

Direct Macrophage to NK Cell Lipid Transfer A New Concept in NK Cell Regulation



A thesis submitted to the University of Dublin for the degree of
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Publications

***De novo* polyamine synthesis supports metabolic and functional responses in activated murine NK cells**

Katie L O'Brien, Nadine Assmann, Eimear O'Connor, **Cathal Keane**, Jessica Walls, Chloe Choi, Peter J Oefner, Clair M Gardiner, Katja Dettmer, David K Finlay

Research article in European Journal of Immunology, 2021

Natural killer loops: pyruvate in, lactate out

Cathal Keane, David K Finlay

News and views article in Nature Metabolism, 2025

The Immunometabolism of Natural Killer Cell Responses

Cathal Keane, Clair M Gardiner, David K Finlay

Book chapter in Natural Killer Cells: At the Forefront of Modern Immunology, 2025

Oral Presentations

Immunometabolism Forum (Local)

Short Talk – Trinity College Dublin

December 2022

Society for Natural Immunity NK2023

Poster Presentation – Oslo, Norway – Best Poster Winner

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Immunometabolism Forum (Global)

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British Society for Immunology Congress – Immunometabolism

Short Talk – Belfast, UK

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Summary

Natural killer (NK) cells are innate, cytotoxic lymphocytes that kill virally infected and transformed cells, as well as exerting direct bactericidal activity. Their ability to quickly kill their targets in the absence of antigen recognition makes them a favourable option for the development of immunotherapies. Understanding the metabolic requirements of NK cells is essential to achieve optimal responses of NK cell-based therapies. Current understanding of NK cell metabolism is based on studies of cultured NK cells in isolation and has provided a lot of information on their fuel preferences and metabolic regulators. For example, sterol regulatory element-binding proteins (SREBPs) have been shown to be important to support glucose metabolism in NK cells following activation. The SREBP target genes *Slc25a1* and *Acly* in particular are essential to support NK cell glycolysis and OxPhos through a non-canonical TCA cycle, called the citrate malate shuttle (CMS). However, in complex tissue niches, there is dynamic interplay between NK cells and other cells in the environment. These extrinsic signals have the potential to shape NK cell metabolism, leading to alterations in their effector function.

Macrophages are innate, phagocytic myeloid cells that have wide ranging functions depending on the environment they are found in. They are found ubiquitously, meaning they are constantly interacting with other immune cells. Following LPS-stimulation, macrophages have been shown to produce an oxidised derivative of cholesterol called 25-hydroxycholesterol (25HC). Many oxysterol species, including 25HC have important roles in controlling cellular lipid homeostasis through inhibiting the activation of SREBP transcription factors. It has been shown that oxysterols can inhibit NK cell metabolism and effector functions in response to cytokine stimulation through inhibition of SREBP, though data in this report also highlights that they have other SREBP-independent inhibitory effects on NK cells through modulating plasma membrane structure. Therefore, it was hypothesised that macrophages could limit NK cell metabolism and effector functions through their production of 25HC. While data in this thesis shows that LPS-stimulated macrophages can limit NK cell effector responses, this was independent of any changes in levels of 25HC. It was shown that direct

contacts between NK cells and LPS-stimulated macrophages dampened IFN γ production by NK cells. Analysis of other metabolic parameters revealed that in the presence of LPS-stimulated macrophages, cultured NK cells adopt a metabolic configuration that involves lipid storage, an effect which is not seen in NK cells cultured in isolation. This was characterised by an increase in lipid droplet number in NK cells and increased expression of genes involved in handling fatty acids. The increase in lipid accumulation was mirrored *in vivo* with peritoneal NK cells increasing their lipid content following LPS-immunisation.

Direct contacts with macrophages were also required for NK cells to express the lipid scavenging receptor CD36, which was hypothesised to be required for lipid uptake and storage by NK cells. While it was shown that the lipid accumulation in NK cells was not dependent on CD36-mediated uptake of exogenous fatty acids, the use of macrophages deficient in CD36 revealed that NK cells were obtaining this protein from macrophages through trogocytosis, rather than endogenous expression. Given that CD36 can be transferred from macrophages to NK cells through trogocytosis, it was hypothesised that NK cells could also be obtaining lipids from macrophages through a similar mechanism.

The use of pharmacological inhibitors of lipid synthesis revealed that lipid accumulation in NK cells is dependent on *de novo* fatty acid synthesis by LPS-stimulated macrophages. The *de novo* synthesised fatty acids are then transferred to the NK cells through direct contacts. Using an irreversible inhibitor of fatty acid synthase to specifically block *de novo* fatty acid synthesis in macrophages, resulted in a rescue in IFN γ production by co-cultured NK cells. This reveals a novel metabolic communication axis between macrophages and NK cells that can control NK cell IFN γ production. Given the role of IFN γ in activating macrophages, this may represent a negative feedback mechanism to limit sustained stimulation of macrophages and prevent progression to hyperinflammatory conditions such as sepsis.

Abbreviations

2DG	2-deoxyglucose
25HC	25-hydroxycholesterol
27HC	27-hydroxycholesterol
7KC	7-ketocholesterol
ABC	ATP-binding cassette transporter
ACAT	Acetyl CoA acetyltransferase
ACC	Acetyl CoA Carboxylase
ACLY	ATP citrate lyase
ACSS	Acetyl CoA synthetase short chain
ADCC	Antibody-dependent cellular cytotoxicity
ADP	Adenosine diphosphate
AHR	Aryl hydrocarbon receptor
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BMDM	Bone marrow-derived macrophage
C75	4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid
CACT	Carnitine acyltransferase
CAR	Chimeric antigen receptor
CCL	C-C motif chemokine ligand
CFSE	Carboxyfluorescein succinimidyl ester
CH25H	Cholesterol 25 hydroxylase
CMS	Citrate-malate shuttle
CoA	Coenzyme A
CpG	Cytosine and guanine triphosphate oligodeoxynucleotides
CPT	Carnitine palmitoyltransferase
CTL	Cytotoxic T lymphocyte
CXCL	C-X-C motif chemokine ligand
CYP	Cytochrome p450

DAPI	4',6-diamino-2-phenylindole
DAG	Diacylglyceride
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECAR	Extracellular acidification rate
eIF	Eukaryotic translation initiation factor
ER	Endoplasmic reticulum
ETC	Electron transport chain
EV	Exocytic vesicle
FABP	Fatty acid binding protein
FAD	Flavin adenine dinucleotide
FasL	Fas ligand
FASN	Fatty acid synthase
FCS	Foetal calf serum
FFA	Free fatty acid
FIA/MS	Flow injection analysis/mass spectrometry
FSC	Forward scatter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAAS	Graphite furnace atomic absorption spectrometry
GLUT	Glucose transporter
GLP	Glucagon-like peptide
HK	Hexokinase
HLA	Human leukocyte antigen
HMGCS	3-hydroxy-3-methylglutaryl CoA synthase
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IFNAR	Interferon α/β receptor
IL2-NK	IL2-expanded NK
INSIG	Insulin-induced gene
KO	Knockout

LC/MS	Liquid chromatography/mass spectrometry
LCMV	Lymphocytic choriomeningitis virus
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LPM	Large peritoneal macrophage
LPS	Lipopolysaccharide
LXR	Liver X receptor
MCMV	Murine cytomegalovirus
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MTOC	Microtubule organising centre
mTOR	Mammalian target of rapamycin
Myc	Myelocytomatosis
NAD	Nucleotide adenine dinucleotide
NADP	Nucleotide adenine dinucleotide phosphate
NCR	NK cell receptor
NLRP	NLR family pyrin domain containing
NK	Natural killer
NKG	Natural killer group
OCR	Oxygen consumption rate
OPP	O-propargyl puromycin
OSBP	Oxysterol-binding protein
OxPhos	Oxidative phosphorylation
OxLDL	Oxidised low density lipoprotein
PBS	Phosphate buffered saline
PDK	Pyruvate dehydrogenase kinase
PK	Pyruvate kinase
PLIN	Perilipin
PolyI:C	Polyinosinic:polycytidylic acid
PPAR	Peroxisome proliferator-activated receptor
PPP	Pentose phosphate pathway
PPRE	Peroxisome proliferator-responsive element

PRR	Pattern recognition receptor
R848	Resiquimod
ROS	Reactive oxygen species
RT	Room temperature
S1P	Site-1 protease
S2P	Site-2-protease
SCAP	SREBP cleavage-activating protein
SCARB	Scavenger receptor class B
SCENITH	Single-cell energy profiling by translation inhibition
SCFA	Short chain fatty acid
SEM	Standard error of the mean
SERM	Selective oestrogen receptor modulator
SLC	Solute carrier
SPM	Small peritoneal macrophage
SQS	Squalene synthase
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
SSC	Side scatter
TAG	Triacylglyceride
TAM	Tumour-associated macrophage
TCA	Tricarboxylic acid
TGF	Tumour growth factor
TIL	Tumour-infiltrating lymphocyte
TINK	Tumour-infiltrating NK cell
TLR	Toll-like receptor
TME	Tumour microenvironment
TNF	Tumour necrosis factor
TOFA	5-tetradecyloxy-2-furoic acid
TRAIL	TNF-related apoptosis-inducing ligand
UPR	Unfolded protein response
WT	Wild type
ZA	Zaragozic acid

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Chapter 1

Introduction

1.1 Natural Killer Cells

Natural killer (NK) cells are innate, granular, cytotoxic lymphocytes that provide defence against virally infected and transformed cells (Caligiuri, 2008). They were first identified because of their ability to kill tumour cells without any prior exposure to tumour antigens (Herberman *et al.*, 1975), and have since been described in maintaining tissue homeostasis (Hanna *et al.*, 2006), exhibiting bactericidal activity (Garcia-Peñarrubia *et al.*, 1989) and removing senescent cells (Brighton *et al.*, 2017). Unlike CD8⁺ cytotoxic T lymphocytes (CTLs), NK cells do not require antigen presenting cells (APCs) to recognise their targets, nor do they require recombination of receptor genes for high antigen specificity (Caligiuri, 2008). Instead, NK cells recognise characteristic ligands of tumour or virally infected cells using receptors inherited through the germline (Raulet, 2006). This innate characteristic of NK cells means that their cytotoxicity must be tightly regulated to prevent the killing of healthy cells.

