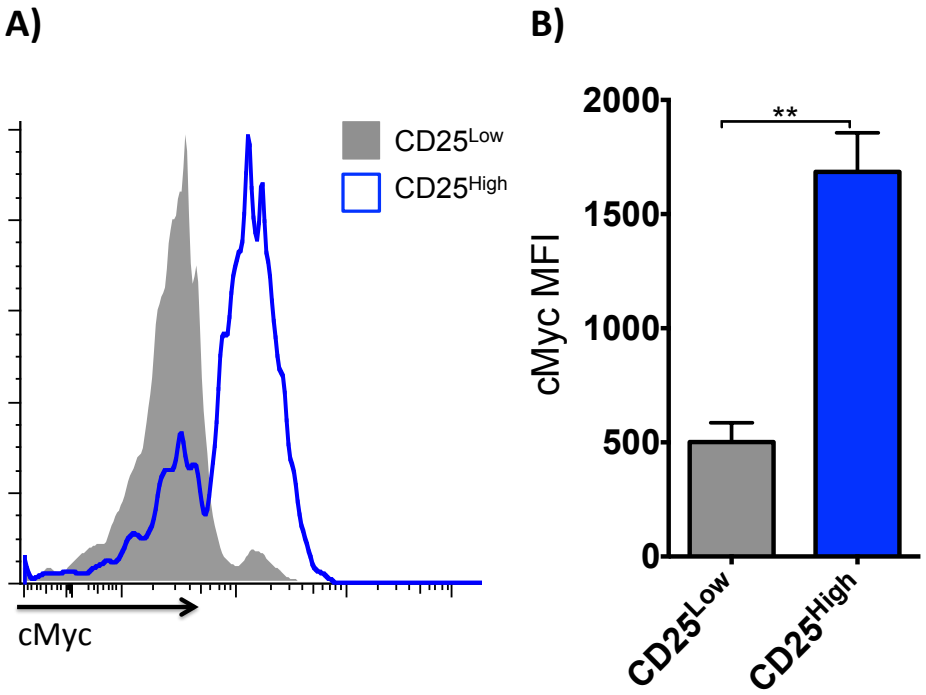


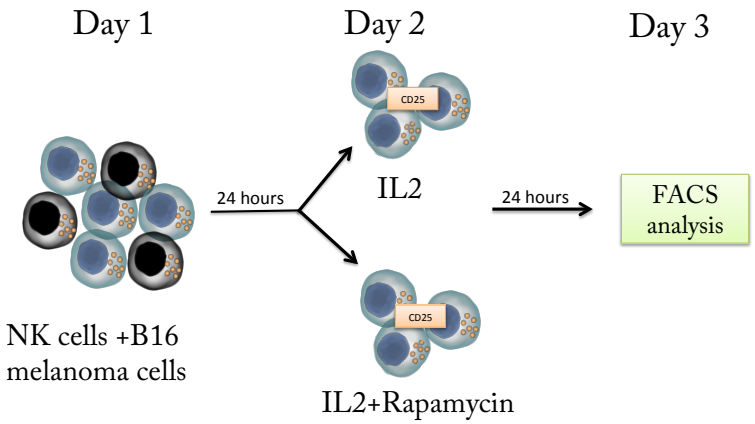
Figure 5.7 CD25 ^{high} NK cells have increased expression of cMyc

(A-B) Cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells for 18 hours, washed and stimulated with IL2 (20ng/ml) for a further 24 hours. CD25^{high} cells were compared to CD25^{low} cells by flow cytometry. cMyc expression was analysed by flow cytometry. Data is representative (A) or mean +/- SEM (B) of 3 independent experiments. Data was analysed using paired student's t-test. (**$p < 0.01$)

5.2 mTORC1 is required for elevated IL2-induced responses in CD25^{high} NK cells

To investigate whether mTORC1 signalling was required for the elevated IL2-induced NK cell reponses observed in CD25^{high} NK cells, the co-culture expriments were performed in the presense or absense of the mTORC1 specific inhibitor rapamycin.

To investigate the impact of mTORC1 inhibition on CD25^{high} NK cells, cultured NK cells were purified by magnetic bead cell sorting and co-cultured with B16 melanoma cells for 18 hours. Following the 18-hour incubation, the cells were stimulated with IL2 in the presence or absence of rapamycin for an additional 24 hours. Inhibition of mTORC1 signalling was confirmed in rapamycin treated CD25^{high} NK cells as a reduction in pS6 levels **(Fig 5.8).** Rapamycin treated CD25^{high} NK cells showed decreased cell size, indicative of reduced cell growth **(Fig 5.9A),** and a significant reduction in the expression of the transferrin receptor CD71 **(Fig 5.9B,C)**. mTORC1 inhibition also led to reduced NK cell effector functions in CD25^{high} NK cells with a significant decrease in IFN-γ production **(Fig 5.10 A,B)** and in Granzyme B expression **(Fig 5.10 C,D).**



Experimental Approach 5.2

On Day 1, cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells overnight, washed and supplemented with low dose IL15 (7.5ng/ml) and were stimulated with IL2 (20ng/ml) in the presence or absence of Rapamycin (20nM) on Day 2 for a further 24 hours. On Day 3, various functional outputs were measured by FACS analysis.

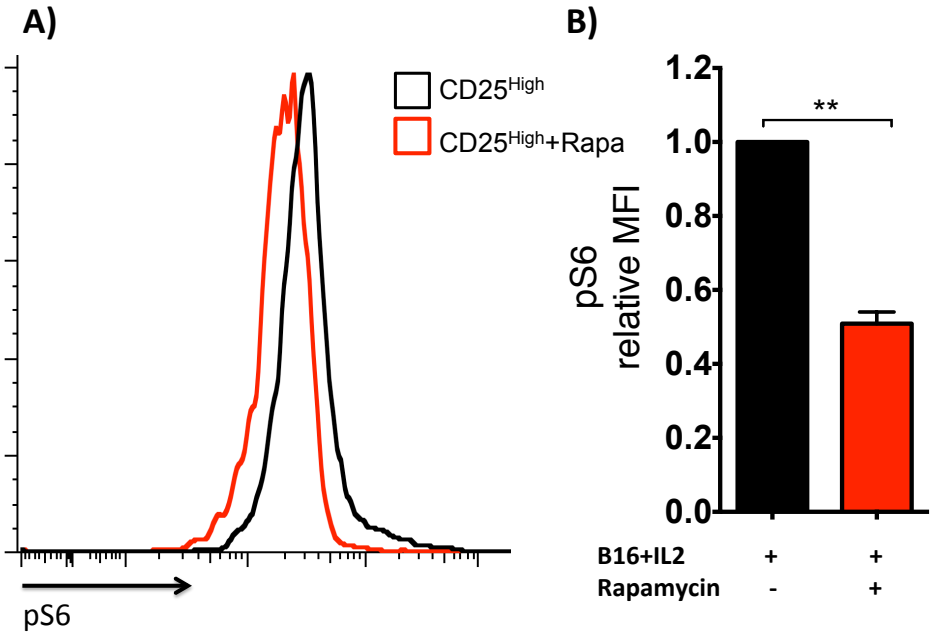


Figure 5.8 Rapamycin treatment results in decreased phosphorylation of the mTORC1 substrate S6

(A-B) Cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells for 18 hours, washed and stimulated with IL2 (20ng/ml) in the presence or absence of Rapamycin (20nM) for a further 24 hours. Phosphorylation of S6 in $CD25^{high}$ cells was analysed by flow cytometry. Data is expressed as relative to untreated cells (B). Data is representative (A) or mean +/- SEM (B) of 3 independent experiments. Data was analysed using a one-sample students t-test against a theoretical value of 1. (**$p < 0.01$)

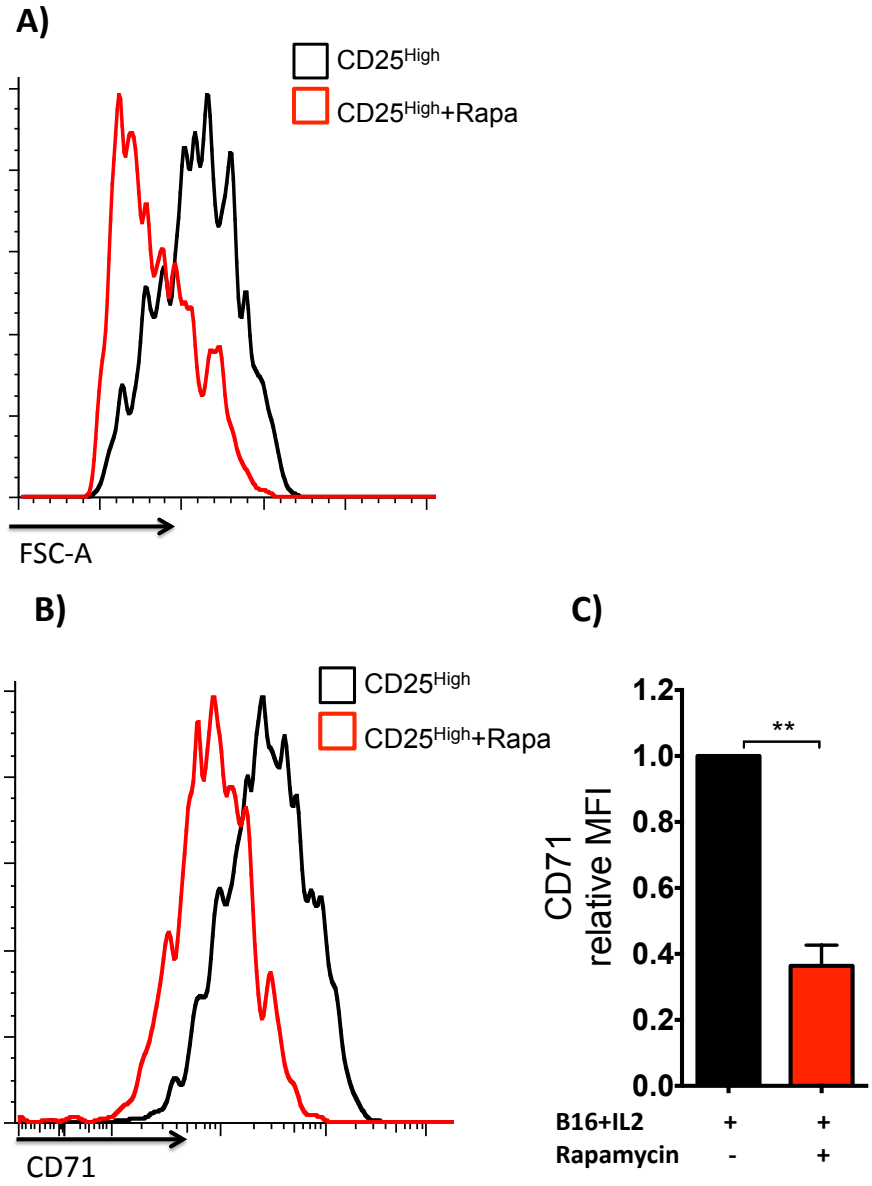


Figure 5.9 mTORC1 inhibition results in impaired blastogenesis and CD71 expression in CD25^{high} NK cells.

(A-C) Cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells for 18 hours, washed and stimulated with IL2 (20ng/ml) in the presence or absence of Rapamycin (20nM) for a further 24 hours. Cell size (FSC-A) and CD71 expression in CD25^{high} cells was analysed by flow cytometry. Data is expressed as relative to untreated cells (C). Data is representative (A,B) or mean +/- SEM (C) of 4 independent experiments. Data was analysed using a one-sample students t test against a theoretical value of 1. (**$p < 0.01$)

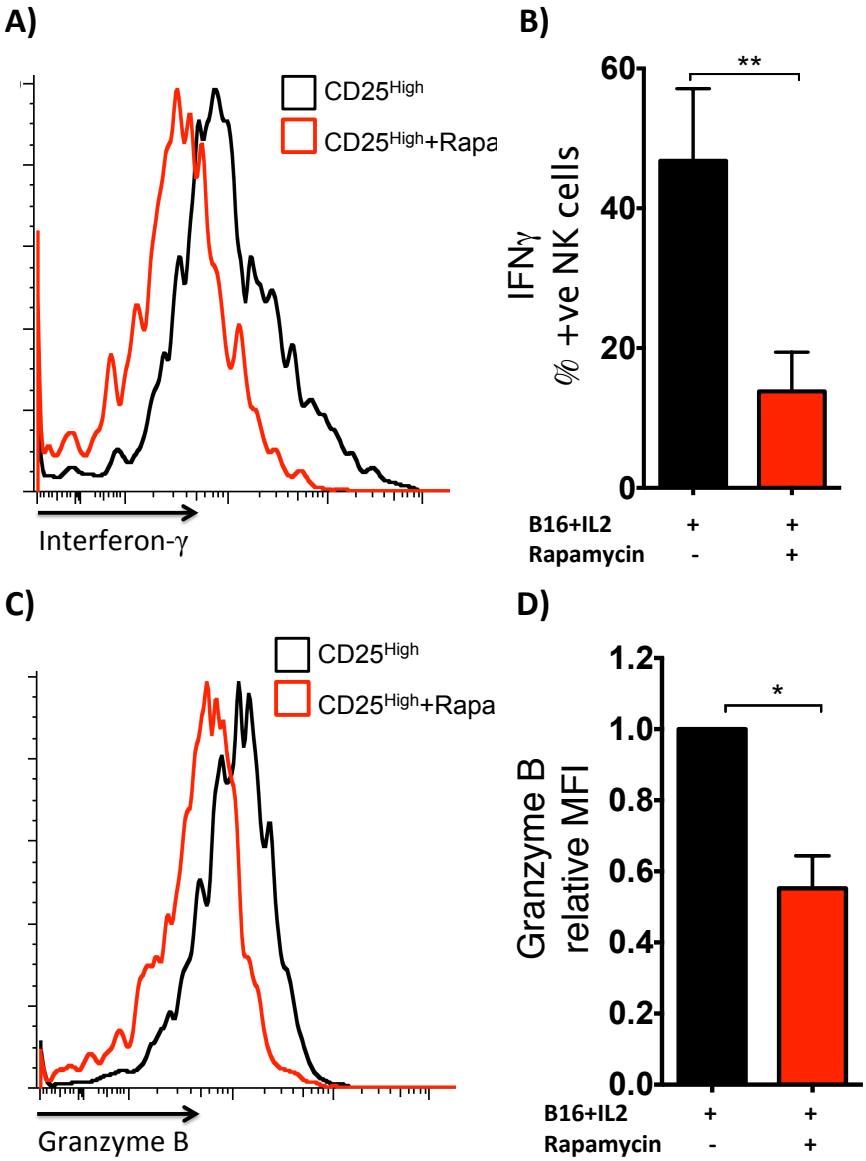


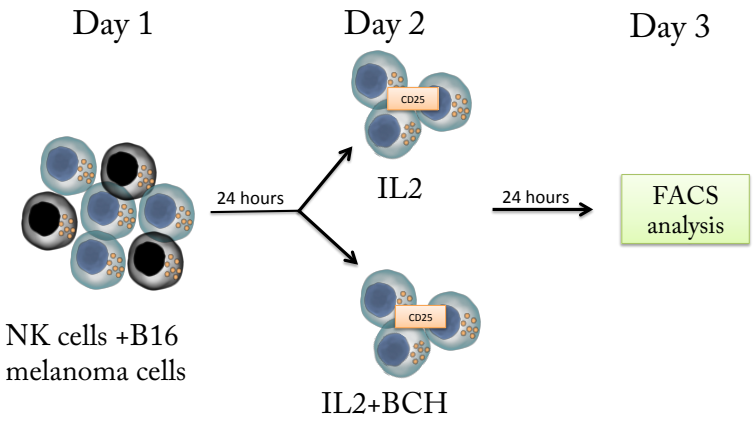
Figure 5.10 mTORC1 inhibition results in impaired effector responses in CD25 ^{high} NK cells

(A-D) Cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells for 18 hours, washed and stimulated with IL2 (20ng/ml) in the presence or absence of Rapamycin (20nM) for a further 24 hours. IFN-γ and Granzyme B expression in CD25^{high} cells was analysed by flow cytometry. Data is expressed as relative to untreated cells (B,D). Data is representative (A,C) or mean +/- SEM (B) of 4 independent experiments. Data was analysed using a one-sample students t test against a theoretical value of 1. (**$p < 0.01$; ns, non-significant).