1.1.1 Receptor-Mediated NK Cell Activation

The 'missing self' hypothesis describes the mechanism by which NK cell cytotoxicity is regulated. The recognition of target cells is based on the balance of signalling between the germline encoded natural killer activating receptors and inhibitory receptors. Activation can occur based on an increase in signalling through activating receptors or a decrease in signalling through inhibitory receptors. Various inhibitory receptors will recognise self-major histocompatibility complex (MHC) molecules and prevent an immune response against self-cells (Ljunggren and Kärre, 1990; Raulet, 2006). In humans there are a variety of inhibitory receptors that can recognise human leukocyte antigen (HLA) variants, these include killer immunoglobulin-like receptors (KIRs) or members of the NKG2 family. In mice Ly49 recognises MHC class I (Lanier, 2005). These receptors signal through an immunoreceptor tyrosine-based intracellular motif (ITIM) located in the cytoplasmic domain that leads to the recruitment of phosphatases which act on several downstream molecules required for NK cell

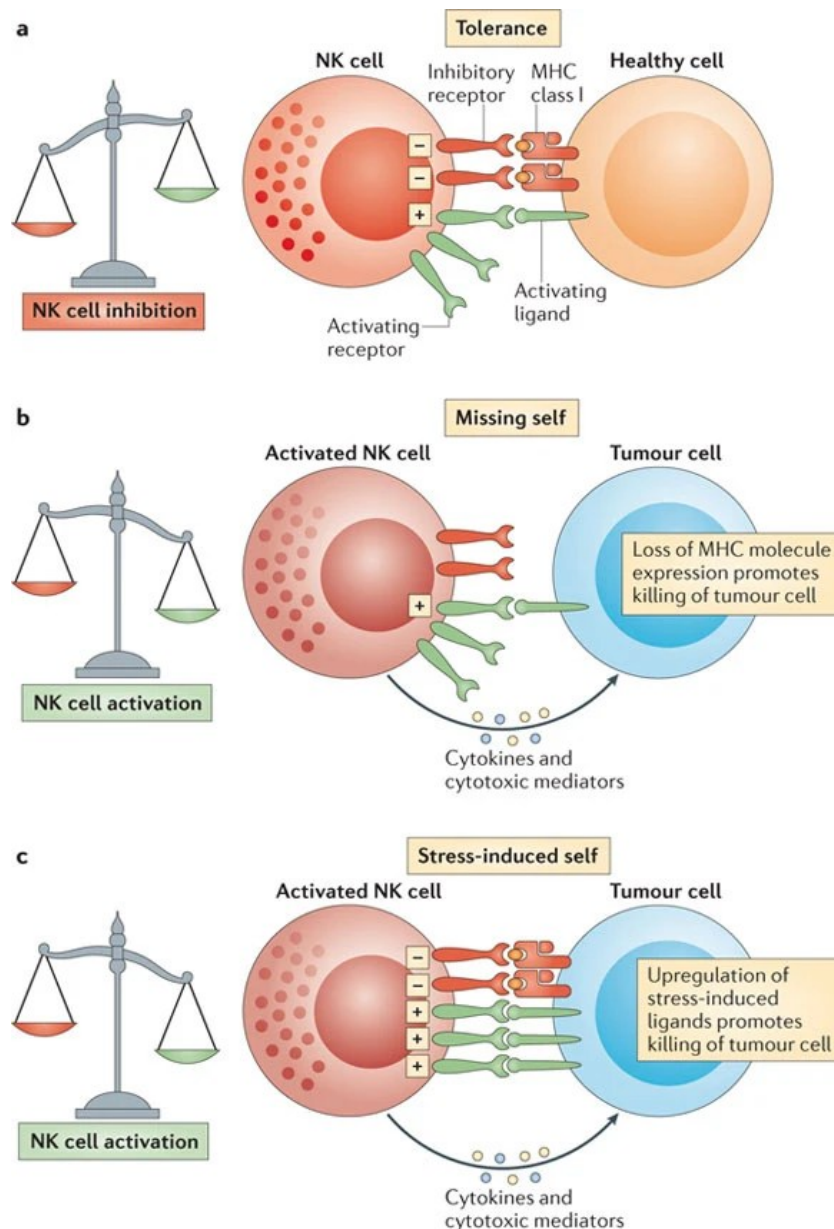


Figure 1.1 Recognition of target cells by NK cells

The 'missing self' hypothesis states that NK cells will not initiate an immune response against cells that express class I MHC molecules (a). However, in order to evade T cell responses, some transformed cells will decrease expression of class I MHC, making them susceptible to NK cell cytotoxicity (b). Many transformed or virally infected cells express stress ligands on their surface which can overcome inhibitory interactions at the immunological synapse and will result in activation of NK cell effector functions (c). *Taken from Vivier et al., 2012.*

activation such as ZAP70 and Syk. The process of 'educating' NK cells occurs in the bone marrow where they are exposed to the host repertoire of MHC class I molecules, generating mature NK cells that will not become activated following detection of healthy tissue. In order to evade CTL responses, many infected cells and tumour cells decrease their expression of MHC class I molecules, and in doing so make themselves vulnerable to a cytotoxic response from NK cells (Lanier, 2008).

In addition, the binding of activating receptors to stress ligands on target cells can overcome NK cell inhibition. NK cells express a wide range of activating receptors that can recognise these ligands. These include natural killer cell group (NKG) 2D and DNAX accessory molecule (DNAM)-1 and natural cytotoxicity receptors (NCRs) such as NKp30, NKp44 and NKp46 (Pegram *et al.*, 2011). Another activating receptor, cluster of differentiation (CD) 16 is important for the killing of opsonised targets as it recognises the fragment crystallisable (Fc) region of immunoglobulin (Ig) G antibodies. This initiates a process called antibody-dependent cellular cytotoxicity (ADCC) (Anegon *et al.*, 1988; O'Leary *et al.*, 2006). Binding of these activating receptors provides NK cells with strong signals through intracellular tyrosine-based activating motifs (ITAMs) (Chan *et al.*, 2014). Upon ligation, phosphorylation of tyrosine residues in this intracellular domain causes recruitment of kinases such as ZAP70 and Syk, which initiate a signalling cascade that results in calcium influx, degranulation and transcription of genes required for NK cell functional responses (Lanier, 2005).

1.1.2 Cytokine-Mediated NK Cell Activation

Aside from activation through target recognition, robust NK cells responses can be induced following the integration of signals produced by other cells in their environment. Cytokines and chemokines can act to recruit NK cells to the site of infection and increase their cytotoxic potential. NK cells are known to respond to IL2, IL12, IL15 and IL18, as well as type I interferons, which can induce activation in different combinations. The production of IL15 by dendritic cells (DCs) is essential for the survival of NK cells demonstrated by their virtual absence in IL15-deficient mice (Lodolce *et al.*, 1998).

The production of IL12 by DCs and macrophages following TLR activation increases the expression of the IL2 receptor (CD25) on NK cells (Nguyen *et al.*, 2000; Duggan *et al.*, 2018). The primary source of IL2 *in vivo* is T cells, revealing an important link between NK cells and adaptive immunity (Owen *et al.*, 2018). In addition to the increase in expression of CD25, IL12 alone is also an inducer of IFN γ expression, though there are synergistic effects observed when NK cells are exposed to IL12 in combination with IL2 or with IL18 (Nakahira *et al.*, 2002). Supporting evidence for this stimulus comes from IL12-deficient mice that show decreased NK cell-derived IFN γ in a murine cytomegalovirus (MCMV) model (Orange *et al.*, 1996). The type I interferon production by plasmacytoid dendritic cells (pDC) can also activate NK cells due to their expression of interferon α/β receptor (IFNAR)1 and IFNAR2. This IFNAR signalling culminates in an increase in NK cell cytotoxicity and IFN γ production (Della Chiesa *et al.*, 2006; Walzer *et al.*, 2005).

Similar to NK cell activation with receptors, there are also mechanisms in place to prevent activation of NK cells in immunoprivileged sites using cytokines. These include tumour growth factor β (TGF β) which has long been described to reduce NK cell development, proliferation, and effector function (Bellone *et al.*, 1995). Its production by T regulatory (T_{reg}) cells has been linked with homeostatic control of inflammation (Trzonkowski *et al.*, 2004; Ghiringhelli *et al.*, 2005; Ralainirina *et al.*, 2007). Many tumour cells are also known to produce TGF β , in particular in breast cancer (MacCallum *et al.*, 1994; Sminia *et al.*, 1998). Murine tumour models have shown that increased TGF β signalling led to inhibition of NK cell immunosurveillance (Gao *et al.*, 2017).