5.3 Slc7A5-mediated mTORC1 and cMyc siganlling is required for metabolism, function and survival of CD25^{high} NK cells

In chapter 4, it was shown that NK cells stimulated via NK1.1 receptor ligation plus IL2 express high levels of cMyc and the metabolic and functional responses in these cells were highly dependent on cMyc expression. NK cells interacting with tumors upregulate their cMyc expression as seen in Fig 5.7. Therefore, we considered whether cMyc suppression had an effect on signalling, metabolism and function in CD25^{high} cells upon tumor interactions.

As explained in chapter 4, the inhibitor of Slc7A5, the nutrient transporter integrally linked to cMyc expression, was used to suppress cMyc expression.
Cultured NK cells were purified by magnetic bead cell sorting and were co-cultured with B16 melanoma cells for 18 hours, following which the NK cells were washed out and stimulated with IL2 in the presence or absence of the Slc7A5 inhibitor BCH for an additional 24 hours. As expected, BCH treated CD25^{high} NK cells showed reduced expression of cMyc **(Fig 5.11A,B**). There is evidence that leucine that's taken up through the Slc7A5 transporter is essential for sustaining mTORC1 activity (Ananieva et al., 2016; Sinclair et al., 2013; Wolfson et al., 2016). Therefore, we predicted that Slc7A5 inhibition would affect mTORC1 activity as well. Indeed, when mTORC1 activity was assessed by measuring pS6 levels, there was a cosiderable reduction in CD25^{high} NK cells that were treated with BCH compared to untreated NK cells **(Fig 5.11C,D).**



Experimental Approach 5.3

On Day 1, cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells overnight, washed and supplemented with low dose IL15 (7.5ng/ml) and were stimulated with IL2 (20ng/ml) in the presence or absence of BCH (25mM) on Day 2 for a further 24 hours. On Day 3, various functional outputs were measured by FACS analysis.

Due to limited cell numbers, detailed metabolic analyses using the Seahorse analyser could not be performed. Therefore, effects of BCH induced cMyc suppression on cell growth was assessed as an indication of its impact on metabolism. Cell size was measured by analysing forward scatter-area (FSC-A) by flow cytometry. BCH treatment had a pronounced effect on cell growth in $CD25^{high}$ NK cells **(Fig 5.12 A).** Next, we looked at CD71, which, apart from being a marker for increased metabolic demands, is also an established cMyc target (O'Donnell et al., 2006). Therefore, as a confirmation of cMyc suppression, CD71 was assessed in BCH treated $CD25^{high}$ NK cells, and indeed, there was a sharp decrease in CD71 expression in BCH treated NK cells **(Fig 5.12B,C).**

This led us to consider whether NK cell functional responses in $CD25^{high}$ NK cells were also impacted by cMyc inhibition. Indeed, cMyc inhibition in $CD25^{high}$ NK cells led to a substantial decrease in IFN-γ **(Fig 5.13 A,B)** and a concommitant decrease in Granzyme B production **(Fig 5.13 C,D).**

It is well evidenced, that NK cell receptor interaction with target cells causes activation induced cell death (Asea and Stein-Streilein, 1998; Jewett and Bonavida, 1995; Jewett and Bonavida, 1996; Taga et al., 1996). It was also demonstrated in chapter 3 that NK1.1 receptor ligation causes active cell death in NK cells that is rescued by the addition of IL2. Therefore we considered what the impact of inhibition of IL2 associated signalling pathways was on NK cell viability following tumor interaction. There was a drastic effect on cell viability in BCH treated NK cells. However, NK cells treated with Rapamycin had no effect on cell viability **(Fig 5.14).** Indeed, it has been shown in other cell types that mTORC1 inhibition increases cell viability in hypoxic conditions by inducing autophagy, reducing oxidative damage and inhibiting BAX, the pro-apoptotic protein and upregulating its counterpart, Bcl2 (Park et al., 2017; Zimmerman et al., 2018).

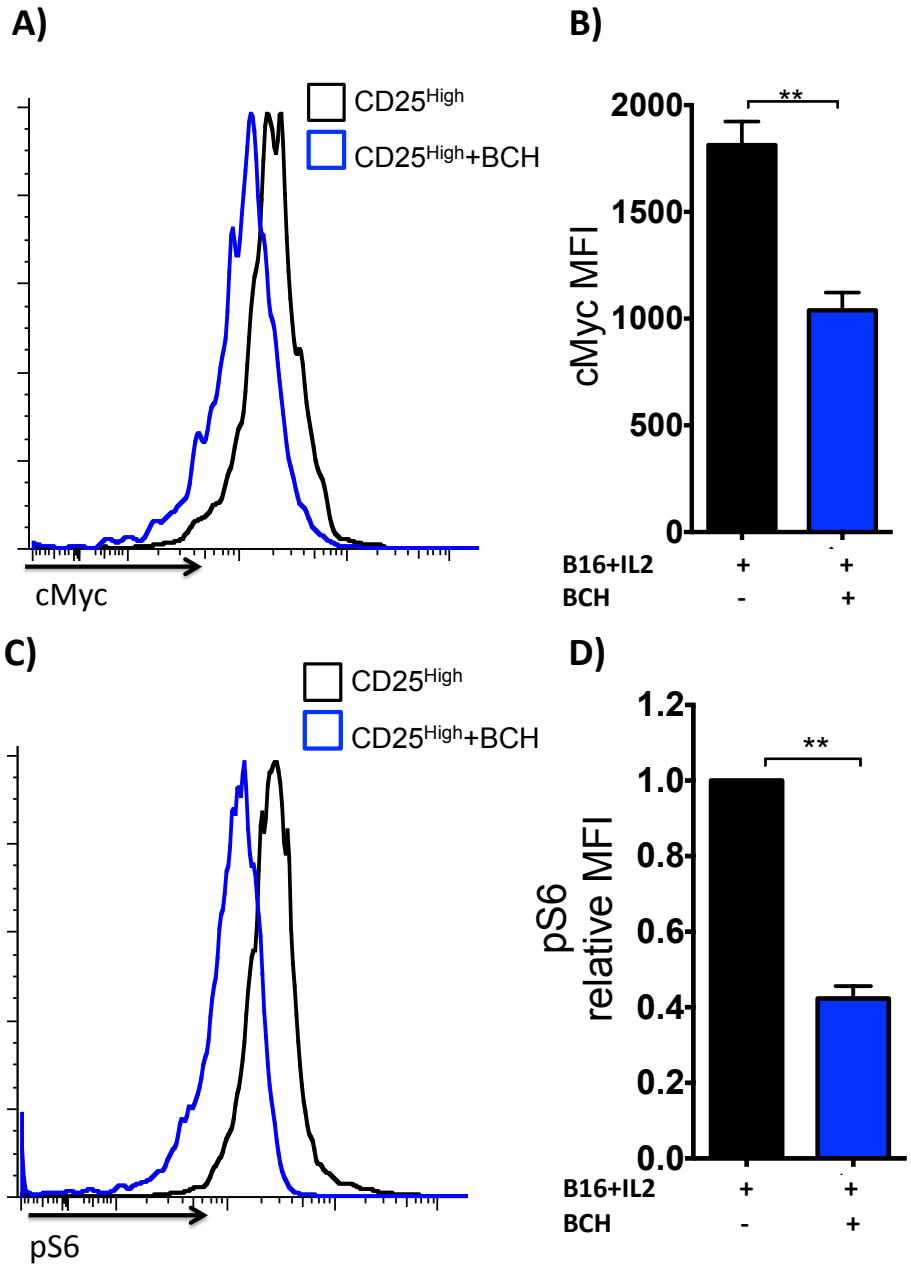


Figure 5.11 Slc7A5 inhibition results in decreased cMyc expression and decreased mTORC1 acitivity in CD25^{high} NK cells

(A-D) Cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells for 18 hours, washed and stimulated with IL2 (20ng/ml) in the presence or absence of BCH (25mM) for a further 24 hours. cMyc and pS6 expression in CD25^{high} cells was analysed by flow cytometry. Data is expressed as relative to untreated cells (C). Data is representative (A,C) or mean +/- SEM (B,D) of 3 independent experiments. Data was analysed using a one-sample students t-test against a theoretical value of 1. (**$p < 0.01$)

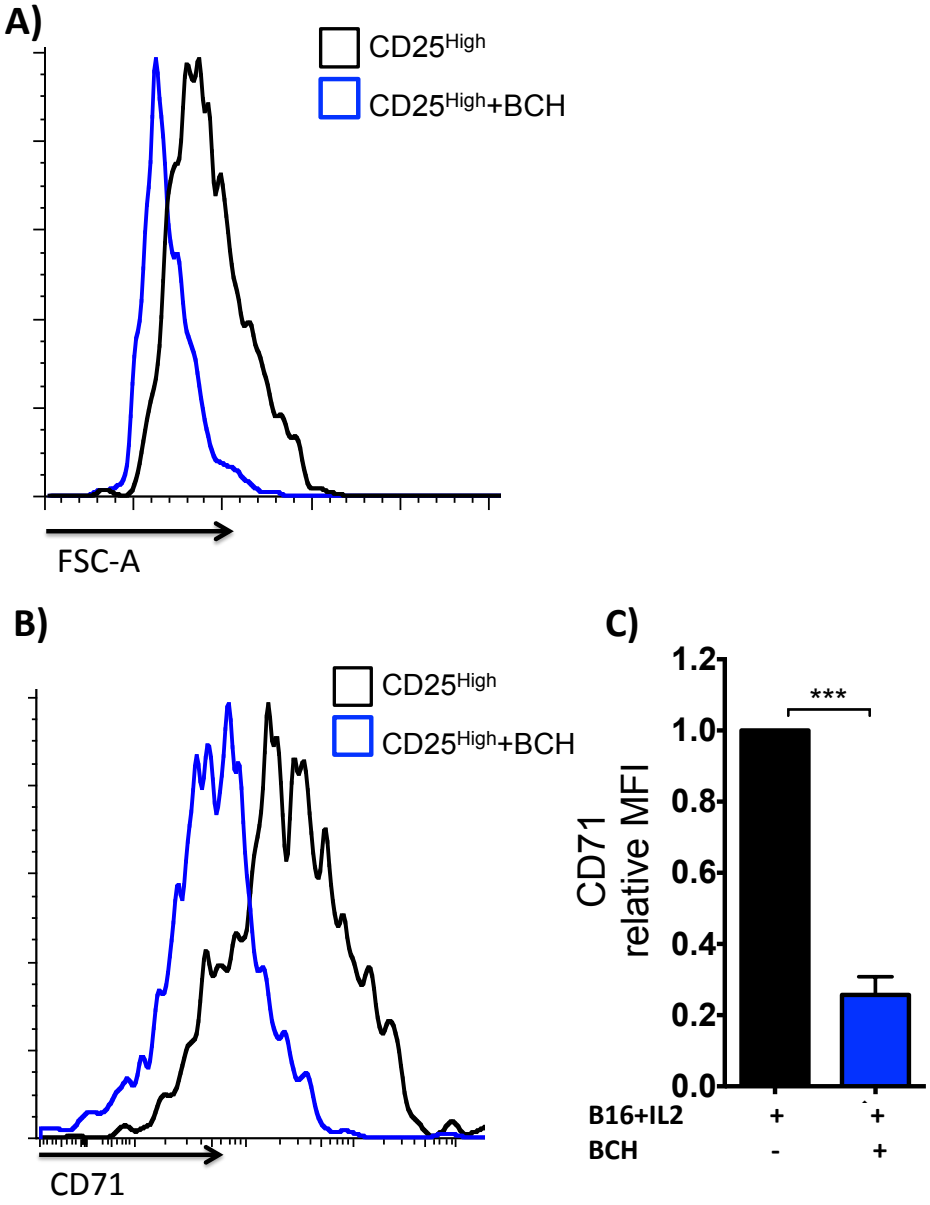


Figure 5.12 Slc7A5 inhibition results in impaired blastogenesis and CD71 expression in CD25^{high} NK cells.

(A-C) Cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells for 18 hours, washed and stimulated with IL2 (20ng/ml) in the presence or absence of BCH (25mM) for a further 24 hours. Cell size (FSC-A) and CD71 expression in CD25^{high} cells was analysed by flow cytometry. Data is expressed as relative to untreated cells (C). Data is representative (A,B) or mean +/- SEM (C) of 4 independent experiments. Data was analysed using a one-sample students t test against a theoretical value of 1. (***$p < 0.001$).