1.1.3 Cytotoxicity

NK cells are potent killers of transformed and virally infected cells through a number of mechanisms including degranulation, cytokine-mediated induction of apoptosis and apoptosis using death ligands. The release of pre-made cytolytic granules containing proteins that induce apoptosis in the target cell, through a process called degranulation, is a method NK cells use to effectively kill target cells while preventing damage to surrounding tissue. To achieve this, degranulation is directed to a contact interface with the target cell called the

immunological synapse (Gwalani and Orange, 2018; Prager and Watzl, 2019). Following the recognition of target cells by binding CD16, or the synergistic binding of multiple activating receptors, NK cells will increase integrin binding affinity for their ligands (Abram and Lowell, 2009; Luo *et al.*, 2007). For example, following binding of the natural killer cell receptor 2B4, NK cells were observed to induce increased binding of the integrin LFA-1 to the intercellular adhesion molecules (ICAMs) 1, 2, and 3 on HeLa cells (Hoffmann *et al.*, 2011).

Once conjugates are formed and the immunological synapse is established, NK cells will direct their pre-formed granules to the interface using the microtubule organising centre (MTOC) (Prager and Watzl, 2019; Gwalani and Orange, 2018). The contents of these granules include perforin and granzymes (Prager and Watzl, 2019; Morvan and Lanier, 2016). Perforin is a pore-forming protein that disrupts the target cell membrane. If this disruption cannot be locally resolved by the target cell, death can occur by necroptosis, a process involving rapid cellular swelling and lysis (Voskoboinik *et al.*, 2006; Voskoboinik *et al.*, 2015). However, the more commonly accepted means of inducing death of a target cell is granzyme-mediated apoptosis (Voskoboinik *et al.*, 2005). The granzymes found in NK cell granules include granzyme A, B, H, K and M. Entry of granzymes into target cells has primarily been described to occur through perforin pores, though recently there is evidence that granzymes can also be endocytosed independently of perforin (Pardo *et al.*, 2007; Voskoboinik *et al.*, 2015). Granzyme B is the most effective pro-apoptotic granzyme as it mimics caspases by cleaving proteins after acidic residues, in particular at aspartate residues. Granzyme A is also well known to kill by this mechanism though it does not induce apoptosis as quickly as granzyme B (Voskoboinik *et al.*, 2006; Voskoboinik *et al.*, 2015). Because of its ability to cleave proteins after aspartate residues, many caspases are activated by granzyme B, initiating caspase-dependent apoptosis. These include caspase-3, 6, 7, 8, 9 and 10 (Darmon *et al.*, 1995; Martin *et al.*, 1996; Duan *et al.*, 1996; Fernandes-Alnemri *et al.*, 1996; Medema *et al.*, 1997). In addition, granzyme B has also been described to work independently of caspases by disrupting the mitochondrial inner membrane, resulting in cytochrome c release and the formation of the apoptosome (Alimonti *et al.*, 2001).

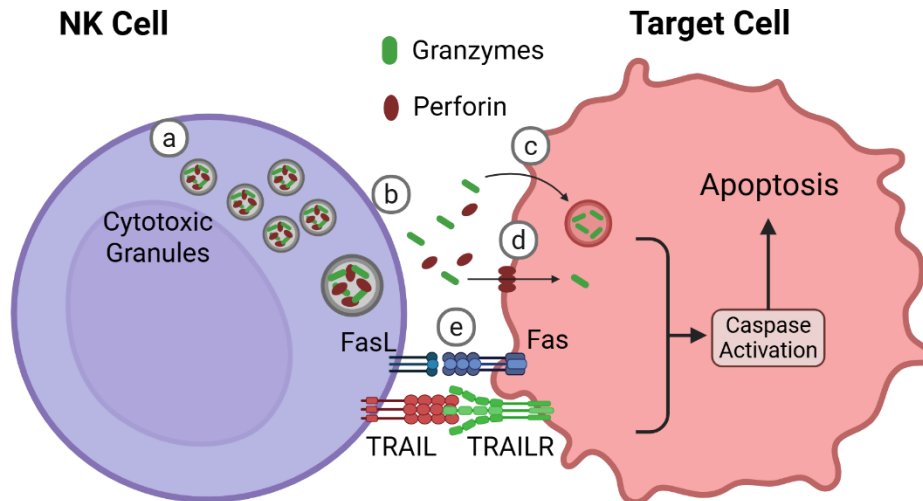


Figure 1.2 Summary of NK cell cytotoxicity

Upon cytokine stimulation or receptor-mediated activation NK cells increase the expression of perforin and granzymes, stored in cytotoxic granules (a). Following the recognition of target cells, NK cells polarise their MTOC to direct the release of cytotoxic molecules from granules into the immunological synapse (b). Granzymes can be endocytosed into the target cells (c). Alternatively, perforin can oligomerise and form pores in the target cell membrane which can cause death due to necroptosis. Alternatively, granzymes can also enter the target cell through these perforin pores (d). Following their entry into the cell, granzyme can cleave intracellular proteins and activate caspases to induce apoptosis in the target cell. Alternatively, NK cells can also induce death in target cells through their expression of FasL and TRAIL which can engage the corresponding death receptors, Fas and TRAILR (e). This can also induce caspase activation in the NK cells leading to apoptosis of the target cell. *This figure was created using Biorender.com.*

The induction of apoptosis in the target cell can also be mediated through production of tumour necrosis factor α (TNF α), which acts on target cells by binding its receptor, triggering a signalling pathway that activates caspases-2 and -8 (Chau *et al.*, 2004; Michaeu and Tschopp, 2003). Other receptor mediated cytotoxic methods used by NK cells include an increase in the expression of death ligands. These include Fas ligand (FasL) and tumour necrosis factor-

related apoptosis inducing ligand (TRAIL), which are expressed at the immunological synapse. The receptors are stored in secretory granules and are brought to the cell surface upon degranulation or cytokine stimulation (Kayagaki *et al.*, 1999; Smyth *et al.*, 2001; Liu *et al.*, 2011). Following degranulation, these ligands can bind to their receptors on the surface of target cells and induce the activation of caspases-8 and -10 (Peter and Krammer, 2003; von Karstedt *et al.*, 2017). There is also some evidence that following non-directed targeting of TRAIL to the plasma membrane, it is shed and can potentially kill nearby target cells in its soluble form, a characteristic that is not shared with FasL (Wiley *et al.*, 1995).

1.1.4 Cytokine and Chemokine Production by NK Cells

The effector functions of NK cells also involve the production of cytokines and chemokines that can act on other immune cells, to coordinate an appropriate immune response. The role of TNF α in inducing apoptosis of target cells has already been described. However, many other cytokines produced by NK cells act to promote killing of transformed and virally infected cells through non-cytolytic mechanisms.

One way NK cells can communicate with other immune cells to induce stronger immune responses is through the expression of chemokines that will recruit other immune cells to sites of infection, or into tumours. Furthermore, they may spatiotemporally regulate the positions of immune cells, such as DCs in lymphoid organs to regulate appropriate production of other cytokines (Ghilas *et al.*, 2021). Among the chemokines produced by NK cells are IL8, XCL1, CCL1, CCL3, CCL4, CCL5, CCL22, CXCL8 and CXCL10 (Robertson, 2002). These chemokines have been shown to have diverse functions in some infections outside their roles in chemoattraction. For example, CCL3, CCL4 and CCL5 produced by NK cells can inhibit replication of human immunodeficiency virus (HIV) *in vitro* (Song *et al.*, 2014).

NK cells are known to produce large amounts of IFN γ which has been consistently shown to be important for creating an anti-viral state in neighbouring cells in MCMV infections. The contribution made by NK cells is significant as depletion of NK cells, but not T or B cells led to decreases in IFN γ which

correlated with increased viral loads (Orange *et al.*, 1996). It has also been shown to induce an antiviral state in HBV, measles virus, SINV and West Nile virus infections (Suri *et al.*, 2001; Patterson *et al.*, 2002; Burdeinick-Kerr and Griffin, 2005; Zhang *et al.*, 2010). The specific mechanism and associated effector proteins in this induced antiviral state are still largely unknown. However, it is known that IFN γ can also act on other immune cells and shape the immune response. For example, IFN γ derived from NK cells is known to promote the differentiation of monocytes into inflammatory DCs at the site of infection with *Toxoplasma gondii*. The DCs then serve as the major source of IL12 for NK cells in the infected tissue (Goldszmid *et al.*, 2012). This has also been described *in vitro* with IFN γ observed to promote CD86 on cultured DCs (Gerosa *et al.*, 2002). Furthermore, NK cell-derived IFN γ was also shown to be important for activation of macrophages in the lung following aspergillosis infections (Park *et al.*, 2009). In fact, IFN γ is commonly used in combination with TLR agonists to synergistically activate macrophages *in vitro*, highlighting its importance as an endogenous macrophage activator in infections (Müller *et al.*, 2017). Aside from shaping the innate immune, IFN γ can also shape the adaptive immune response through promoting differentiation of CD4⁺ T cells into T_H1 cells (Scharton and Scott, 1993; Martín-Fontecha *et al.*, 2004; Morandi *et al.*, 2006). This effect was first described in *Leishmania* infection and was shown to require NK cell-derived IFN γ (Scharton and Scott, 1993). Further interplay between NK cells and DCs has been described with conventional type 1 DCs (cDC1) in the spleen, where granulocyte-macrophage colony stimulating factor (GM-CSF) production by NK cells was shown to be essential to support the response to MCMV infection. The production of this cytokine was regulated spatiotemporally by cDC1-derived IL12. The interplay between both cell types promoted CCR7 expression on the cDC1 and was also essential for IFN γ production by the NK cells (Ghilas *et al.*, 2021).

Further to the production of these pro-inflammatory cytokines, NK cells are also known to be producers of IL10 in a variety of bacterial and protozoan infections, following exposure to IL12 (Perona-Wright *et al.*, 2009). This mechanism likely exists in a negative feedback loop as the increase in IL10 production from NK cells was associated with decreased production of IL12 from DCs in co-culture. It is known that IL10 can also inhibit macrophage activation (O'Farrell *et al.*,

1998). Furthermore, the culture of macrophages with IL10 increased NKG2D expression, leaving the macrophages susceptible to NK cell-mediated lysis (Schulz *et al.*, 2010). This may exist as a potential mechanism for NK cell-mediated editing of the immune system to prevent hyperinflammation. In fact, the process of NK cell cytotoxicity against macrophages is essential to prevent hyperinflammation. Individuals with inherited defects in cytotoxic machinery develop a life threatening condition called hemophagocytic lymphohistiocytosis, which involves uncontrolled activation of macrophages leading to severe tissue damage (Sepulveda and de Saint Basile, 2017).

1.1.5 NK Cell Immunotherapy

The features of NK cell biology that have been described so far make them favourable targets for immunotherapeutic strategies. Many existing immunotherapies harness these protective effects of NK cells. For example, the ability of NK cells to recognise and kill opsonised targets mean that they are important for the efficacy of many monoclonal antibody (mAb) therapies such as trastuzumab and rituximab (Sliwkowski *et al.*, 1999; Carson *et al.*, 2001; Cartron *et al.*, 2002). Preclinical models using the murine leukaemia virus FrCasE suggest that mAb therapies could also be used to treat viral infections through harnessing NK cells responses (Naranjo-Gomez *et al.*, 2021).

However, NK cells are also being explored for use in allogeneic therapies, especially because they do not induce graft versus host disease (GVHD) unlike T cell therapies. Some 'off the shelf' NK cell therapies that are being investigated include the adoptive transfer of cytokine activated NK cells, chimeric antigen receptor (CAR) NK cells and the engagement of CD16 to promote ADCC, such as bispecific and trispecific killer engager therapies. To date these therapies have mainly been successful in patients that have haematological malignancies (Ruggeri *et al.*, 2002; Miller *et al.*, 2005; Bachanova *et al.*, 2018). One of the main reasons for this is the immunosuppressive environment that exists within solid tumours characterised by elevated levels of TGF β or inhibitory metabolites such as lactate and kynurenine. These factors have been shown to inhibit NK cell proliferation and cytotoxicity and can directly impact NK cell metabolism (Frumento *et al.*, 2002; Chiesa *et al.*, 2006; Brand *et al.*, 2016; Zaiatz-Bittencourt

et al., 2018; Harmon *et al.*, 2019). Therefore, to improve the function of tumour-infiltrating lymphocytes (TILs), including tumour-infiltrating NK cells (TINKs) in the tumour microenvironment (TME), immunotherapeutic strategies should also consider the metabolic landscape that exists in the TME and how this could be altered to promote the metabolism and function of TINKs.

1.2 Macrophages

1.2.1 Macrophage Development

Macrophages are innate, phagocytic myeloid cells that have wide ranging functions in maintaining tissue homeostasis or defence against pathogens, depending on the environment they are found in (Zhao *et al.*, 2018). They exist in almost all tissues and are present from as early as embryonic development. These macrophage populations arise from yolk sac progenitor cells and are seeded throughout tissues as the embryo develops. They then become persistent tissue-resident macrophage populations that are self-replenishing in adulthood (Hoeffel and Ginhoux, 2015). Aside from tissue-resident macrophage subsets, macrophages can also differentiate from circulating monocytes following their recruitment into tissues. These circulating monocytes develop in the bone marrow and are derived from common myeloid progenitor (CMP) cells through the monocyte-macrophage DC progenitor (MDP). This give rise to Ly6C^{high} monocytes, which are considered pro-inflammatory and Ly6C^{low} monocytes, which are considered anti-inflammatory (Hettinger *et al.*, 2013; Kawamura *et al.*, 2017). These bone marrow-derived monocytes can then replenish macrophage populations during injury or infection (Wolf *et al.*, 2019).

A good example of this process can be seen in peritoneal macrophage populations. In the peritoneum, macrophages are separated into two populations that differ in size, leading to them being termed large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs). Aside from their differences in size these two macrophage populations also exhibit very different functions. The LPMs are tissue-resident yolk sac-derived macrophages that are self-renewing and are involved in maintenance of tissue homeostasis (Cain *et al.*, 2013; Yona *et al.*, 2013). SPMs are derived from circulating monocytes and are generally present in very low numbers in the peritoneum before the onset of

inflammation. Following infection, LPMs largely disappear and SPMs increase their frequency following the differentiation of circulating monocytes and produce pro-inflammatory cytokines such as IL12, TNF α and macrophage inflammatory protein (MIP) 1 α (Cain *et al.*, 2013). This decrease in the numbers of LPMs in the peritoneum following LPS immunisation has been attributed to migration of these macrophages to the omentum (Meza-Perez and Randall, 2017; Vega-Pérez *et al.*, 2021), or their removal from the peritoneal through the formation of clots (Zhang *et al.*, 2019). This shift in the frequency of macrophage populations demonstrates how the lineage of macrophage differentiation is important to elicit an appropriate inflammatory response.

1.2.2 Macrophage Polarisation

Macrophage activation, or more accurately, polarisation is controlled by the signals they receive in their environment. Pro-inflammatory cytokines and pathogen-associated molecular patterns (PAMPs) can polarise macrophages to a pro-inflammatory M1 phenotype, whereas M2 macrophages are more associated with the resolution of inflammation through removal of cellular debris and promoting tissue repair (Martinez *et al.*, 2008). Macrophages can recognise PAMPs using germline-encoded pattern recognition receptors (PRRs), which act as innate immune sensors, and initiate a number of different signalling pathways to initiate the subsequent inflammatory response. These PRRs include TLRs, NOD-like receptors (NLRs), c-type lectin receptors (CLRs) and RIG-I-like receptors (RLRs) which are expressed on the plasma membrane, cytosol and several intracellular membranes (Li and Wu, 2021). *In vitro* M1 macrophages can be polarised by treating with TLR agonists alone or often in combination with IFN γ (Murray *et al.*, 2014).

M1 macrophages are professional APCs along with DCs. It is generalised that macrophage antigen-presenting functions support the activation of effector T cells at the site of infection, whereas DCs are described to migrate to the lymph nodes to present antigen to naïve T cells there (Muntjewerff *et al.*, 2020). Their ability to engulf pathogens and remove them from tissues also makes them important for clearance of microbes in infection. The production of nitric oxide synthase by macrophages results in the synthesis of inducible nitric oxide, which

has microbicidal activity through generation of reactive nitrogen species or induction of mitochondrial dysfunction (Palmieri *et al.*, 2020). Through this mechanism macrophages can kill mycobacteria or opsonised bacteria.

M2 macrophages, or alternatively activated macrophages can be induced by cytokines such as IL4 and IL13 (Gordon and Martinez, 2010; McCormick and Heller, 2015). They are commonly induced through stimulation with IL4 *in vitro*. Their effector functions primarily include the production of anti-inflammatory cytokines such as IL10 and TGF β to resolve inflammation (Mills *et al.*, 2000). They can also clear damaged tissue and promote tissue repair through efferocytosis. This involves an increase in expression of scavenging receptors such as MerTK (Dalli and Serhan, 2017; Gerlach *et al.*, 2021).

This concept of macrophage polarisation is not as simple as previously believed. Even *in vitro* there is an increase in the number of activation states depending on the stimuli provided (Chen *et al.*, 2023). *In vivo* it is even more complicated because of the diverse signals from metabolites, cytokines, chemokines and receptor-ligand interactions that exist in specific tissue niches. Advancements in single cell techniques have shown the heterogeneity in macrophage subsets that exist in many tissues (Xu *et al.*, 2024). One example of this, is tumour-associated macrophages (TAMs). Many studies have linked increased macrophage infiltration to tumours with poor outcomes (Ding *et al.*, 2009; Qing *et al.*, 2012; Poh and Ernst, 2018). This is because macrophage polarisation in the tumour has an M2-like skew due to re-education of the infiltrating macrophages by the TME. An increase in the production of C-C motif chemokine ligand (CCL) 2 and colony stimulating factor (CSF) 1 by tumour cells promotes the infiltration of macrophages into the tumour, where tumour-derived metabolites can drive their polarisation to pro-tumour macrophages. Succinate, lactate and kynurenine production have been shown to play an important role in modulation of TAM functions (Colegio *et al.*, 2014; Wu *et al.*, 2020). The polarisation of TAMs is also linked with other environmental perturbations, such as hypoxia (Casazza *et al.*, 2013). Once the macrophages have been polarised to their pro-tumour phenotype they can promote angiogenesis through the production of soluble factors such as vascular endothelial growth factor (VEGF), facilitating metastasis (Fu *et al.*, 2020). Furthermore, they can suppress the immune response in the

tumour through production of IL10 and TGF β (Rui Wang *et al.*, 2011; Cai *et al.*, 2019), or induction of T_{reg} responses through the transfer of micro RNAs (miRNAs) in exosomes (Zhou *et al.*, 2018). Because of all of these pro-tumour effects, TAMs are being targeted in cancer immunotherapy. For example, blocking the CSF1-dependent recruitment of TAMs with pexidartinib has shown efficacy in some solid tumours and significantly reduces the number of tumour-infiltrating macrophages (Wesolowski *et al.*, 2019).

1.3 NK Cell-Macrophage Interactions

Certain interactions between NK cells and macrophages are important to shape immune responses in infection or in tumours. They are known to work collaboratively to identify and eliminate pathogens in infection, though the plasticity of macrophages means that their interactions are more far-reaching than this. In fact, the heterogeneity of macrophage populations across various tissue niches and disease states, means that NK cell-macrophage interactions can be either beneficial or detrimental for NK cell functions, depending on the environment.