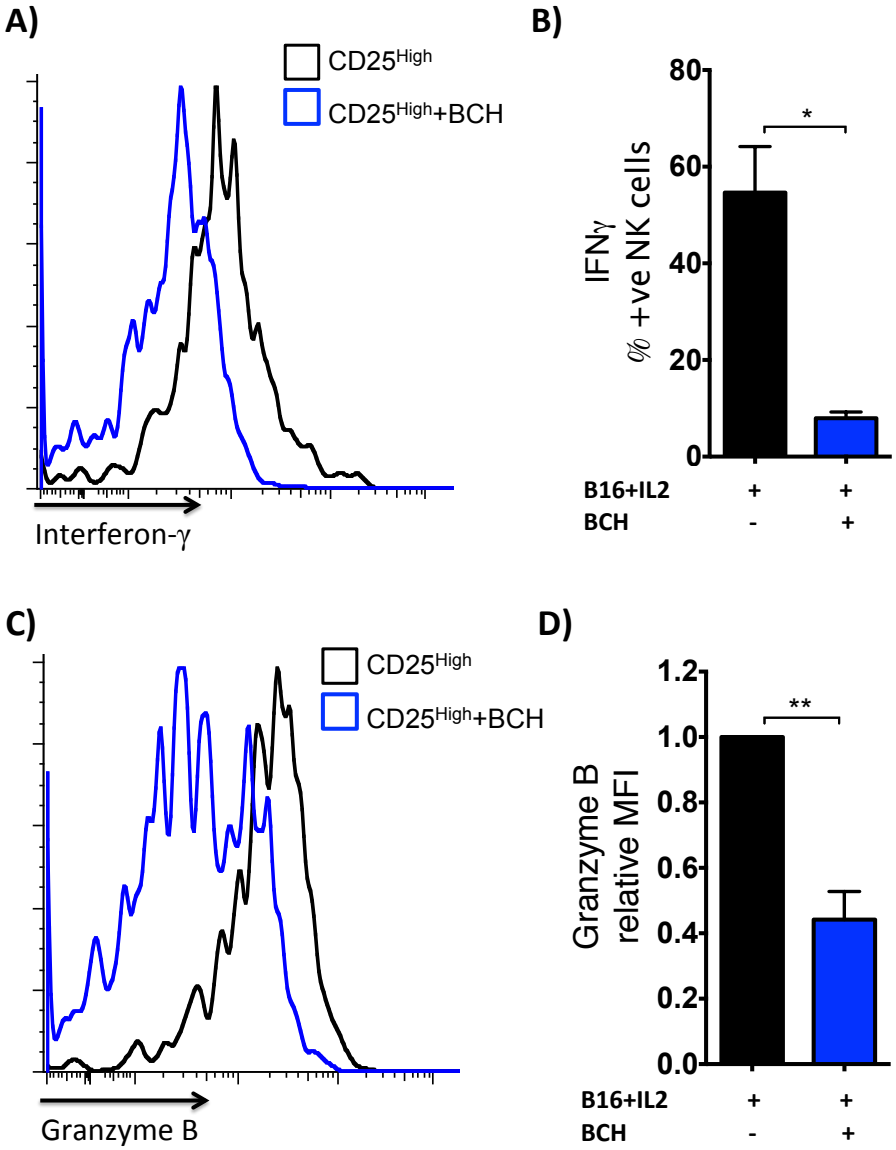


Figure 5.13 Slc7A5 inhibition results in impaired effector responses in CD25^{high} NK cells

(A-D) Cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells for 18 hours, washed and stimulated with IL2 (20ng/ml) in the presence or absence of BCH (25mM) for a further 24 hours. IFN-γ and Granzyme B expression in CD25^{high} cells was analysed by flow cytometry. Data is expressed as relative to untreated cells (B,D). Data is representative (A,C) or mean +/- SEM (B) of 4 independent experiments. Data was analysed using a paired student's t-test (B) or a one-sample students t test against a theoretical value of 1(D). (*$p < 0.05$, **$p < 0.01$).

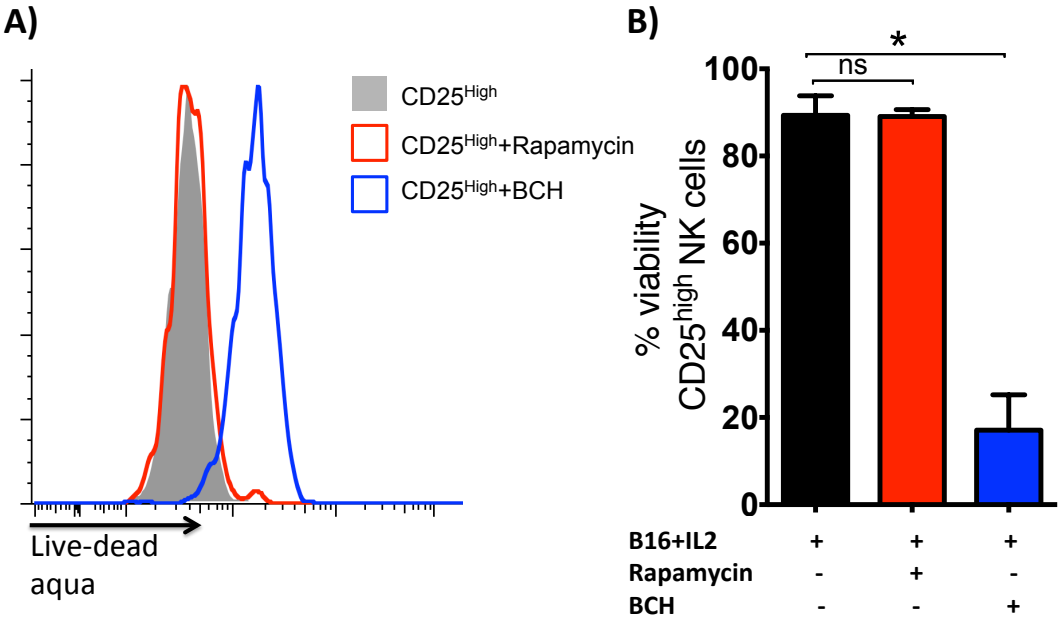


Figure 5.14 Slc7A5 inhibition but not mTORC1 inhibition results in cell death in CD25^{high} NK cells

(A-B) Cultured NK cells (6 days in low dose IL15) were MACS-purified and co-cultured with B16 melanoma cells for 18 hours, washed and stimulated with IL2 (20ng/ml) in the presence or absence of Rapamycin (20nM) or BCH (25mM) for a further 24 hours. Cell viability was measured by Live-Dead Aqua staining. Data is representative (A) or mean +/- SEM (B) of 4 independent experiments. Data was analysed using one-way ANOVA followed by Tukey's multiple comparison post-test. (*$p < 0.05$; ns, non-significant).

Taken together these data highlight the importance of CD25 expression upon NK cell-target cell interaction. CD25 expression is essential for making NK cells more responsive to IL2 cytokine. Increased IL2 sensitivity allows for enhanced signalling through the IL2 associated signalling pathways involving mTORC1 and cMyc, the master regulators of metabolism in NK cells. This study also reveals the impact of inhibition of these pathways on NK cell responses against tumour cells. This would be especially relevant for tumour infiltrating lymphocytes that highly depend on metabolic reprograming to execute their functional responses but are severely nutrient deprived and hence compromised.

Chapter 5 Discussion

In chapters 3 and 4, we studied the metabolic and functional responses in NK cells stimulated through the NK1.1 receptor, the archetypal NK cell activatory receptor. Next, we used a broader approach to study NK cell responses following interactions with tumour cells. Co-culturing NK cells with B16 melanoma cells resulted in a substantial increase in CD25 expression on a proportion of NK cells leading us to predict that these cells would be more responsive to IL2 cytokine. IL2 has been shown to enhance NK cell responses in many instances. For example, IL2 was shown to increase serial killing by NK cells (Bhat and Watzl, 2007). IL2 was also shown to be necessary for NK1.1 induced proliferation (Reichlin and Yokoyama, 1998). IL2 activated NK cells that were stimulated with anti-NK1.1 antibody had an increased specificity in terms of cytotoxic targets (Karlhofer and Yokoyama, 1991). NK cells were made more efficient at killing weak targets in the presence of IL2 achieved by depletion of Tregs (Gasteiger et al., 2013b). However, the mechanisms by which receptor ligated NK cells become more responsive to IL2 haven't been fully explained.

In this report, for the first time we show that a proportion of NK cells co-cultured with tumour cells up-regulate CD25 expression, the high affinity IL2 receptor subunit. Moreover, these $CD25^{high}$ NK cells demonstrated heightened metabolic and functional responses to IL2 when compared to their $CD25^{low}$ counterparts in the same co-culture. Following the addition of IL2, $CD25^{high}$ NK cells produced high levels of IFN-γ and expressed higher amounts of Granzyme B compared to $CD25^{low}$ NK cells. The experiments also show the importance of metabolic signalling through mTORC1 and cMyc for these elevated effector functions. These new data highlight a mechanism through which NK cells can span the innate and adaptive immune system. Innate and adaptive immune responses are known to collaborate and the idea that innate and adaptive immune systems work independently is almost out-dated.

Especially in case of NK cells, there is a growing body of evidence, demonstrating their crosstalk with adaptive lymphocytes. Indeed, IFN-γ from NK cells restricts B cell transformation by Epstein Barr virus (Strowig et al., 2008). IL12 activation of NK cells is important for the induction of Th1 responses against *Leishmania major* infection (Scharton-Kersten et al., 1995). Not only is the early IFN-γ known to promote Th1 responses, it is also directly involved in controlling *P.falciparum* infection (Horowitz et al., 2010). IL2 itself has been described to link NK cells to the adaptive immune system. IL12 induced CD25 expression on NK cells makes them more responsive to the T cell derived cytokine IL2 (Lee et al., 2012). Donnelly et al showed that IL12 facilitated IL2 signalling in NK cells makes them highly glycolytic in an mTORC1 dependent manner (Donnelly et al., 2014). There is subset of CD56 bright NK cells that are more responsive to T cell derived IL2, possibly because they express high CD25 (Fehniger et al., 2003). Apart from activation, the regulation of NK cells is also linked to the adaptive immune system. Tregs also express high CD25, and they compete with NK cells for the T cell derived IL2, thus contributing in NK cell homeostasis (Gasteiger et al., 2013a).

Although in normal physiological conditions Treg competition for IL2 is beneficial in containing NK cell mediated immune response, it is often a stumbling block for IL2 based immunotherapies. High-dose IL2 is approved as a therapy for melanoma and renal carcinoma (Rosenberg et al., 1985). However IL2 therapy has been reported to have toxic effects due to its action on other immune cells that results in the inevitable cytokine secretion (Ljunggren and Malmberg, 2007; Smyth et al., 2002). Additionally, high dose IL2 therapy causes vascular leak syndrome, which leads to accumulation of intravascular fluid in organs such as lungs and liver eventually leading to pulmonary oedema (McDermott and Atkins, 2004; Nakagawa et al., 1996). Moreover, IL2 treatment has low efficacy rates due to its effect on Treg cells which also express CD25 and can compete with NK cells to block anti-tumour immune response (Sakaguchi et al., 2001).

However, a combination therapy where IL2 is administered in conjunction with checkpoint inhibitor blockade has shown promising results (Caudana et al., 2019; Porichis et al., 2018). To counter Tregs competition for IL2, new genetically modified IL2 cytokine has been designed that has higher affinity for IL2Rβ and works independently of CD25 (Levin et al., 2012). Another version of IL2 called IL2Cx also bypasses activation through CD25, and was designed to avoid the activation of lung endothelial cells that caused pulmonary oedema, adding to the toxic effects of high dose IL2 therapy (Caudana et al., 2019). My research suggests that these kind of mutant-IL2 therapies might prove extremely beneficial in inducing anti-tumour responses in most NK cells instead of only a subset of NK cells that express high CD25. Although a caveat in these therapies might be a risk of uncontrollable immune response with no Tregs to contain it. A novel fusion receptor protein was also designed in combination with IL2, that works by selectively activating IL2 cells only on NKG2D bearing cells (Ghasemi et al., 2016).

Although promising in haematological malignancies, these strategies fall short in solid tumour microenvironments. Tumour microenvironments are extremely hypoxic and nutrient deprived. Our research highlights the importance of availability of nutrients for proper activation of immune cells. This report demonstrates that the NK cells that were the most responsive against tumour cells, the $CD25^{high}$ NK cells, were larger than $CD25^{low}$ NK cells and had elevated levels of mTORC1 and cMyc signalling and increased expression of the cMyc target gene CD71, the transferrin receptor. This suggests that the highly responsive $CD25^{high}$ are also metabolically more active. This supports the idea that tumour infiltrating NK cells have elevated demands for nutrients to support high metabolic rates and optimal functional responses. One key reason that cancer immunotherapies fail in solid tumours is because the tumour microenvironment is a metabolically restrictive site that impairs the metabolism and function of infiltrating immune cells.

My research and that of others (Assmann et al., 2017; Donnelly et al., 2014; Loftus et al., 2018) support the idea that IL2 is a key molecule for driving the signalling pathways that control the metabolic and functional responses in activated NK cells. mTORC1 which is upregulated in NK cells that have interacted with tumour cells, is sustained by IL2 signalling. In chapter 3, it was demonstrated that pS6 levels were significantly higher in NK cells stimulated by NK1.1+IL2 compared to those stimulated by either NK1.1 or IL2 alone. That result highlighted the importance of integration of these two signals. In this chapter, we show that NK cells become more responsive to IL2 cytokine having been in contact with tumour cells. Indeed, our data shows that inhibiting the IL2 dependent signalling pathways in NK cells that have been primed with tumour cell interactions results in a failure to induce key effector functions. Inhibition of mTORC1 with rapamycin resulted in significant decreases in functional responses in the highly responsive $CD25^{high}$ NK cells with reduced IFN-γ and Granzyme B expression. Rapamycin treatment also impeded growth and resulted in reduced CD71 expression in these cells. This result emphasizes the importance of mTORC1 activity in NK cells for anti-tumour responses. mTORC1 activation is highly dependent on nutrient availability in the microenvironment (Hara et al., 1998; Laplante and Sabatini, 2009). Indeed, glucose, glutamine, leucine and arginine deprivation can lead to the inhibition of mTORC1 signalling in mammalian cells (Hara et al., 1998; Sancak et al., 2008; Son et al., 2019; Wang et al., 1998).

The other key metabolic regulator that we have shown to be critical for receptor mediated NK cell metabolic and functional responses was cMyc. cMyc is also known to be driven by IL2 signalling (Lord et al., 2000). The tumour interacting $CD25^{high}$ cells express high levels of cMyc in response to IL2. Based on the findings by (Sinclair et al., 2013) and (Loftus et al., 2018) that cMyc expression is sustained by amino acid transport through the Slc7A5 transporter and inhibition of this transporter results in the loss of cMyc, we hypothesized that Slc7A5 inhibition of NK cells primed with tumour cells and stimulated with IL2

would result in the loss of cMyc in these cells. Indeed, $CD25^{high}$ NK cells activated under Slc7A5 inhibition failed to sustain cMyc expression. Inhibition of Slc7A5 that transports leucine into the cells also resulted in an expected loss of mTORC1 activity. Slc7A5 dependent loss of cMyc activity also resulted in impaired effector functions along with reduced cellular growth and an expected suppression of CD71 expression, a cMyc target (O'Donnell et al., 2006). This finding is corroborated by other research that identified cMyc as being critical for NK and T cell activation and effector responses (Loftus et al., 2018; Wang and Green, 2012).

Together these data suggest that in solid tumours where the environment is metabolically restrictive, it is likely that NK cells would fail to sustain cellular metabolism leading to functional exhaustion, as has been observed (Baginska et al., 2013).

NK cell receptor interaction with target cells is known to cause activation induced cell death (Asea and Stein-Streilein, 1998; Jewett and Bonavida, 1995; Jewett and Bonavida, 1996; Taga et al., 1996). However, IL2 is known to increase NK cell longevity and enhance serial killing by NK cells (Lord et al., 2000; Watzl and Long, 2010). We also demonstrated in chapter 3 that NK1.1 receptor ligation causes active cell death in NK cells that is rescued by the addition of IL2. In addition to functional exhaustion, NK cells from solid tumours have been demonstrated to display poor persistence and short life span (Barth et al., 2016; Konjevic et al., 2012). We hypothesized that IL2-induced signalling and metabolism would be important for the viability of tumour interacting $CD25^{high}$ NK cells. Indeed, inhibition of amino acid uptake through Slc7A5, which resulted in reduced mTORC1 and cMyc signalling, resulted in the death of $CD25^{high}$ NK cells. In contrast, the inhibition of mTORC1 alone with rapamycin treatment had little effect on NK cell viability.

This chapter demonstrates that tumour interactions with NK cells initiates signalling pathways that make it possible for these innate cells to integrate with the adaptive immune system. This collaboration of innate and adaptive immune responses leads to a more potent and long-lived anti-tumour immune response. This chapter also underscores the importance of nutrient availability and metabolic responses for effective NK cell anti-tumour responses. Therefore, there is a need to consider the metabolic requirements of tumour infiltrating NK cells to design better NK cell-based immunotherapies.

Chapter 6

Cellular metabolism is essential for trained NK cell responses

6.1 Introduction- Innate immune training

Classical immunological memory is described based on the following criteria- memory cells are long lived, antigen specific and are intrinsically and irreversibly changed at the genome level, so that the changes are inherited by daughter cells (Farber et al., 2016). Indeed, adaptive cells fulfil all these criteria. A subset of T and B cells become memory cells following primary encounter with a pathogen, that leads to irreversible changes in the DNA during and after the rearrangement of antigen receptor sequences (Natoli and Ostuni, 2019). Moreover, these changes are passed onto daughter cells after a secondary challenge that leads to clonal expansion.

Although not originally defined in innate cells, recent evidence suggests that memory like characters are expressed by innate cells as well. Evolutionarily, the appearance of the adaptive immune system is thought to coincide with the evolution of the early jawed vertebrates. There is so far no evidence of adaptive immune system in any known species of invertebrates (Sun et al., 2014). However, the presence of innate immune memory was first demonstrated in an invertebrate species, copepod *Macrocyclops albidus.* Despite the lack of an adaptive immune system, the copepod, following a primary infection, became resistant to a second challenge with an antigenically similar parasite (Kurtz and Franz, 2003). This became one of the first and important findings in the field of innate immune memory.

NK cells although traditionally classified as part of the innate immune system, also have been proven to show some adaptive characteristics that include the presence of memory like functions. Indeed, studies in murine cytomegalovirus (MCMV) have provided a great deal of evidence in terms of adaptive nature of NK cells. The hallmark of adaptive cells is antigen specificity due to gene-recombination. However, several MCMV specific receptors have been identified

on NK cells such as Ly49H which is a receptor for the MCMV protein m157 (Smith et al., 2002). Moreover, m157 antigen has been also shown to induce long term NK cell responses and memory responses in B6 mice wherein primed NK cells were able to respond rapidly and more efficiently upon re-infection with MCMV. Exposure to m157 results in NK cell expansion and these NK cells are detectable up to 70 days post infection (Holder et al., 2018; Sun et al., 2009; Sun et al., 2014; Tripathy et al., 2006). Similarly, the human counterpart of MCMV, human CMV, was shown to modify NK cell receptor repertoire with increased frequency of NK cells expressing CD57 and NKG2C (Gumá et al., 2004). Indeed, expansion of $CD57^{pos}$, $NKG2C^{pos}$ also known as "adaptive" NK cells was blocked in an HCMV/NK cell in vitro co-culture system by an NKG2C blocking antibody (Rölle et al., 2014). Moreover, there is some evidence that these adaptive NK cells partly rely on specific recognition of HLA-E loaded HCMV peptides and polymorphisms in these HLA-E peptides define the heterogeneity and effector responses of these NKG2C+ adaptive cells in HVMV seropositive patients (Hammer et al., 2018) (Rölle et al., 2018). NK cells were also shown to acquire memory like characteristics in a model of contact hypersensitivity (CHS) in the absence of T and B cells (O'Leary et al., 2006). The same group also demonstrated that administration of an NKG2D specific antibody abrogated the effects of CHS (O'Leary et al., 2006). Additionally, NK cells that had been primed with live Vaccinia virus were shown to confer protection against fatal dose of Vaccinia virus in the absence of adaptive cells (Gillard et al., 2011). It was also demonstrated that hepatic NK cells develop specific memory to viral antigens including those from, influenza, vesicular stomatitis virus and human immunodeficiency virus type 1 and adoptive transfer of these primed NK cells increased survival in mice after they were re-challenged with the same virus but not when rechallenged with a different virus (Paust et al., 2010a). Furthermore, this hepatic NK cell memory depended on an NK cell specific chemokine CXCR6 (Paust et al., 2010a).

Another model described to study NK cell memory like responses is called cytokine induced memory like NK cells or CIML NK cells. In this model, NK cells are primed with cytokines such as IL2, IL12, IL18 and IL15, instead of pathogens. Following this initial stimulation, they are allowed to quiesce and then re-stimulated with cytokines (Cooper et al., 2009b). This memory-like characteristic of NK cells is however antigen-unspecific and cytokine pre-activated NK cells are able to respond to different cytokines or receptor stimulation alike. The CIML NK cells are also characterised by enhanced proliferation, increased *in-vivo* persistence and enhanced IFN-γ production upon re-stimulation. This model was first described by Cooper et al, who also demonstrated that these memory-like characteristics are not affected by proliferation and the capacity of enhanced IFN-γ production is adopted by daughter NK cells too (Cooper et al., 2009b). Murine CIML NK cells were then shown to have enhanced responses against tumours (Ni et al., 2012). Later on, Todd Fehniger's group proved the capacity of human NK cells to generate CIML NK cells (Romee et al., 2012). The same group then went on to show enhanced anti-leukaemia activity of CIML NK cells in xenografted mice and also proved efficacy of these cells in patients of Acute Myeloid Leukaemia (AML) (Romee et al., 2016). The current report is based on a similar model where CIML NK cells are generated by stimulating them with IL12/IL18 and IL15.

Studying metabolism in immune cells is now a prerequisite to completely understand immune cell responses. Recent researches in the field have now revealed the importance of metabolism in trained immune responses.

β-glucan trained macrophages were shown to have increased glucose consumption (Cheng et al., 2014). Genes encoding glucose metabolism enzymes- hexokinase and pyruvate kinase- were found to be epigenetically up regulated following β-glucan priming of macrophages.

M1 macrophages are known to up-regulate their rates of glycolysis and re-purpose the TCA cycle intermediates in the synthesis of various pro-inflammatory mediators (O'Neill et al., 2016). In β-glucan trained macrophages, although there is a glycolytic shift, with decreased rates of OxPhos, TCA cycle is not completely shut down, but there is a continual replenishment of TCA cycle intermediates. The concentrations of TCA cycle metabolites like citrate, fumarate and succinate are found to be increased in trained versus non-trained macrophages (Arts et al., 2016a). Indeed, fumarate and succinate are now being implicated in the control of trained immune responses due to their role in methylation and acetylation of histones (Arts et al., 2016b). Another TCA cycle metabolite that is of interest in the field of innate immune training is citrate. Citrate derived from glucose can be converted to Acetyl Co-A by the action of the enzyme ATP citrate lyase that has been shown to be crucial for histone acetylation. This finding also proves to be an important link between glucose consumption and histone acetylation, thus linking metabolism to epigenetics (Wellen et al., 2009). Indeed cytokine activated NK cells engage in citrate malate shuttle that promotes citrate metabolism into acetyl Co-A and the transcription factor Srebp was found to be crucial for the adoption of this metabolic profile (Assmann et al., 2017). In support of the possible involvement of Srebp transcribed genes, cholesterol synthesis pathway has been implicated in the induction of training in monocytes. One of the metabolites in the cholesterol synthesis pathway, mevalonate that is downstream of acetyl-Co-A, induced training in monocytes by epigenetic re-programming. Furthermore, patients with hyper immunoglobulin D syndrome (HIDS) that lacked mevalonate kinase and therefore accumulated mevalonate showed a similar trained monocyte phenotype (Bekkering et al., 2018). Although the relationship between metabolism and epigenetics has been investigated in great detail in macrophages and monocytes, little is known about the metabolic requirements for the induction of NK cell training.

6.2 IL12/IL15/IL18 cytokine 'training' enhances NK cell IFN-γ production and cytotoxicity.

As discussed in the introduction to this chapter, there has been considerable research on cytokine induced NK cell training in both mice and humans (Cooper et al., 2009b; Romee et al., 2016; Romee et al., 2012). Trained NK cells have also been applied in cancer immunotherapies with positive results (Berrien-Elliott et al., 2015; Romee et al., 2016).

Given that the generation of memory like NK cells involves an initial cytokine stimulation for 12 to 18 hours that induces substantial increases in cellular metabolism we considered whether this metabolic response is important for the formation of cytokine induced memory like NK cells (CIML) and if so whether CIML generation is defective when NK cell metabolism is perturbed in metabolically defective NK cells such as those from obese individuals or NK cells from a cancer patient. Therefore, we considered what the effects of metabolic inhibition during NK cell training would be on secondary NK cell responses upon re-simulation.

To generate CIML cells, splenocytes were isolated from a murine spleen and stimulated for 18 hours with IL12/IL15/IL18 -hereafter referred to as trained- in the presence or absence of various metabolic inhibitors, or left unstimulated, hereafter, untrained. Following the 18-hour stimulation, all the conditions were washed thoroughly out of any cytokines or inhibitors, and cultured in low dose IL15 for 6 days to allow them to quiesce. On day 7, all the conditions, including the untrained and trained conditions with/without inhibitors were either re-stimulated with cytokines IL12 and high dose IL15 or co-cultured with K562 cancer cells for 4 hours. It is to be noted that no metabolic inhibitors were added at this time point. Following the 4-hour incubation, cytokine re-stimulated cells were assessed for IFN-γ production and cells co-cultured with K562 cells were assessed for their killing capacity **(Experimental approach Fig 6.1).**

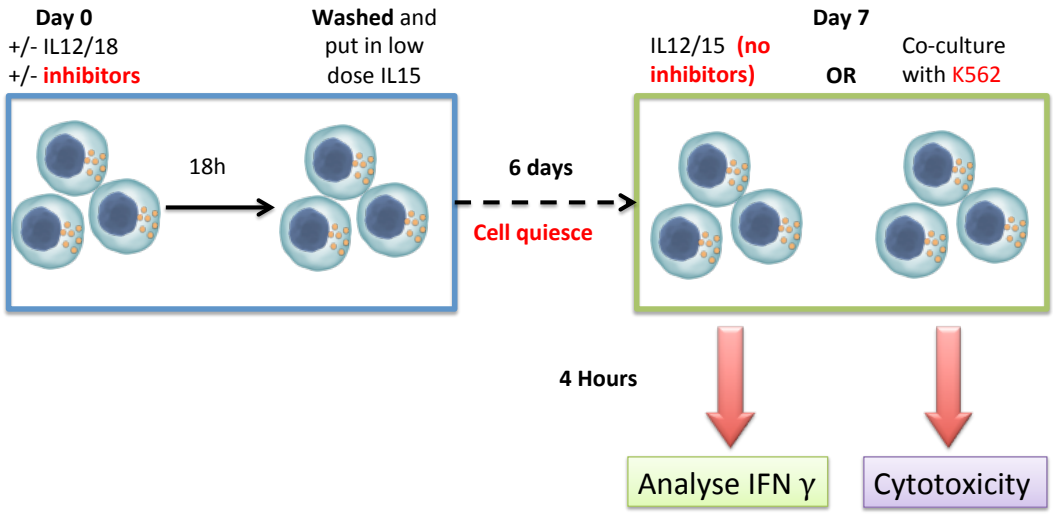


Figure 6.1 Experimental approach

Murine splenocytes were put in low dose IL15 (10ng/ml) and stimulated with the cytokines IL12(20ng/ml) plus IL18 (50ng/ml) (trained) in the presence or absence of metabolic inhibitors or left unstimulated(untrained) for 18 hours, Following the18 hour incubation, the cells were washed thoroughly out of cytokines and inhibitors and cultured in low dose IL15 for 6 days during which time they quiesce. On day 7, the trained and untrained conditions were either re-stimulated with IL12(20ng/ml) and high dose IL15 (100ng/ml) for 4 hours or co-cultured with K562s. Following the 4-hour incubation, the cytokine re-stimulated cells were analyzed for IFN-γ by flow-cytometry and the K562 co-cultured cells were analyzed for specific lysis by spectroscopy.

To ascertain that NK cells trained on day 0 had a normal IFN-γ response following 18-hour stimulation, murine splenocytes were stimulated with IL12, IL15 plus IL18 or left unstimulated for 18 hours. These cells were analysed by flow cytometry for IFN-γ. Indeed IL12/IL15/IL18 stimulated NK cells had a robust IFN-γ response **(Fig6.2).** Before looking at the effects of inhibitors on NK cell trained responses, it was important to ascertain that the trained NK cell indeed quiesced and were able to induce enhanced functional responses upon re-stimulation. Neither untrained NK cells nor trained NK cells produced IFN-γ on day 7 before re-stimulation, indicating that the trained NK cells had quiesced over the course of the 6 days **(Fig 6.3A).** Following re-stimulation the trained NK cells had an increased IFN-γ response compared to untrained NK cells **(Fig 6.3B,C).**

Similarly, when the cytotoxicity of trained NK cells was compared with that of untrained NK cells, trained NK cells were found to be significantly more efficient at killing cancer cells **(Fig 6.4).** This training response could be due to altered signalling in the trained cells or alternatively due to epigenetic changes in gene loci. To bypass any differences in signalling, NK cells were stimulated with PMA/Ionomycin to investigate whether the training response was still evident if both trained and untrained cells received a strong activating signal. Indeed, the trained NK cells had increased IFN-γ production in response to PMA/Ionomycin stimulation, which suggests that the training involves epigenetic changes **(Fig 6.5).** Consistent with this, NK cells stimulated with IL12/IL15 plus IL18 for 18 hours had increased levels of Histone 3 acetylation **(Fig 6.6).** Taken together these data show that NK cells undergo training leading to an enhanced response upon subsequent re-stimulation that likely involves epigenetic modifications that occur during the primary response.