There are a number of receptor-ligand interactions that have been described *in vitro* which can activate NK cells following co-culture with macrophages. For example, in mice it has been shown that expression of CD40L on NK cells, led to binding of CD40 on macrophages, which enhanced NK cell activation (Scott *et al.*, 2004; Atochina and Harn, 2005). Activation of peritoneal macrophages *in vitro* with a variety of TLR agonists was shown to increase expression of RAE-1 by macrophages which is a ligand for the NKG2D receptor (Hamerman *et al.*, 2004). In fact, it was subsequently shown that following *Bacillus anthracis* infection, BMDMs increased expression of RAE-1, and this interaction was important to induce the secretion of IFN γ by NK cells (Klezovich-Bénard *et al.*, 2012). However, persistent exposure to NKG2D ligands has been associated with desensitised NK cells. Enhanced expression of RAE-1 by TAMs has been shown to reduce expression of NKG2D by TINKs in B16 tumours. In this model, deletion of RAE-1 or NKG2D in mice significantly enhanced NK cell anti-tumour functions (Deng *et al.*, 2015). Although primarily associated with direct cell-cell contacts,

macrophages are also known to shed ligands for NK cell receptors and use these as soluble messengers to either activate or inhibit NK cell responses.

The initial studies investigating the role of cytokines as messengers between NK cells and macrophages saw that following infection of peritoneal macrophages *in vitro* with *Listeria monocytogenes*, the subsequent IL12 and TNF α production were sufficient to induce IFN γ production by NK cells (Tripp *et al.*, 1993). Since then, a number of NK cell-activating cytokines have been shown to be secreted by macrophages in bacterial infections leading to enhanced IFN γ production and CD69 expression by NK cells. These macrophage-derived cytokines include IL12, IL15 and IL18 (Haller *et al.*, 2002; Small *et al.*, 2008; Lapaque *et al.*, 2009; Bellora *et al.*, 2012). With regard to NK cell-macrophage interactions in the TME, TAMs are known to be producers of TGF β which can be secreted into the environment or alternatively, presented on the cell surface. Increased expression of TGF β by TAMs isolated from murine breast tumours was shown to significantly impair NK cell cytotoxicity following co-culture (Krnetá *et al.*, 2017). Because of the abundance of macrophages in almost every tissue, these interactions should be considered when considering the use of NK cells for immunotherapies.

1.4 NK Cell Immunometabolism

The term 'immunometabolism' has emerged to describe the advances made in understanding immune cell metabolism, and importantly, how that is integrally linked to immune responses. The metabolic configurations employed by cells are critical for their viability and to support their biological functions. The cells of the immune system are no different requiring specific use of their fuels through different metabolic pathways to support their effector functions. These metabolic configurations can produce metabolites that regulate intracellular signalling, alter gene expression, post-translationally modify proteins or can be used in anabolic processes such as DNA replication or protein synthesis. Furthermore, increases in energy metabolism can make adenosine triphosphate (ATP) available to the cell which will support these processes. The activation of NK cells is a very dynamic process involving integration of signals from the external environment to elicit an appropriate response to cancer or infection (O'Brien and Finlay, 2019; Keane *et al.*, 2025). In order to rapidly respond to these stimuli, NK cells evolved

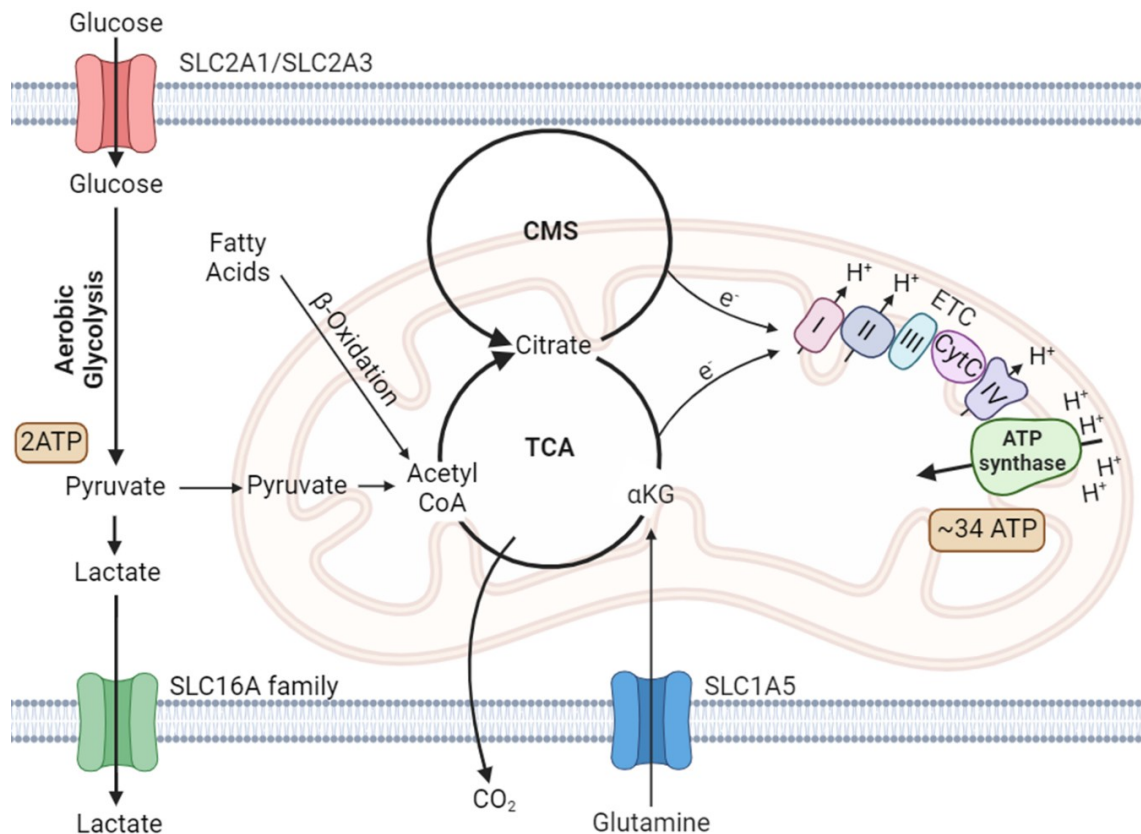


Figure 1.3 Summary of glycolysis and OxPhos-mediated ATP production in NK cells.

Glucose is transported into NK cells through SLC2A1 and SLC2A3. Lactate, produced through aerobic glycolysis is exported by the SLC16A family of transporters. This metabolic configuration supports rapid ATP production, generating 2 ATP per molecule of glucose. Alternatively, glucose can be metabolised to pyruvate which can be used to fuel OxPhos. Pyruvate can be transported into the mitochondria and converted to acetyl CoA, which generates citrate following condensation with oxaloacetate. Citrate then enters the tricarboxylic acid (TCA) cycle or the citrate-malate shuttle (CMS) (Section 1.4.1). These metabolic cycles generate NADH, a reducing equivalent that provides electrons to complex I of the electron transport chain (ETC). The ETC generates a proton (H^+) electrochemical gradient across the inner mitochondrial membrane which drives the activity of ATP synthase, generating about 34 ATP. Glutamine, transported by SLC1A5, is another fuel that supports the TCA cycle, but not flux through the CMS. Similarly, β -oxidation of fatty acids produces acetyl-CoA which can fuel these mitochondrial metabolic cycles. *Taken from Keane et al., 2025.*

to quickly and effectively alter their metabolism through changes in expression of nutrient transporters and metabolic machinery. Glycolysis and glucose-fuelled oxidative phosphorylation (OxPhos) are the primary metabolic pathways used to support ATP production in many cell types. In cells with increased biosynthetic demands such as activated lymphocytes, increased metabolism of glucose to lactate, means glycolytic intermediates can be channelled into biosynthetic metabolic pathways while simultaneously producing ATP. For more efficient ATP synthesis, cells can metabolise glucose to pyruvate which enters the mitochondria and supports oxidative metabolism. Pyruvate can be converted to acetyl CoA which enters the tricarboxylic acid (TCA) cycle. This pathway generates the reducing equivalents: NADH and FADH₂ which donate electrons to the electron transport chain (ETC), generating a proton electrochemical gradient which leads to more efficient production of ATP through OxPhos. Our current understanding of what fuels NK cell functional responses is based on studies that have primarily focused on glucose metabolism because of the use of cultured NK cells in glucose-rich medium. Therefore, it is likely that glucose is not the only fuel that NK cells have the potential to use but rather represents what is currently understood in the absence of more advanced culturing techniques and metabolic analysis.

1.4.1 NK Cell Metabolic Configurations

Glycolysis

Studies on NK cell metabolic signatures following activation are primarily based on glucose metabolism, whereby glycolysis and glucose-fuelled OxPhos are the primary pathways used to generate ATP. The utilisation of glucose to support NK cell metabolism is supported by the expression of glucose transporters which increase upon activation. Glucose uptake is primarily facilitated by certain isoforms from the GLUT family of glucose transporters: SLC2A1 (GLUT1) and SLC2A3 (GLUT3) (Pfeifer *et al.*, 2009; Keating *et al.*, 2016; Salzberger *et al.*, 2018; Sun *et al.*, 2022; Brenes *et al.*, 2023). Some studies have sought to investigate whether the availability of other sugars in glucose-restricted environments can support NK cell activation, or if there is indeed reliance on

glucose as a fuel. Galactose has been investigated, but it did not support effector molecule production by NK cells (Donnelly *et al.*, 2014; Keating *et al.*, 2016).