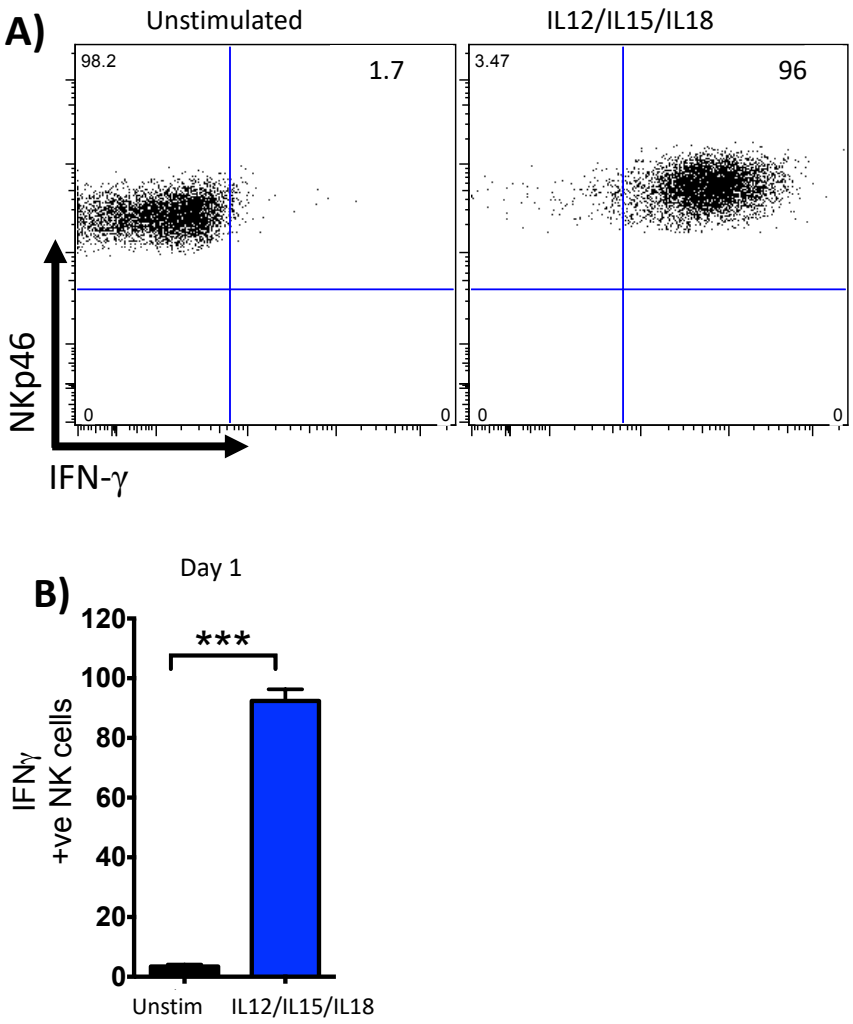


Figure 6.2 Splenic NK cells trained with IL12/IL15/IL18 have a robust IFN-γ response following 18-hour stimulation.

(A-B) Splenocytes were put in low dose IL15 (10ng/ml) and stimulated for 18 hours with cytokines IL12(20ng/ml) plus IL18 (50ng/ml) (trained) or left unstimulated (low dose IL15 at 10ng/ml only; untrained). IFN-γ was analysed by flow cytometry. Data is representative (A) or mean +/- SEM (B) of 4 independent experiments. Data was analysed using a paired student's t-test(C) or (***$p<0.001$).

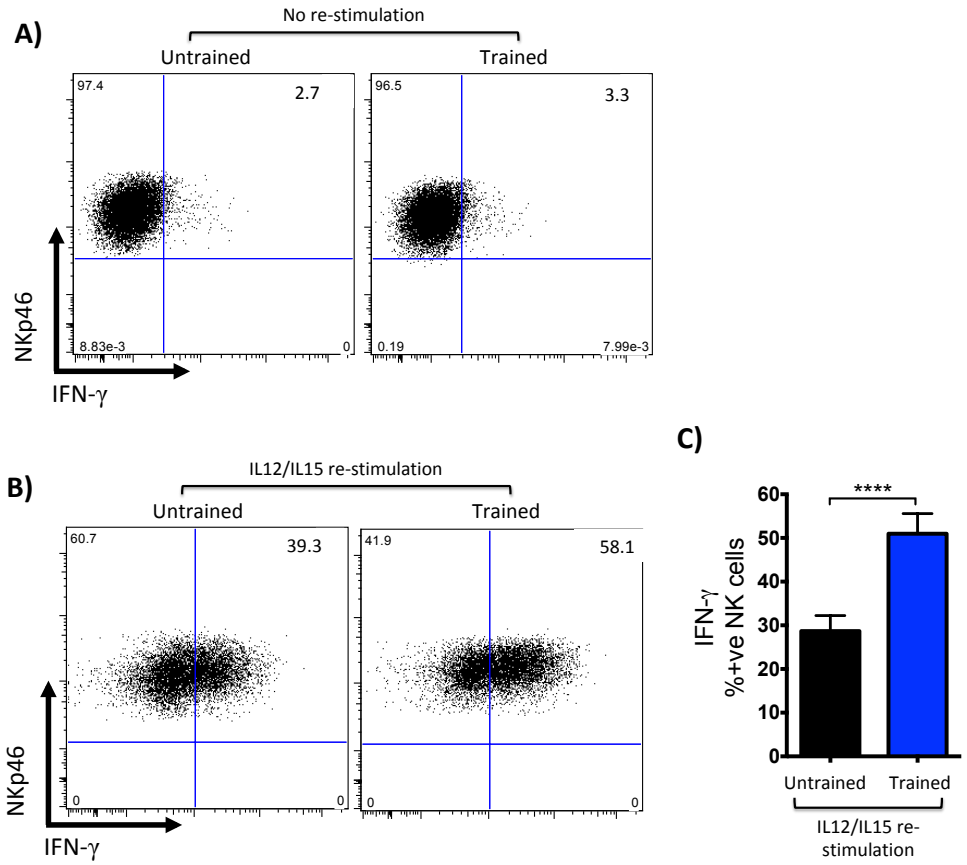


Figure 6.3 NK cells trained with IL12/IL15/IL18 have an enhanced IFN-γ response upon re-stimulation with cytokines.

(A-C) Splenocytes were stimulated for 18 hours with cytokines IL12(20ng/ml) plus IL15(10ng/ml) plus IL18 (50ng/ml) (trained) or left unstimulated (low dose IL15 at 10ng/ml only; untrained). The cells were thoroughly washed after 18 hours and quiesced in low dose IL15 for 6 days. On day 7, untrained and trained NK cells were re-stimulated with cytokines IL12 (20ng) plus IL15 (100ng) for 4 hours. IFN-γ was analysed by flow cytometry. Data is representative (A, B) or mean +/- SEM (C) of 6 independent experiments. Data was analysed using a student's t test(C) or (****p<0.0001).

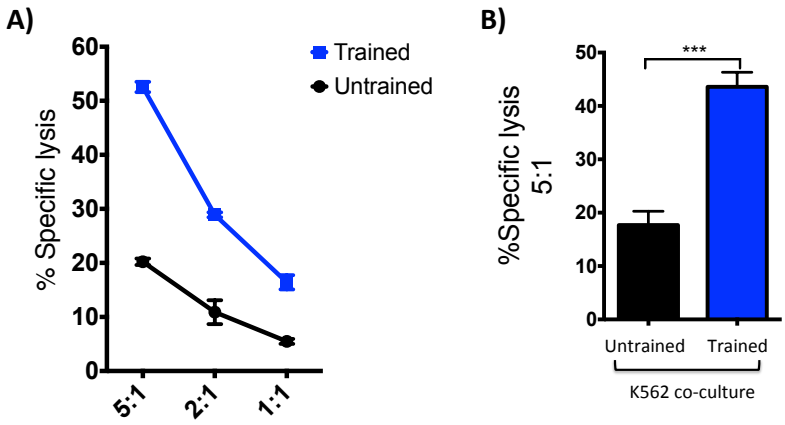


Figure 6.4 NK cells trained with IL12/IL15/IL18 have an enhanced target cell killing capacity

(A-B) Splenocytes were stimulated for 18 hours with cytokines IL12 (20ng/ml) plus IL15 (10ng/ml) plus IL18 (50ng/ml) (trained) or left unstimulated (low dose IL15 at 10ng/ml only; untrained). The cells were thoroughly washed after 18 hours and quiesced in low dose IL15 for 6 days. On day 7, untrained and trained NK cells were co-cultured with Calcein AM stained K562 cancer cells for 4 hours. Killing capacity was analysed by measuring the Calcein AM release by spectroscopy. Data is representative (A) or mean +/- SEM (B) of 4 independent experiments. Data was analysed using a paired student's t- test (***$p<0.001$).

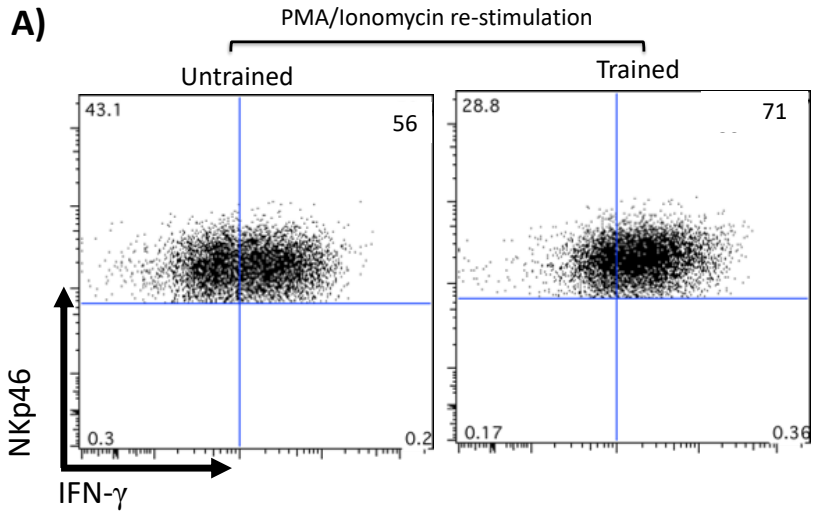


Figure 6.5 NK cells trained with IL12/IL15/IL18 have an enhanced IFN-γ response upon re-stimulation by PMA/Ionomycin

Splenocytes were stimulated for 18 hours with cytokines IL12 (20ng/ml) plus IL15 (10ng/ml) plus IL18 (50ng/ml) (trained) or left unstimulated (low dose IL15 at 10ng/ml only; untrained). The cells were thoroughly washed after 18 hours and quiesced in low dose IL15 for 6 days. On day 7, untrained and trained NK cells were re-stimulated with PMA (50ng/ml) and Ionomycin (1μg/ml) for 4 hours. IFN-γ was analyzed by flow cytometry. Data is representative of two individual experiments.

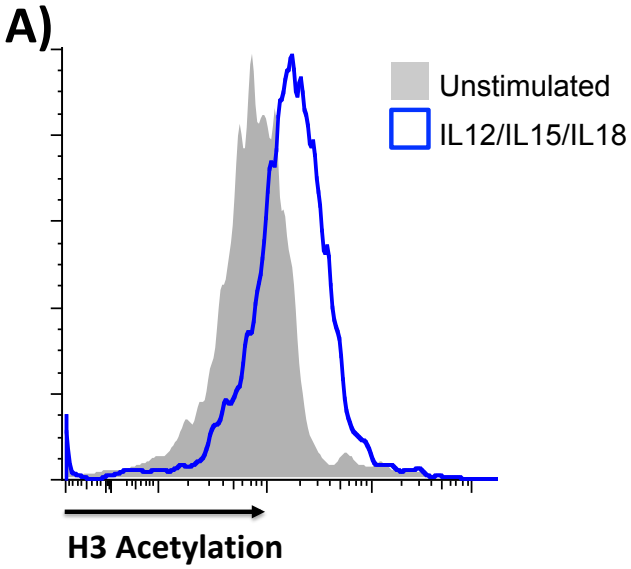


Figure 6.6 NK cells trained with IL12/IL15/IL18 have an enhanced acetylation of histone core protein H3

Splenocytes were stimulated for 18 hours with cytokines IL12 (20ng/ml) plus IL15 (10ng/ml) plus IL18 (50ng/ml) or left unstimulated (low dose IL15 at 10ng/ml only). The cells were then stained for acetylated H3 and analyzed by flow cytometry. Data is representative of 2 independent experiments.

6.3 NK cell metabolic responses are essential for NK cell trained responses

Cellular metabolism is known to play an important role in effector NK responses but not much is known about metabolic control of NK cell trained responses. Several links have been found between metabolism and epigenetics in case of trained macrophages (Arts et al., 2016a). For instance, glucose consumption was increased in β-glucan trained macrophages (Cheng et al., 2014). ATP citrate lyase, which breaks down glucose-derived citrate into Acetyl Co-A, has been implicated in the control of histone acetylation (Wellen et al., 2009). Therefore we hypothesized that metabolic response during the initial activation of NK cells would be important for the formation of trained NK cells. To look at the importance of metabolism for NK cell trained responses, NK cells were trained in the presence of various metabolic inhibitors, including those used for suppressing glycolysis and OXPHOS. Inhibitors were added at low dose to limit the flux through the metabolic pathways and only during the initial activation of NK cells, but not during re-stimulation.

When NK cells were trained in the presence of a low dose of 2DG, an inhibitor of the glycolytic pathway, there was a significant decrease in the trained responses 7 days later, when they were re-stimulated with IL12/IL15 **(Fig 6.7).** NK cells trained in the presence of 2DG also had reduced cytotoxicity against K562 cancer cells **(Fig 6.8)**. Similarly, NK cells that were trained in the presence of nanomolar concentrations of oligomycin, an inhibitor of ATP synthase, trained NK cells produce substantially less IFN-γ upon re-stimulation and had reduced cytotoxicity against K562s **(Fig 6.9, 6.10).**

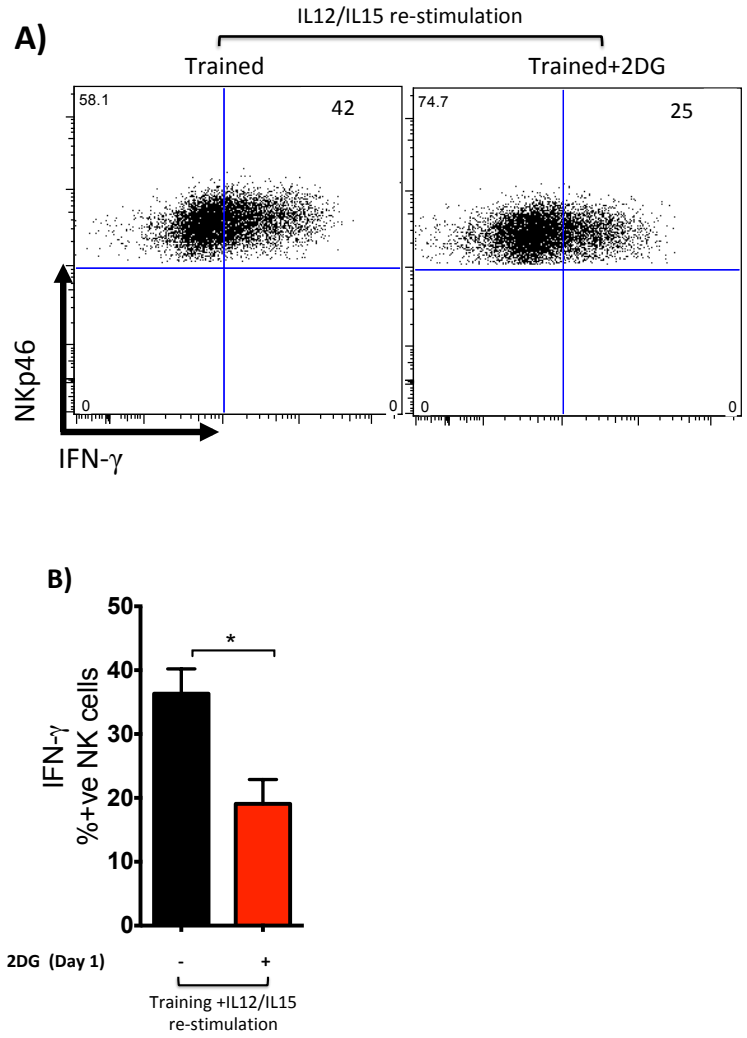


Figure 6.7 NK cells trained under glycolytic inhibition fail to have an enhanced IFN-γ response

(A-B) Splenocytes were stimulated for 18 hours with cytokines IL12 (20ng/ml) plus IL15 (10ng/ml) plus IL18 (50ng/ml) (trained) in the presence or absence of 2-deoxyglucose (2DG, 2mM) or left unstimulated (low dose IL15 at 10ng/ml only; untrained). The cells were thoroughly washed after 18 hours and quiesced in low dose IL15 for 6 days. On day 7, untrained and trained NK cells were re-stimulated with cytokines IL12 (20ng) plus IL15 (100ng) for 4 hours. IFN-γ was analysed by flow cytometry. Data is representative (A) or mean +/- SEM (B) of 4 independent experiments. Data was analysed using a paired student's t-test(C) (*$p<0.05$).

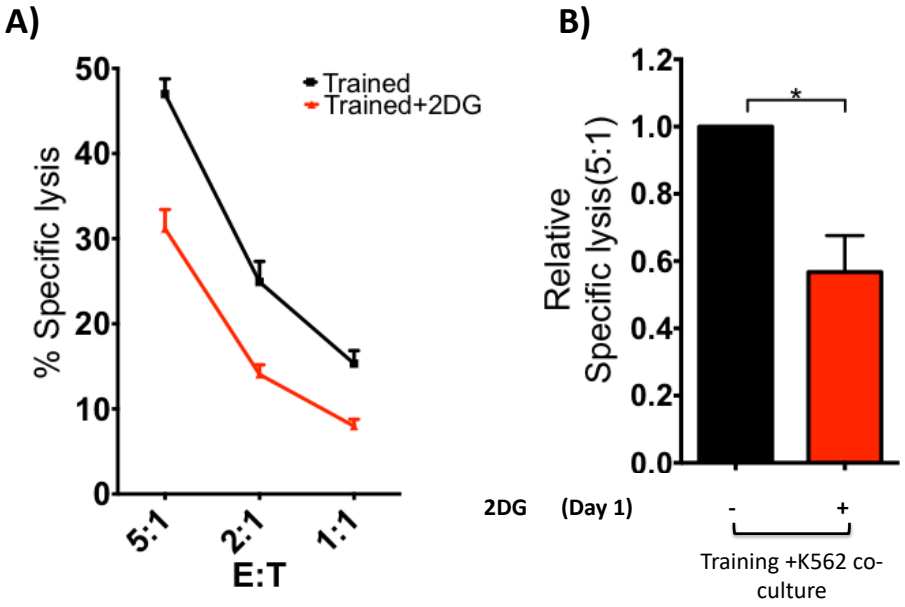


Figure 6.8 NK cells trained under glycolytic inhibition fail to have an enhanced killing capacity

(A-B) Splenocytes were stimulated for 18 hours with cytokines IL12 (20ng/ml) plus IL15 (10ng/ml) plus IL18 (50ng/ml) (trained) in the presence or absence of 2-deoxyglucose (2DG, 2mM) or left unstimulated (low dose IL15 at 10ng/ml only; untrained). The cells were thoroughly washed after 18 hours and quiesced in low dose IL15 for 6 days. On day 7, untrained and trained NK cells were co-cultured with Calcein AM stained K562 cancer cells for 4 hours. Killing capacity was analysed by measuring Calcein AM release by spectroscopy. Data is representative (A) or mean +/- SEM (B) of 4 independent experiments. Data was analysed using a paired student's t-test (*$p<0.05$).

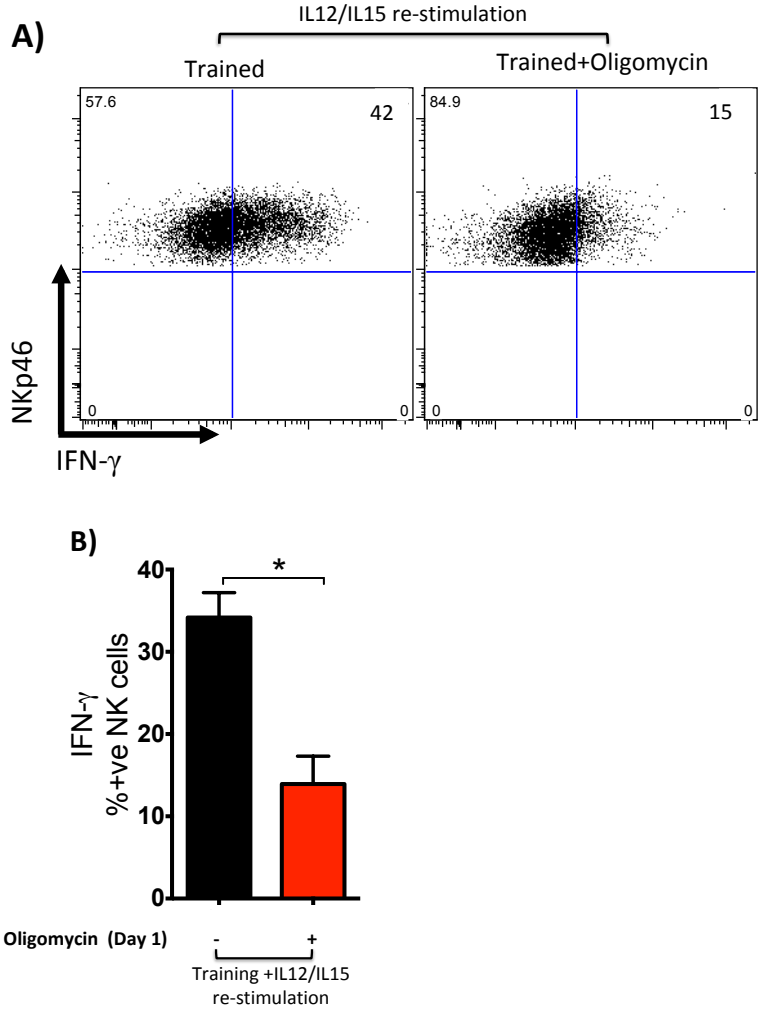


Figure 6.9 NK cells trained in the presence of Oligomycin fail to have an enhanced IFN-γ response

(A-B) Splenocytes were stimulated for 18 hours with cytokines IL12 (20ng/ml) plus IL15 (10ng/ml) plus IL18 (50ng/ml) (trained) in the presence or absence of Oligomycin (2nM) or left unstimulated (low dose IL15 at 10ng/ml only; untrained). The cells were thoroughly washed after 18 hours and quiesced in low dose IL15 for 6 days On day 7, untrained and trained NK cells were re-stimulated with cytokines IL12 (20ng) plus IL15 (100ng) for 4 hours. IFN-γ was analysed by flow cytometry. Data is representative (A, B) or mean +/- SEM (C) of 6 independent experiments. Data was analysed using a paired student's t test. (*$p<0.05$).

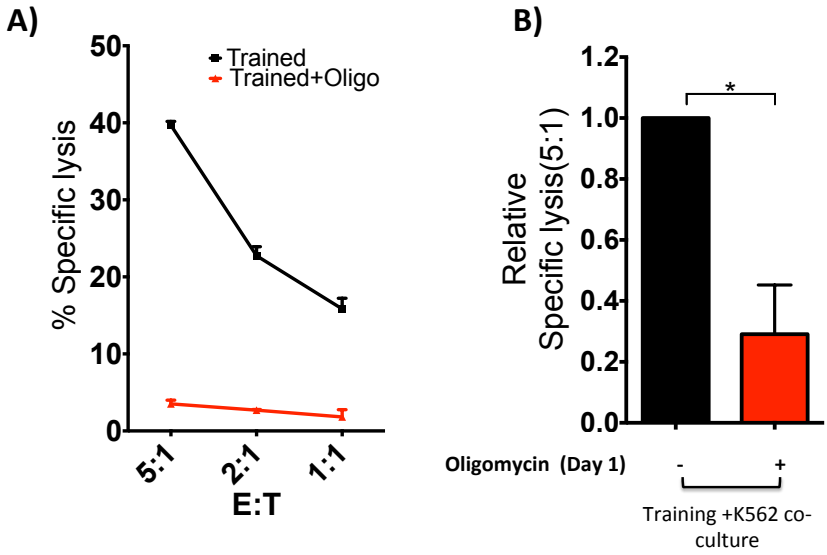


Figure 6.10 NK cells trained in the presence of Oligomycin fail to show enhanced killing capacity

(A-B) Splenocytes were stimulated for 18 hours with cytokines IL12 (20ng/ml) plus IL15 (10ng/ml) plus IL18 (50ng/ml) (trained) in the presence or absence of Oligomycin (2nM) or left unstimulated (low dose IL15 at 10ng/ml only; untrained). The cells were thoroughly washed after 18 hours and quiesced in low dose IL15 for 6 days. On day 7, untrained and trained NK cells were co-cultured with Calcein AM stained K562 cancer cells for 4 hours. Killing capacity was analysed by measuring Calcein AM release by spectroscopy. Data is representative (A) or mean +/- SEM (B) of 4 independent experiments. Data was analysed using a paired student's t test. (*$p<0.05$).

After establishing that upregulation of the glycolytic pathway and rates of OxPhos are important for NK cell training responses, I next investigated whether the key metabolic regulators discussed in chapters 4-5 that are essential for NK cell effector responses, also controlled NK cell trained responses.

The activation of Srebp transcription factor is required for cytokine induced NK cell metabolism (Assmann et al., 2017). To study the role of Srebp for trained NK cell responses NK cells were trained in the presence of the inhibitors of Srebp activation, 25-hydroxycholesterol or PF429242. NK cells trained in the presence of inhibitors of Srebp activation had reduced IFN-γ production upon re-stimulation on day 7 **(Fig 6.11).** Similarly, these cells had reduced cytotoxicity against K562 cells **(Fig 6.12).** This shows that the activity of the transcription factor Srebp is essential for NK cell training.

The amino acid transporter Slc7A5 has been shown to be important for cytokine induced NK cell effector responses as it is required for the expression of the transcription factor cMyc (Loftus et al., 2018). The inhibitor of Slc7A5, BCH was used to block amino acid uptake in NK cells during training. NK cells trained in the presence of BCH had reduced IFN-γ production compared to untreated NK cells upon re-stimulation on day 7 **(Fig 6.13).** While there was also a trend towards reduced cytotoxicity against K562 cells the differences did not reach statistical significance **(Fig 6.14).**

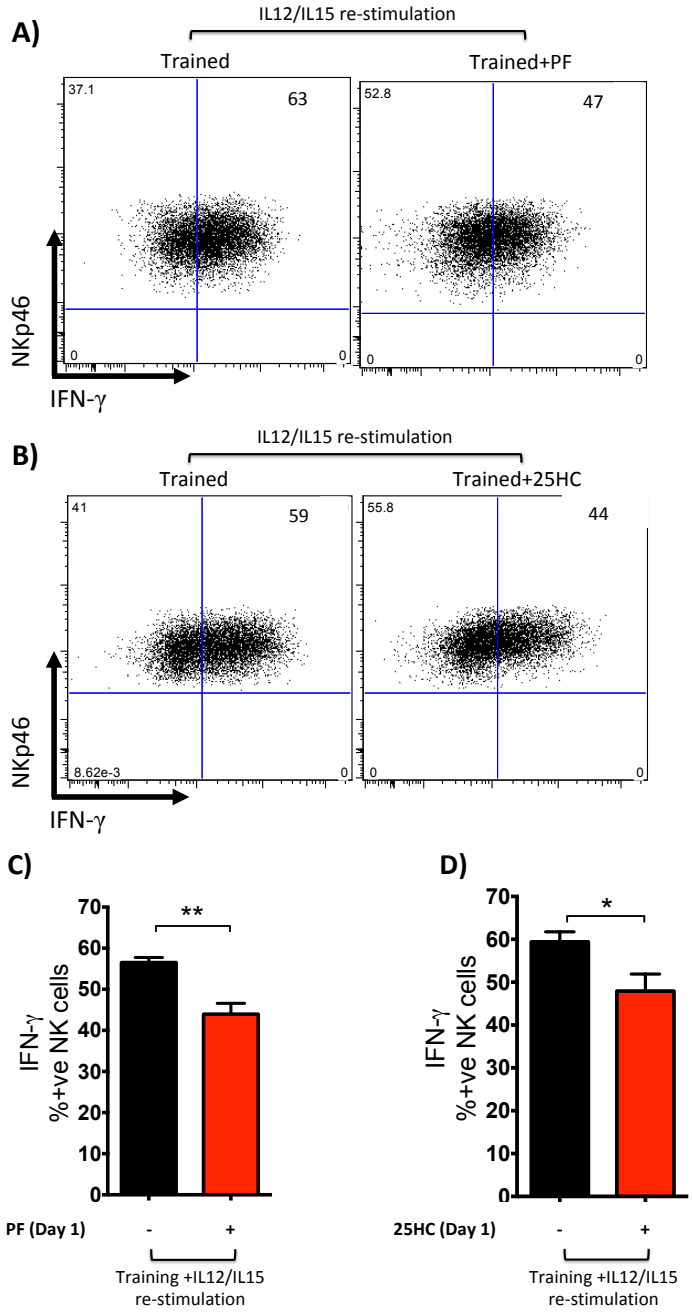


Figure 6.11 NK cells trained under Srebp inhibition fail to have trained IFN-γ response.

(A-D) Splenocytes were stimulated for 18 hours with cytokines IL12 (20ng/ml) plus IL15(10ng/ml) plus IL18 (50ng/ml) (trained) in the presence or absence of PF(A,C) or 25HC(C,D) or left unstimulated(low dose IL15 at 10ng/ml only; untrained). The cells were thoroughly washed after 18 hours and quiesced in low dose IL15 for 6 days On day 7, untrained and trained NK cells were re-stimulated with cytokines IL12 (20ng/ml) plus IL15 (100ng/ml) for 4 hours. IFN-γ was analysed by flow cytometry. Data is representative (A, B) or mean +/- SEM (C,D) of 3-5 independent experiments. Data was analysed using a paired student's t test. (*$p<0.05$, **$p<0.01$).