Current technologies used to assess flux through glucose metabolism use extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) as measures of glycolysis and OxPhos respectively. Consistent with the levels of glucose transporter expression, NK cells have basal levels of glycolysis and OxPhos which dramatically increase following cytokine stimulation in both murine and human NK cells (Donnelly *et al.*, 2014; Keating *et al.*, 2016; Assmann *et al.*, 2017; Terrén *et al.*, 2021). By limiting flux through glycolysis with 2-deoxyglucose (2DG), an inhibitor of glycolysis, murine NK cells were unable to activate in response to cytokine stimulation (Donnelly *et al.*, 2014; Mah *et al.*, 2017). Activation of murine NK cells with anti-NK1.1 has also been shown to be dependent on increased rates of glycolysis (Kedia-Mehta *et al.*, 2019). 2DG injected into mice is primarily taken up by highly glycolytic cells. In the context of MCMV infection, this has been shown to limit the proliferative capacity of NK cells and also caused significant mortality in comparison to control mice (Mah *et al.*, 2017). It should also be considered that inhibition of glycolysis not only limits ATP production but has other off-target effects such as inhibition of protein glycosylation.

Aside from the glucose transporters, flux through glycolysis can also be controlled by the expression of enzymes in the pathway. The phosphorylation of glucose to glucose-6-phosphate is essential to make glucose available for use in the cell and maintain the glucose concentration gradient. Therefore, hexokinase is an essential enzyme required to support NK cell functions. Its isoforms HK1, HK2 and HK3, have all been shown to be expressed at comparable levels in NK cells, however, the ways in which they individually regulate glucose metabolism has not been investigated (Seiler *et al.*, 2022). Another essential glycolytic rate limiting enzyme in NK cells is lactate dehydrogenase (LDH)-A which is important to support aerobic glycolysis. Metabolic tracing analysis using radiolabelled glucose showed that a considerable proportion of glucose is converted into lactate in NK cells following cytokine stimulation (Assmann *et al.*, 2017; Sheppard *et al.*, 2021). In mice with an NK cell-specific deletion in LDH-A expression, there were no observed alterations to NK cell development. However, upon cytokine

stimulation NK cells from these mice had decreased rates of glycolysis and were unable to appropriately respond to MCMV infection (Sheppard *et al.*, 2021).

Pyruvate is also produced in glycolysis and is subsequently converted to acetyl CoA which then enters the mitochondria to fuel the TCA cycle. Pyruvate kinase is another enzyme that controls the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP. There are two isoforms that are expressed after alternative splicing: PKM1 and PKM2. PKM2 can exist as a tetramer which efficiently creates pyruvate and ATP, or alternatively as a catalytically inefficient monomer or dimer which slows down the rate of glycolysis and results in diversion of glycolytic intermediates into biosynthetic pathways such as the pentose phosphate pathway (PPP) or the glucose-6-phosphate pathway (Christofk *et al.*, 2008; Hitosugi *et al.*, 2009). Following cytokine stimulation, NK cells significantly increased expression of PKM2 in its monomeric form, suggesting an increased biosynthetic demand. In NK cells that had a specific deletion of PKM2 there were no metabolic or functional impairments due to compensatory expression of PKM1 (Walls *et al.*, 2020). Pyruvate uptake has been shown to be essential to support the steady state functions of human CD56^{dim} NK cells (Kern Coquillat *et al.*, 2025). The uptake of exogenous pyruvate regenerated NAD⁺ through its metabolism to lactate, which supported enhanced rates of glycolysis. However, the carbons from glucose are largely diverted into the pentose phosphate pathway and serine production, essential processes to support NK cell proliferation. This highlights the importance of the SLC16 family of transporters, not only for their role in exporting lactate but also taking up exogenous pyruvate (Lin *et al.*, 1998).

Citrate Malate Shuttle (CMS)

The canonical TCA cycle involves the conversion of acetyl CoA to citrate, which will then be converted to mitochondrial oxaloacetate. However, unlike other lymphocytes, in NK cells this citrate was not metabolised to mitochondrial oxaloacetate and other TCA cycle intermediates but rather was shown to be exported from the mitochondria to the cytosol. Metabolic tracing analysis using radiolabelled glucose revealed that this cytosolic citrate is then converted to oxaloacetate and acetyl CoA in the cytosol before the oxaloacetate is converted

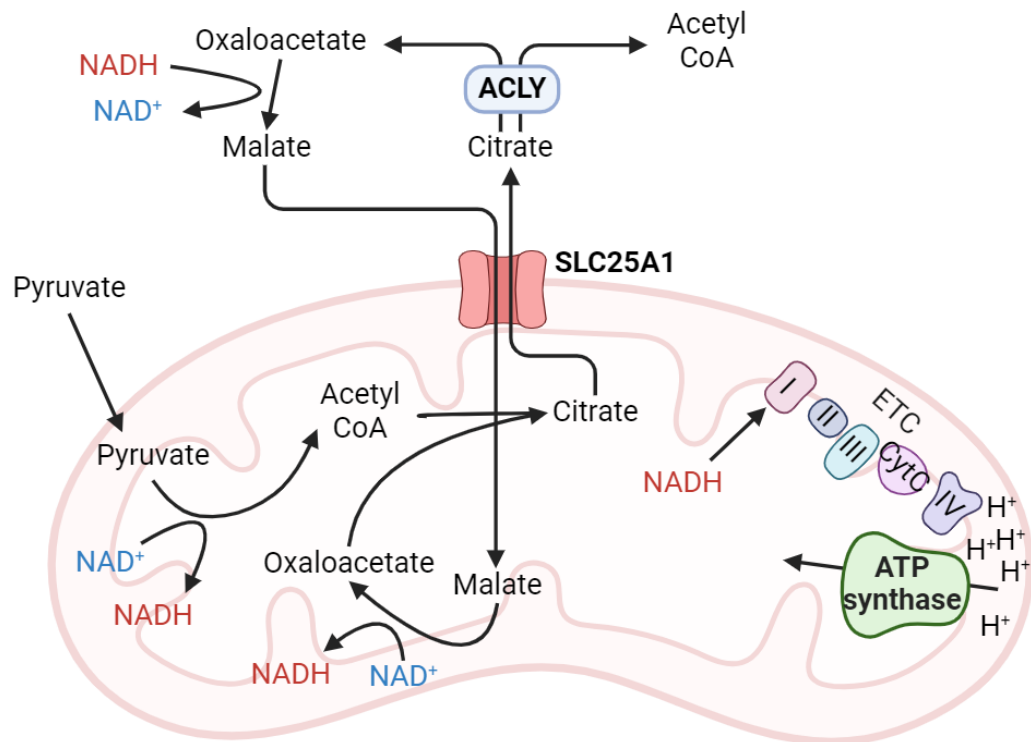


Figure 1.4 The citrate-malate shuttle (CMS)

Pyruvate generated from glycolysis is transported into the mitochondria, metabolised to acetyl CoA, converting one NAD⁺ into NADH. Acetyl CoA and oxaloacetate are combined to give citrate, which is transported into the cytosol via SCL25A1, in exchange for malate. ACLY converts citrate to oxaloacetate and acetyl CoA. Oxaloacetate is metabolised to malate converting one cytosolic NADH to NAD⁺, an essential co-factor for GAPDH. Malate is transported back into the mitochondria through SLC25A1 and metabolised to oxaloacetate, completing the cycle and generating a second mitochondrial NADH. Both NADH molecules generated transfer electrons to complex I of the ETC. While the TCA cycle generates 4 NADH plus 1 FADH₂ all in the mitochondria, the CMS generates 2 NADH in the mitochondria and 1 NAD⁺ plus 1 acetyl CoA molecule in the cytosol. *Taken from Keane et al., 2025.*

to malate which can then re-enter the mitochondria. Oxaloacetate is then regenerated in the mitochondria (Assmann *et al.*, 2017; Kern Coquillat *et al.*, 2025). This metabolic configuration has been termed the citrate malate shuttle (CMS) or a non-canonical TCA cycle and has been associated with increases in

lipid synthesis, nucleotide synthesis and acetylation reactions, in addition to production of NADH and NAD⁺ which can support flux through OxPhos and glycolysis respectively (Assmann *et al.*, 2017; Arnold *et al.*, 2022; Kern Coquillat *et al.*, 2025). There are key proteins that are essential for this shuttle system to occur. One is SLC25A1, a citrate-malate antiporter found on the inner mitochondrial membrane, which enables that export of mitochondrial citrate to the cytosol and the transport of cytosolic malate into the mitochondria. Expression of this protein and the use of the CMS has primarily been described in rapidly proliferating cells such as embryonic stem cells and tumour cells (Eboli and Galeotti, 1981; Lei *et al.*, 2020; Arnold *et al.*, 2022), but has also been described to support NK cell glucose metabolism. Pharmacological inhibition of SLC25A1 caused impaired OxPhos and glycolysis in cultured murine NK cells following cytokine stimulation. This was associated with decreases in IFN γ and granzyme B expression in both murine and human NK cells. Inhibition of succinate dehydrogenase using dimethyl malonate had no impact on NK cell OxPhos, suggesting that increased flux through a complete TCA cycle is not occurring in these activated NK cells (Assmann *et al.*, 2017). An important metabolic advantage for NK cells to use this metabolic configuration includes the regeneration of NAD⁺ in the cytosol and the generation of NADH from the reverse reaction in the mitochondrial matrix, which can support glycolytic flux and OxPhos respectively.