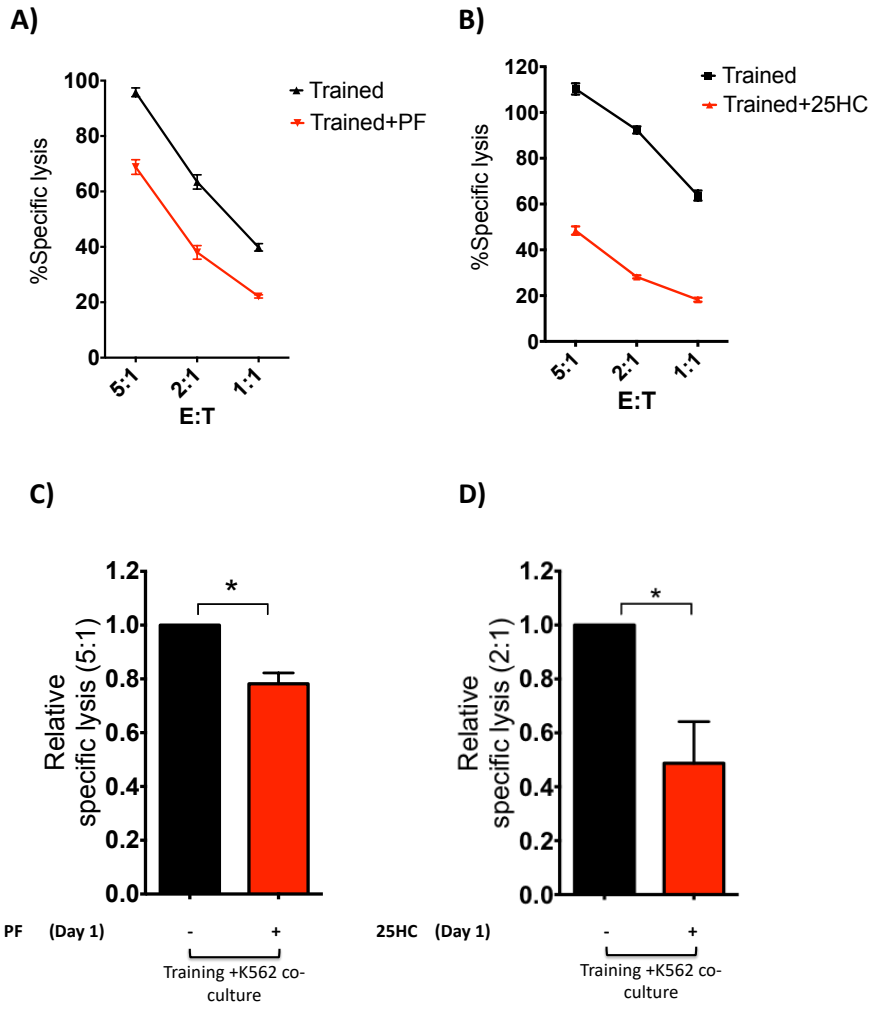


Figure 6.12 NK cells trained under Srebp inhibition fail to show enhanced killing capacity

(A-D) Splenocytes were stimulated for 18 hours with cytokines IL12 (20ng/ml) plus IL15(10ng/ml) plus IL18 (50ng/ml) (trained) in the presence or absence of PF(A,C) or 25HC(C,D) or left unstimulated (low dose IL15 at 10ng/ml only; untrained). The cells were thoroughly washed after 18 hours and quiesced in low dose IL15 for 6 days. On day 7, untrained and trained NK cells were co-cultured with Calcein AM stained K562 cancer cells for 4 hours. Killing capacity was analysed by measuring Calcein AM release by spectroscopy. Data is representative (A,B) or mean +/- SEM (C,D) of 4 independent experiments. Data was analysed using a paired student's t test. (*$p<0.05$).

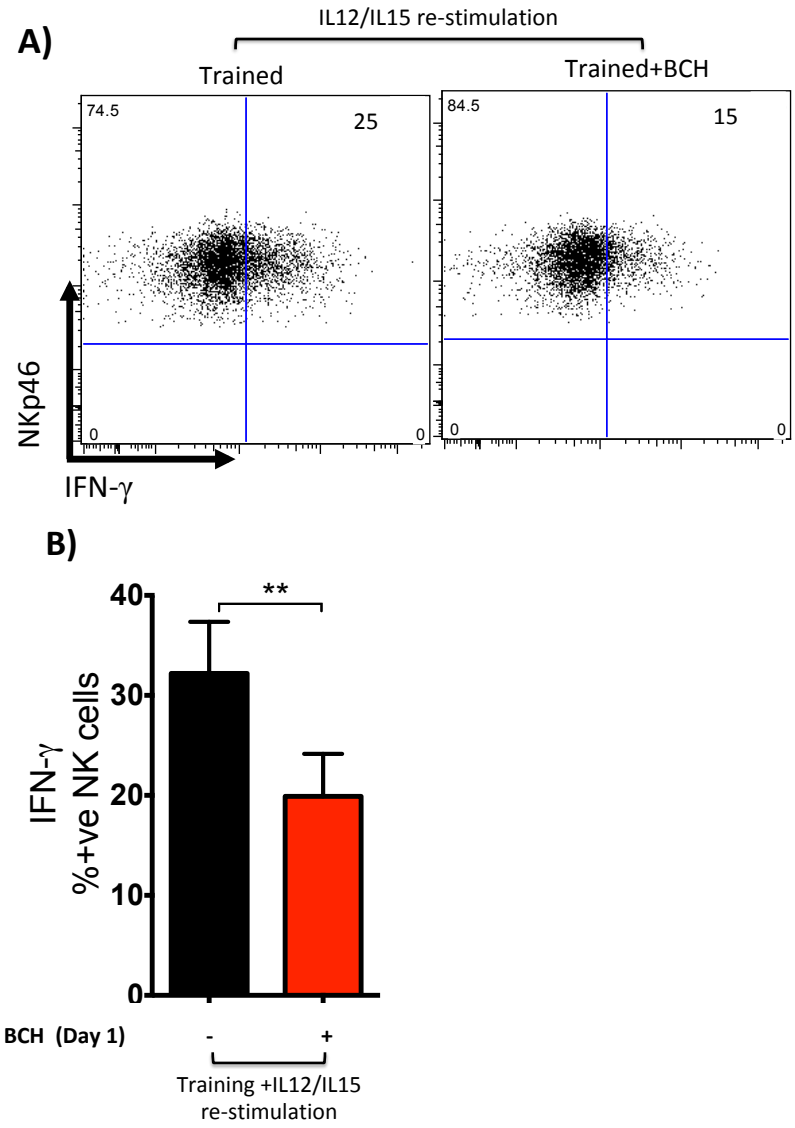


Figure 6.13 NK cells trained in the presence of BCH, inhibitor of Slc7A5 fail to show enhanced IFN-γ production.

(A-B) Splenocytes were stimulated for 18 hours with cytokines IL12 (20ng/ml) plus IL15 (10ng/ml) plus IL18 (50ng/ml) (trained) in the presence or absence of BCH (25mM) or left unstimulated (low dose IL15 at 10ng/ml only; untrained). The cells were thoroughly washed after 18 hours and quiesced in low dose IL15 for 6 days On day 7, untrained and trained NK cells were re-stimulated with cytokines IL12 (20ng/ml) plus IL15 (100ng/ml) for 4 hours. IFN-γ was analysed by flow cytometry. Data is representative (A) or mean +/- SEM (B) of 4 independent experiments. Data was analysed using a paired student's t test (**$p<0.01$).

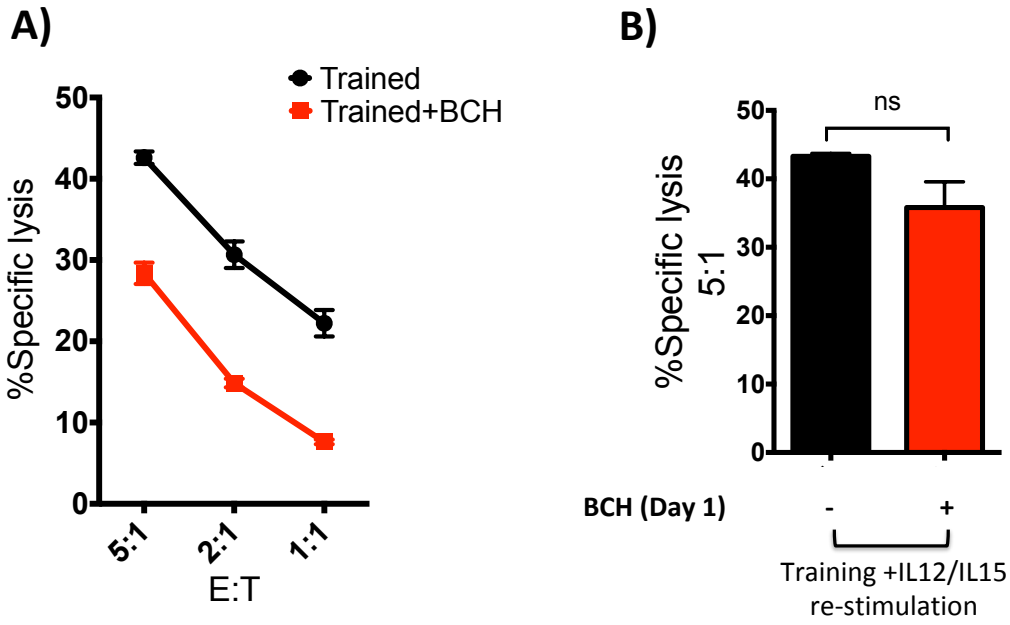


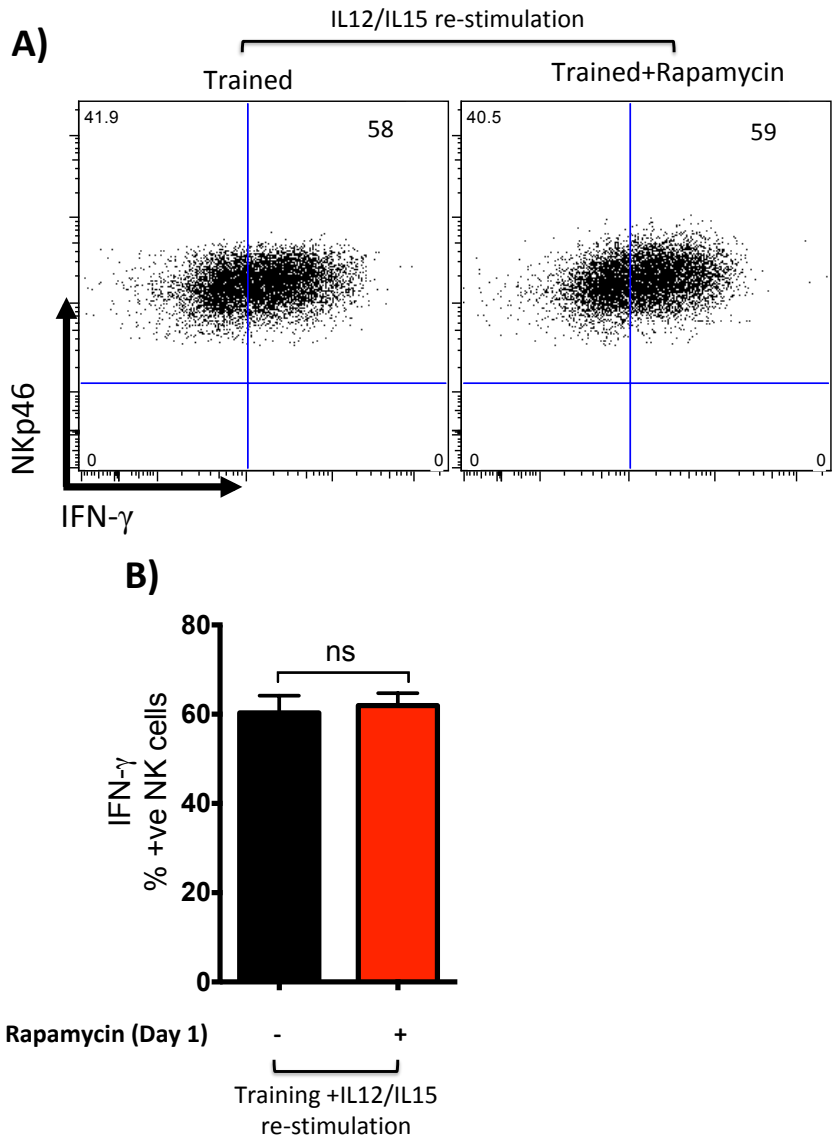
Figure 6.14 NK cells trained under SLc7A5 inhibition have reduced killing capacity

(A-B) Splenocytes were stimulated for 18 hours with cytokines IL12 (20ng/ml) plus IL15 (10ng/ml) plus IL18 (50ng/ml) (trained) in the presence or absence of BCH (25mM). The cells were thoroughly washed after 18 hours and quiesced in low dose IL15 for 6 days. On day 7, trained NK cells and NK cells trained with BCH were co-cultured with Calcein AM stained K562 cancer cells for 4 hours. Killing capacity was analysed by measuring Calcein AM release by spectroscopy. Data is representative (A) or mean +/- SEM (B) of 3 independent experiments. Data was analysed using a paired student's t test. (*$p<0.05$).

6.4 mTORC1 is dispensable for formation of trained NK cells

The kinase mTORC1 has been described as a key metabolic regulator in NK cells (Assmann et al., 2017; Donnelly et al., 2014). Therefore, we tested whether inhibition of mTORC1 during the initial activation period using the specific inhibitor rapamycin affected the formation of trained NK cells. Surprisingly, rapamycin did not inhibit CIML NK cell formation in murine NK cells. NK cells trained in the presence of rapamycin produced equivalent levels of IFN-γ NK cells trained in the absence of rapamycin **(Fig 6.15).**

Together these data show that metabolic pathways engaged during the primary response play a role in NK cell trained responses. If these metabolic pathways are inhibited at the crucial time when NK cells are getting trained, they fail to have an elevated recall response. However, interestingly, the signalling pathways initiated by IL12, IL15 and IL18 together seem to bypass the mTORC1 pathway and its inhibition has no effect on NK cell trained responses.



6.15 NK cells trained in the presence of rapamycin, inhibitor of mTORC1 had no effect on training.

(A-B) Splenocytes were stimulated for 18 hours with cytokines IL12 (20ng/ml) plus IL15 (10ng/ml) plus IL18 (50ng/ml) (trained) in the presence or absence of Rapamycin or left unstimulated (low dose IL15 at 10ng/ml only; untrained). The cells were thoroughly washed after 18 hours and quiesced in low dose IL15 for 6 days On day 7, untrained and trained NK cells were re-stimulated with cytokines IL12 (20ng/ml) plus IL15 (100ng/ml) for 4 hours. IFN-γ was analysed by flow cytometry. Data is representative (A) or mean +/- SEM (B) of 4 independent experiments. Data was analysed using a paired student's t test (ns; non-significant).

Chapter 6 Discussion

In chapters 4 and 5, the importance of metabolism in NK cell effector function was investigated. However, little is known about the role cellular metabolism plays in trained NK cell responses. Training has been described in cytokine activated NK cells in murine and human models where NK cells pre-activated with IL12/IL15/IL18 have enhanced IFN-γ response to a secondary stimulus 7 days after they were first activated (Cooper et al., 2009b; Keppel et al., 2013; Romee et al., 2012). Cytokine trained NK cells were also shown to have enhanced cytotoxicity against K562s (Romee et al., 2012).

The memory like property of cytokine pre-activated NK cells was shown to be intrinsic and was inherited by daughter cells that had not been exposed to cytokines (Keppel et al., 2013). This indicates that there might be an epigenetic reprogramming event involved in the formation of memory. To confirm this, trained and untrained NK cells were activated with PMA/Ionomycin that would circumvent any differences in the signalling. Indeed, cytokine trained NK cells had an enhanced IFN-γ response following PMA/Ionomycin treatment which suggests that cytokine training of NK cells enhances accessibility at the IFN-γ gene locus. This is supported by the report showing that the gene promoter CNS1 at the *Ifng* gene locus was shown to be de-methylated in cytokine activated NK cells (Luetke-Eversloh et al., 2014; Ni et al., 2016). Furthermore, data in the current report alludes to epigenetic changes as cytokine stimulation resulted in increased H3 acetylation.