Amino Acid Oxidation

Aside from glucose, there are other fuels that can support OxPhos in immune cells. Glutamine has been shown to be taken up by many immune cell subsets such as T cells, B cells, macrophages and NK cells based on expression of glutamine transporters or through uptake of radiolabelled glutamine (Cantor *et al.*, 2009; Carr *et al.*, 2010; Tavakoli *et al.*, 2017). Following uptake, glutamine can be converted to α -ketoglutarate which enters the TCA cycle and supports OxPhos. In T cells glutamine has been shown to be essential for the differentiation of CTLs through supporting energy metabolism in these cells (Nakaya *et al.*, 2014).

In NK cells the role of glutamine in supporting energy metabolism is not fully understood. NK cells are known to express certain glutamine transporters including SLC1A5 and SLC38A2 (Loftus *et al.*, 2018). Short-term glutamine deprivation had no impact on NK cell metabolism or effector function basally, in both mouse and human NK cells, suggesting glutamine oxidation is not essential to support ATP production (Loftus *et al.*, 2018; Surace *et al.*, 2021).

Fatty Acid Oxidation (β -Oxidation)

In order to initiate the oxidation of fatty acids, they must first be converted to their fatty acyl CoA derivatives by the action of acyl CoA synthetase. This enzyme is tethered to the mitochondrial outer membrane, enabling the efficient transport of small and medium acyl chain molecules into the mitochondrial matrix. These smaller fatty acyl CoA molecules can readily diffuse into the mitochondrial matrix where β -oxidation can occur. Longer chain fatty acyl CoA molecules need to first be conjugated to carnitine by carnitine palmitoyl transferase (CPT) 1, an enzyme that is also tethered to the mitochondrial outer membrane. This generates an acylcarnitine molecule that can be transported across the mitochondrial inner membrane by acylcarnitine translocase (CACT) (Qu *et al.*, 2016). In the mitochondrial matrix the acylcarnitine molecules have the carnitine removed before the CoA is transferred back on by the action of CPT2, regenerating fatty acyl CoA molecules. These are broken down by a repeating sequence of four reactions: oxidation, hydration, oxidation and thiolysis, which remove two carbon units from the acyl CoA molecule in the form of acetyl CoA, generating FADH₂ and NADH in the process (Qu *et al.*, 2016). These molecules can then donate electrons to the ETC to support OxPhos. The acetyl CoA can then enter the TCA cycle and generate even more reducing equivalents. Although not canonically associated with immune cell function, certain immune cells such as M2 macrophages and T_{reg} cells have been shown to use oxidation of fatty acids to support ATP production (Huang *et al.*, 2014; Michalek *et al.*, 2011).

In NK cells there is little evidence suggesting that β -oxidation of fatty acids is required to support metabolism. However, this is largely due to limitations with the use of etomoxir, an inhibitor of the carnitine shuttle system that is required to introduce fatty acids into the mitochondrial matrix as acyl carnitine molecules

(O'Connor *et al.*, 2018; Yao *et al.*, 2018; Raud *et al.*, 2018). Stimulation of human NK cells with IL15 and IL18 in the presence of etomoxir did not result in any changes in effector molecule production (Surace *et al.*, 2021). Similar results were seen in murine NK cells stimulated with IL12 and IL18 or anti-NK1.1 in the presence of etomoxir (Keppel *et al.*, 2015). However, the fuel preference of different NK cell subsets may vary. In murine models of viral infections it has been shown that β -oxidation is essential to support viral clearance, but not production of IFN γ (Schimmer *et al.*, 2023; Sheppard *et al.*, 2024).

Mitochondria

Oxidative metabolism is essential for the maintenance of homeostatic functions in quiescent NK cells. Following activation, increased rates of OxPhos generate large amounts of ATP required to support energy demanding processes such as proliferation, protein synthesis and degranulation. Therefore, functional mitochondria are essential for NK cell responses. Following cytokine activation *in vitro* or in a murine model of viral infection NK cells were shown to increase their mitochondrial mass (Assmann *et al.*, 2017; Khan *et al.*, 2021; Littwitz-Salomon *et al.*, 2021). Human CD56^{dim} NK cells have a greater mitochondrial mass than CD56^{bright} NK cells and this correlates with higher rates of OxPhos (Surace *et al.*, 2021). An important measure of mitochondrial fitness is mitochondrial morphology, which can control how efficiently immune cells can carry out OxPhos. The process of mitochondrial fusion and fission is regulated by mitochondrial GTPases which will generate extensive mitochondrial networks or small individual mitochondria. Cells with high rates of oxidative metabolism tend to have fused mitochondrial networks, whereas cells favouring aerobic glycolysis are often found to have smaller fragmented mitochondria (Guido *et al.*, 2012; Kuznetsov *et al.*, 2021). Upon cytokine stimulation CD56^{dim} NK cells develop more fragmented mitochondria, whereas no changes were observed in CD56^{bright} NK cells (Surace *et al.*, 2021). This does not correlate with rates of OxPhos and may indicate that CD56^{dim} NK cells are more proliferative and use mitochondrial fission to ensure daughter cells receive sufficient mitochondria. The mitochondrial morphology of NK cells in the context of cancer is of particular interest. It has been shown that NK cells within tumours or in the peripheral blood of breast cancer patients have fragmented and dysfunctional mitochondria

(Zheng *et al.*, 2019; Slattery *et al.*, 2021). Strategies to promote fused mitochondrial networks might support NK cell anti-tumour responses.

Iron is another essential nutrient that is essential to support mitochondrial mass in NK cells where it is incorporated into Fe-S clusters in complexes of the ETC. Furthermore, it acts as an important co-factor for mitochondrial enzymes such as aconitase (Cronin *et al.*, 2019). In a murine viral model, it has been shown that depletion of iron *in vivo* significantly impaired NK cell cytokine production and viral clearance (Littwitz-Salomon *et al.*, 2021).

1.4.2 Regulation of NK Cell Metabolism

NK cell metabolism is tightly regulated to ensure that increases in biosynthesis and energy metabolism occur when they are required. This generally occurs following NK cell cytokine activation or receptor-mediated activation to support increased biosynthetic demands and increased demand for ATP.

mTOR

Mammalian target of rapamycin (mTOR) is an important metabolic regulator for NK cells because of its ability to integrate extrinsic signals of nutrient availability and activation signals received by NK cells. The two complexes of mTOR: complex 1 (mTORc1) and complex 2 (mTORc2) generally promote an anabolic response in immune cells, promoting increases in protein synthesis, glycolysis, mitogenesis and lipid synthesis and by doing so will support the increased metabolic rate required for effector functions, proliferation, survival and memory responses. mTORc1 has been shown to be essential for NK cell development in addition to its roles in supporting activation. Murine NK cells undergo several stages of maturation in the bone marrow that can be characterised based on their expression of CD11b and CD27. Pre-NK cells are characterised as CD11b^{low}CD27^{high}, immature NK cells are characterised as CD11b^{high}CD27^{high}, and terminally differentiated NK cells are characterised as CD11b^{high}CD27^{low} (Chiossone *et al.*, 2009). Phosphorylation of mTORc1 as well as its targets has been shown to be highest in pre-NK cells and decreases as NK cells terminally differentiate in the spleen and the bone marrow (Marçais *et al.*, 2014; Marçais *et al.*, 2017). This correlates with the expression of nutrient transporters and nutrient

uptake, suggesting that this increase in mTORc1 signalling is important to support energy metabolism and the biosynthetic demands associated with the proliferation of pre-NK cells. The expansion and development of NK cells is known to be reliant on IL15 signalling; and indeed, in these proliferating pre-NK and immature NK cells, blocking IL15 signalling impaired phosphorylation of mTORC1 targets (Marçais *et al.*, 2014; Li *et al.*, 2021).

Upon maturation, NK cells have low basal mTORc1 activity which is increased upon cytokine or receptor-mediated activation. In murine NK cells, activation *in vivo* with polyI:C in the presence of rapamycin (an inhibitor of mTORc1 activity), impaired expression of glycolytic machinery (Donnelly *et al.*, 2014). This was associated with impaired glycolysis, with no detectable changes in rates of OxPhos. This metabolic defect has been shown to impair NK cell effector functions with rapamycin-treated NK cells also showing decreased cytokine production (Donnelly *et al.*, 2014). Other studies have also shown that impaired mTORc1 activation in NK cells leads to impaired clearance of tumour cells and increased viral loads *in vivo* (Marçais *et al.*, 2014; Yang *et al.*, 2018; Li *et al.*, 2021).

cMyc

c-Myelocytomatosis (cMyc) is a transcription factor that is associated with highly proliferative cells and also has important roles in supporting NK cell metabolism. Because of their enhanced proliferation, pre-NK cells have been shown to require cMyc activity, with defects in NK cell numbers in mice lacking cMyc expression (Khameneh *et al.*, 2023). Following their development to mature NK cells, there is low expression of cMyc, which rapidly increases following cytokine stimulation (Loftus *et al.*, 2018; Khameneh *et al.*, 2023). NK cells that had genetically deleted cMyc showed greatly reduced rates of glycolysis and OxPhos, with decreased expression of glucose transporters and glycolytic enzymes. This was associated with impaired production of IFN γ and granzyme B expression (Loftus *et al.*, 2018). Ribogenesis in mature NK cells is also under the control of cMyc which may attribute to the loss of these effector molecules in addition to the metabolic defects observed (Tang *et al.*, 2021; Khameneh *et al.*, 2023). Another mechanism cMyc has been shown to use to control effector function in NK cells is through

the *de novo* polyamine synthesis pathway. Pharmacological inhibition of this cMyc-controlled pathway led to impaired glycolysis, OxPhos and NK cell functional responses (O'Brien *et al.*, 2021). It has been shown that polyamines are required to support protein translation through eukaryotic translation initiation factor (eIF) 5A. Inhibition of this translation factor in NK cells led to significant impairments in mitochondrial metabolism and in vitro cytotoxicity of NK cells (O'Brien *et al.*, 2021).