Metabolism has been linked to trained immune responses in β- glucan trained macrophages. For instance, trained macrophages were shown to have increased glucose consumption and inhibition of mTOR and HIF1a resulted in a loss of trained responses (Cheng et al., 2014). Furthermore, there's growing evidence that, as well as being important for M1 effector responses, the intermediates of the obstructed TCA cycle in M1 macrophages are important for training in

macrophages (Arts et al., 2016b; Wellen et al., 2009; Xu et al., 2011). Glycolysis and OxPhos were shown to be important for cytokine induced NK cell effector responses (Assmann et al., 2017; Donnelly et al., 2014). This led us to hypothesize that cytokine mediated NK cell metabolic responses during the initial activation may be controlling NK cell trained responses as well. Indeed, inhibiting glycolysis or OxPhos during the initial activation of NK cells led to impaired NK cell trained responses 7 days later when they were re-stimulated without inhibitors. In a recent publication from our group, cytokine activated NK cells were shown to adopt a novel metabolic circuit where OxPhos is fuelled by the Citrate-Malate shuttle (CMS). CMS involves the export of citrate into the cytosol; it's metabolism into acetyl Co-A and oxaloacetate, which gets converted to malate that is imported back into the mitochondria. Acetyl Co-A is the acetyl group donor for the acetylation of histones (Hebbes et al., 1988); and the enzyme that breaks down glucose derived citrate into acetyl Co-A, ATP citrate lyase (ACLy) directly controls histone acetylation as it maintains the pool of acetyl Co-A for histone modification, thus linking metabolism and epigenetic reprogramming (Wellen et al., 2009). Onco-metabolites are known to enter cells and interfere with epigenetic programs (Xu et al., 2011). Acetate, which is one such onco-metabolite, can also add to the pool of acetyl CoA (Gao et al., 2016). Owing to the importance of acetyl Co-A in histone modifications (Hebbes et al., 1988) and on the basis of reports from our lab showing increased accumulation of the acetyl co-A precursor, citrate in cytokine activated NK cells (Assmann et al., 2017), we hypothesized that inhibiting the CMS that generates citrate would affect NK cell trained responses. To inhibit CMS, inhibitors of Srebp - the transcription factor that was shown to control Acly and Slc25A1 gene transcription, were used. Indeed, inhibition of Srebp resulted in impaired trained NK cell responses.

In a recent publication, increased transport of methionine in TCR activated T cells was found to be important in the control of DNA and histone methylation which in turn ensured T cell differentiation (Sinclair, 2018). Methionine is taken up by the Slc7A5 transporter (Kanai et al., 1998), which was shown to be important for T cell and NK cell effector responses (Loftus et al., 2018; Sinclair et al., 2013). The pharmacological inhibitor of Slc7A5, BCH was shown to disrupt metabolic and functional responses in cytokine activated NK cells (Loftus et al., 2018) as well as in receptor activated NK cells as demonstrated in chapters 4 and 5. Therefore, we hypothesized that inhibiting this transporter would affect NK cell trained responses. Indeed, we saw a significant decrease in the IFN-γ response upon re-stimulation, and a trend towards impaired cytotoxicity in NK cells that had been trained in the presence of the Slc7A5 inhibitor.

Based on our findings in effector NK cells that were stimulated by cytokines (Donnelly et al., 2014), and those by another group that suggested mTOR was crucial for training in macrophages (Cheng et al., 2014), we next considered mTORC1 requirement for trained NK cell responses. Surprisingly, there was no impact of mTORC1 inhibition on NK cell trained responses. It is noteworthy that we trained NK cells with the cytokine cocktail that included IL18. A recent publication demonstrated that although IL18 induces mTORC1 activation in NK cells, it could induce nutrient transporters independently of mTORC1 (Almutairi et al., 2019). This is suggestive of some mTORC1 independent mechanisms being involved in IL18 induced metabolic responses. Additionally, unpublished data from our lab strongly indicates that metabolism and function in NK cells activated in the presence of IL18 is mTORC1 independent.

Taken together the data in this chapter demonstrates that the initial metabolic responses induced upon cytokine activation of NK cells govern NK cell training response. This is likely due to the contribution of certain nutrients and metabolites in bringing about epigenetic changes required for NK cell trained

responses. This study has wider implications in that individuals suffering from conditions such as obesity and cancer where NK cells are metabolically exhausted could have impaired NK cell trained responses.

Chapter 7

Overall Discussion

This study characterizes the role for IL2 induced NK cell metabolic responses that are facilitated by NK cell receptor engagement. The induction of the high affinity IL2 receptor, CD25, upon receptor engagement or co-culture of NK cells with tumour cells uncovers an important link between innate and adaptive immune responses. Although NK1.1 receptor ligation was used as the model system for the present study, deductions can be made for other NK cell activating receptors such natural cytotoxicity receptors (NCRs), NKG2D and CD16 that, like NK1.1, also involve ITAM signalling (Arase et al., 1997; Bauer et al., 1999; Bottino et al., 2000). ITAM signalling activates multiple signalling pathways that would be predicted to promote CD25 expression such as NFκB (Gross et al., 2008) which has been shown to control CD25 expression and maintenance in T cells (Fu et al., 2013). ITAM signalling has been shown to activate the transcription factor NFAT through the activation of Ca^{2+}/calcenurin signalling in T cells (Srikanth and Gwack, 2013). Importantly, CD25 promoter has been shown to be a target of NFAT (Schuh et al., 1998). Additionally, similarities between the NK1.1 model and the B16 co-culture model in terms of CD25 expression despite B16 not being a ligand for NK1.1, increase the probability that the proposed hypothesis that signals from receptor ligation and IL2 signalling integrate for enhanced anti-tumour responses is universally true for most NK cell receptors.

These NK cells that work alongside the adaptive immune cells are highly glycolytic and require glucose and other nutrients such as amino acids for optimal function. In this respect NK cells are similar to activated T lymphocytes that also rely upon glucose and amino acids such as leucine and glutamine (Macintyre et al., 2014; Sinclair, 2018; Sinclair et al., 2013; Wang et al., 2011). The importance of nutrients and metabolism for NK cell responses raises the possibility that nutrient restriction could be a mechanism for disrupting NK cell function in vivo. Competition of nutrients between different immune cells in an immunological micro-environment could result in nutrient depletion and affect

immune cell responses (Lawless et al., 2017). Additionally, bacteria and viruses could also sequester glucose and amino acids such as arginine as a strategy to deplete the nutrient pool available for immune cells, thus escaping an immune attack (Kedia-Mehta and Finlay, 2019). The best-described nutrient deficient site is the solid tumour microenvironment and tumour cells are characterized by their prodigious appetite for glucose and glutamine. In fact, glucose levels in tumours were found to be 10 times less than those in spleen (Ho et al., 2015). The excessive glutamine consumption could be attributed to its role in polyamine and nucleotide biosynthesis that supports the high rates of proliferation in cancer (Cory and Cory, 2006). Our research shows that glucose availability and its metabolism through glycolysis is crucial to sustain NK cell effector functions. Although glutamine is not required as a fuel for NK cell function, it is an important signalling molecule and is crucial to sustain cMyc which facilitates metabolic and functional responses in NK cells (Loftus et al., 2018). Indeed, our research highlights the role for cMyc in sustaining NK cell metabolism and function. Thus, excessive consumption of glutamine by cancer cells could cause NK cell dysfunction. Certainly, glutamine metabolism is being targeted as an anti-cancer therapy (Lukey et al., 2013).

Tumour associated NK cells significantly reduced their expression of activating receptors (Rocca et al., 2013) and these NK cells were found to have a defective IFN-γ response and impaired cytotoxicity (Mamessier et al., 2011; Platonova et al., 2011). Not only do tumour cells take up excess nutrients, they also release immune-modulatory mediators into the intra-tumoral space (Baginska et al., 2013). These immune-modulatory molecules are often important for normal physiological homeostasis. However, in the tumour microenvironment, their presence could lead to immune cell exhaustion. For instance, transforming growth factor-β (TGF-β), which can suppress early-stage cancerous cells, is detrimental to immune cells when over expressed by cancer associated fibroblasts

and tumour cells (Colak and Ten Dijke, 2017). TGF-β signalling was demonstrated to be the cause of NK cell dysfunction in childhood B-acute lymphoblastic leukaemia (ALL) (Rouce et al., 2016).

Oxysterols, which are oxygenated derivatives of cholesterol, could also be accumulated in tumour microenvironments. Although they are known to promote cancer cell death (de Weille et al., 2013), oxysterols like 27-hydroxysterol have been implicated in poor prognosis in breast cancer. Indeed they have been shown to directly increase tumour growth and promote metastasis (Baek et al., 2017; Wu et al., 2013). This report and others (Assmann et al., 2017; Kidani et al., 2013) highlighted the requirement of Srebp for NK and T cell activation induced metabolic and functional responses which were impaired upon inhibition of Srebp by 25-hydroxycholesterol, an oxysterol. Therefore, it could be predicted that presence of oxysterols or high cholesterol would inhibit metabolic and therefore, anti-tumour responses of lymphocytes, thus linking hypercholesterolemia, obesity and cancer.

Additionally, conditions like hypoxia and nutrient deprivation could also activate AMP activated protein kinase (AMPK) that negatively regulates mTORC1. mTORC1 itself is acutely sensitive to the availability of nutrients and growth factors. Removal of glucose, amino acid or cytokines inhibits mTORC1 activity in immune cells (Rolf et al., 2013; Sinclair et al., 2013). As discussed in previous chapters, mTORC1 is an important mediator of immune cell metabolic and effector responses (Donnelly et al., 2014; Finlay et al., 2012; Marçais et al., 2014b). Therefore, altered mTORC1/AMPK signalling due to nutrient restriction in the tumour microenvironment will result in impairment of NK and T cell responses (Kedia-Mehta and Finlay, 2019). Interestingly, TGF-β was shown to repress mTOR signalling in NK cells, inhibiting NK cell activation and function *in vitro* (Viel et al., 2016).

In a nutshell, immunosuppressive conditions in the tumour microenvironment, like low glucose, low glutamine, presence of immunomodulatory metabolites, all

suppress the pathways that govern metabolic and functional responses in tumour infiltrating lymphocytes.

This report also emphasizes the importance of IL2, which is predominantly released by T cells, for optimal NK cell responses. Tumour infiltrating T cells are not only anergic but also over-express immune-checkpoints such as PD-1 and CTLA-4 (Thommen and Schumacher, 2018), which would eventually lead to a lack of T cell-secreted effectors such as IL2. The immune exhaustion in TME is a double-edged sword for NK cells as they face anergy and exhaustion owing to the lack of IL2 from T cells as well as direct detriment from the tumour microenvironment. This report demonstrates the importance of IL2 for NK cells that have interacted with tumour cells as it sustained metabolism, function and longevity in these cells. NK cells stimulated with anti-NK1.1 in the absence of IL2 were shown to have a short life span that was rescued by the addition of IL2. Indeed, IL2 has been shown to increase serial killing by NK cells (Bhat and Watzl, 2007). Furthermore, IL2 signalling is essential for sustenance of cMyc in an Slc7A5 dependent manner in IL2/IL12 activated NK cells, as removal of IL2 resulted in a sharp decline in Slc7A5 mRNA expression (Loftus et al., 2018).

The importance of IL2 is evident from its application in cancer immunotherapies, several IL2 based immunotherapies have been developed and efforts have been made to generate mutant forms of IL2 like "super-2" and IL2Cx to circumvent the toxic effects and low efficacy rates seen in the original IL2 therapy, by making the IL2 cytokine CD25 independent (Caudana et al., 2019; Porichis et al., 2018; Rosenberg et al., 1985). Based on our findings, this is very promising as only a subset of NK cells that came in contact with tumour cells upregulate CD25 and having a CD25 independent IL2 response would enhance NK cell anti-tumour responses by upregulating IL2 associated metabolic and functional pathways is almost all NK cells.

These therapies are being used in combination with checkpoint blockade. Although checkpoint blockade has been promising in haematological malignancies, relatively low efficacy is observed in the treatment of solid tumours (Assmann and Finlay, 2016). This maybe in part due to the immune-restrictive conditions found in the tumour microenvironments. My research highlights a key requirement for the optimal functioning of NK cells that are in direct contact with cancer cells. NK cells that have come in contact with tumour cells express high CD25 and these $CD25^{high}$ NK cells that are highly responsive to IL2 cytokine have elevated cMyc and mTORC1 signalling. In order to sustain, both these pathways need abundant supply of glucose, glutamine and other amino acids.

These findings have important implications for the development of new NK cell-based immunotherapies. Our data suggests that the use of chemo- and radiotherapies in combination with NK cell based anti-cancer immunotherapies could prove extremely beneficial. This research suggests that reducing the tumour load and therefore, nutrient competition within the tumour microenvironment might help re-invigorate immune cells by facilitating nutrient availability.

Another promising immunotherapy is the adoptive transfer of NK cells either taken from the host themselves (autologous) or from a donor (allogeneic). There have been successful reports in case of various types of cancers using this type of therapy including acute myeloid leukaemia (AML) (Ishikawa et al., 2004; Li et al., 2015; Ni et al., 2016). Although NK cells isolated from cancer patients are known to display fundamental defects in their cytotoxic capacity and functional responses, making autologous immunotherapies redundant (Platonova et al., 2011; Rocca et al., 2013). This impairment could be attributed to the metabolic defects that NK cells acquire in cancer environments.

However, newer, more promising NK cell-based therapies have been developed. NK cells with genetically engineered receptors-chimeric antigen receptors

(CAR-NK) have also been developed and have proven to be as effective CAR-T cell therapies. However, CAR-NK cell therapies have proven more advantageous as NK cells don't pose the risk of inducing cytokine release syndrome (Li et al., 2018). Other types of genetically engineered NK cells have been developed like NK-92 and CAR-NK92 that can be expanded easily and indefinitely. These "off-the-shelf" therapies are very exciting as they are not patient specific, and therefore cost-effective and available widely. This creates the possibility that NK92-CAR-NK cells could be manipulated to over-express molecules that could enhance NK cell metabolic responses in a nutrient deprived area. Induced pleuripotent stem cells (iPSC) are another renewable source of NK cells that can be standardized as "off-the-shelf NK" therapy. Indeed, in a recent study NK cell specific CAR constructs were expressed on iPSC derived NK cells and showed impressive results in an ovarian cancer xenograft model (Li et al., 2018). One possibility is the over expression of the LNAA receptor Slc7A5, which our research shows to be important for the sustenance of cMyc, in NK-92 cells or iPSCs and administering them into patients. Another strategy could be the over-expression of CD25, thus making NK cells more competitive for IL2 compared to Tregs. Our research supports that NK cells interacting with tumour cells undergo apoptosis, if not provided with survival factors such as IL15 and IL2. Strategies might be developed to overcome this by creating NK cells that endogenously express IL2 or IL15 cytokine.

In conclusion, this study has expanded our knowledge about NK cell metabolism and its vital role in governing NK cell mediated anti-tumour effector responses. The greatest hurdle in treating cancer remains in overcoming the hostile conditions in the tumour microenvironments. Understanding NK cell metabolism in greater detail will pave the way for more improved immunotherapies that focus on re-invigorating exhausted NK cells and using the "natural killers" to their full potential.

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