The regulation of cMyc is complex and has been shown to involve both mTORc1-dependent and -independent mechanisms, depending on the context (Loftus *et al.*, 2018; Sinclair *et al.*, 2013). For instance, during early cytokine activation (30-60 min) cMyc expression is mTORc1-dependent, but this is not the case following overnight activation. In this context, cMyc expression is highly sensitive to amino acid transporters including SLC1A5, the glutamine transporter and SLC7A5, the large neutral amino acid transporter (Jensen *et al.*, 2017; Loftus *et al.*, 2018; Surace *et al.*, 2021). cMyc protein has a very short half-life (Hann *et al.*, 1983; Hann and Eisenman, 1984), and so continued amino acid uptake and protein synthesis is essential to maintain cMyc protein levels in these activated NK cells. Pharmacological inhibition of SLC7A5 activity or glutamine starvation in murine NK cells resulted in rapid loss of cMyc expression (Loftus *et al.*, 2018). This data reveals an essential role for amino acid uptake in NK cells to support this key metabolic regulator. It also may explain why NK cells increase their glutamine uptake, despite their lack of requirement for glutaminase activity to support energy metabolism.

SREBP

It has been shown that murine NK cells use a non-canonical TCA cycle, also known as the CMS, to support glucose metabolism. Two essential components of the CMS are the citrate-malate antiporter: SLC25A1, and the enzyme responsible for the conversion of cytosolic citrate to oxaloacetate and acetyl CoA: ATP citrate lyase (ACLY) (Assmann *et al.*, 2017; Arnold *et al.*, 2022). The expression of both these proteins is regulated by sterol regulatory element-binding protein (SREBP) transcription factors which have classically been described to maintain lipid homeostasis in cells (Horton *et al.*, 2002). There are

three mammalian isoforms of the transcription factor. These are SREBP1a and SREBP1c which are both associated with expression of genes involved in fatty acid synthesis, and SREBP2 which is associated with expression of genes involved in sterol homeostasis. They are all ubiquitously expressed though their ratios may vary in different cell types (Horton *et al.*, 2002; Horton *et al.*, 2003; Eberlé *et al.*, 2004).

The activation of SREBP transcription factors is a complex process that begins in the endoplasmic reticulum (ER) (Wang *et al.*, 1993; Wang *et al.*, 1994). Inactive precursor molecules are synthesised which contain two hydrophobic transmembrane spanning segments that maintain the proteins at the ER membrane. The N-terminus contains the basic helix-loop-helix leucine zipper region which enables it to bind the sterol regulatory element (SRE). The C-terminus regions can interact with the C-terminus of an ER escort protein called SREBP cleavage-activating protein (SCAP). In order to activate SREBP, SCAP binds to COPII proteins through a protein motif called the MELADL loop. This induces a conformation change in SCAP, allowing it to interact with the SREBP C-terminus, and this complex will escort SREBP to the Golgi. Within the Golgi two proteases: site 1 protease (S1P) and site 2 protease (S2P) will cleave SREBP in two locations, releasing the N-terminus, which is the active form of the transcription factor that binds the SRE to promote the synthesis of target genes.

This mechanism is controlled using intracellular cues from cholesterol and other sterols. When excess cholesterol accumulates in cells and the lipid composition of the ER reaches 4-5%, cholesterol can interact with SCAP and alter the structure of the MELADL loop to prevent its binding to COPII proteins (Brown and Goldstein, 2009). It also induces the binding of SCAP to another ER protein called insulin-induced gene (INSIG) 1. This mechanism maintains SREBP at the ER membrane in its inactive form (Brown and Goldstein, 2009). Other sterols, called oxysterols are oxidised cholesterol derivatives that can prevent the activation of SREBP through binding to INSIG1. This results in the binding of SCAP to INSIG1 which prevents the interaction of SCAP with COPII proteins. However, there are a large number of sterol species all with binding capabilities to SCAP, INSIG1 or both proteins, that can alter the activation of SREBP (Radhakrishnan *et al.*, 2007).

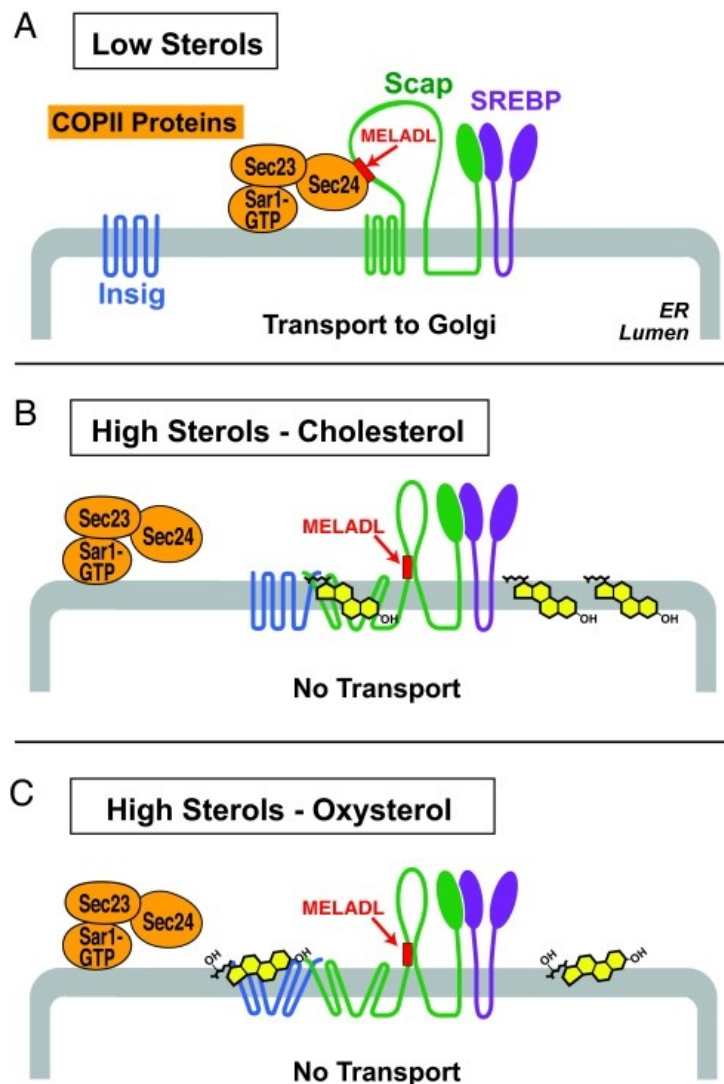


Figure 1.5 Regulation of SREBP activation by cholesterol and oxysterols

SREBP is synthesised at the ER membrane. When ER cholesterol and oxysterols are low, SREBP is trafficked from the ER to the Golgi apparatus in complex with SCAP. This interaction with SCAP allows the MELADL motif to interact with COPII proteins which facilitate transfer to the Golgi for further processing (A). Under conditions of high ER cholesterol, there is increased association of cholesterol molecules with SCAP which changes its conformation and promotes its association with INSIG1. This complex restricts SREBP to the ER, which inhibits further activation (B). Increased oxysterols in the ER will bind to INSIG1, which promotes its association with SCAP. This also promotes a conformational change in SCAP that restricts SREBP to the ER membrane, preventing further processing and activation (C). *Taken from Sun et al., 2007.*

Because NK cells use the CMS, it was investigated whether impairments in SREBP activation would cause metabolic and functional defects, through loss of expression of SLC25A1 and ACLY. Indeed, it was shown that pharmacological inhibition of SREBP activation, or genetic alteration of SREBP activation resulted in dramatic decreases in rates of glycolysis and OxPhos in murine NK cells (Assmann *et al.*, 2017). This occurred despite the cells maintaining expression of glycolytic machinery and mitochondrial mass. In both human and murine NK cells, inhibition of SREBP activation was also shown to impair expression of IFN γ and granzyme B. This impairment in metabolism and effector molecule expression ultimately led to decreased cytotoxicity *in vitro* and *in vivo* following adoptive transfer of NK cells into B16F10 tumours. Through pharmacologically inhibiting fatty acid synthesis there were no observed defects in NK cell function, suggesting that the CMS is the crucial SREBP-regulated process required to support NK cell glucose metabolism (Assmann *et al.*, 2017).

In line with its role as master regulator of cellular metabolism, mTORc1 was also shown to exert control over SREBP activation. NK cells stimulated with IL2 and IL12 in the presence of rapamycin showed impaired expression of SREBP target genes which has also been observed in other cell types (Assmann *et al.*, 2017). Another link between metabolic regulators of NK cell metabolism has been established between SREBP and cMyc. Inhibition of SREBP activation impaired cMyc expression and expression of cMyc target genes (O'Brien *et al.*, 2021), revealing another regulatory axis that has control over NK cell metabolism.

Other metabolic regulators

As discussed, mTORc1 and cMyc signalling are sensitive to the presence of metabolites. This was discussed referring to key fuels such as glucose and amino acids, though additional metabolites have also been shown to impact upon mTORc1 and cMyc activity in NK cells. Butyrate, a short chain fatty acid (SCFA) produced by the microbiota in the gut inhibits NK cell glycolysis and OxPhos and was associated with impaired mTORc1 signalling and reduced expression of cMyc target genes (Zaiatz-Bittencourt *et al.*, 2023). N-acetyl-L-alanine is another metabolite that is found to be elevated in the plasma of HIV⁺ individuals. It was associated with suppression of NK cell metabolism, though whether this is related

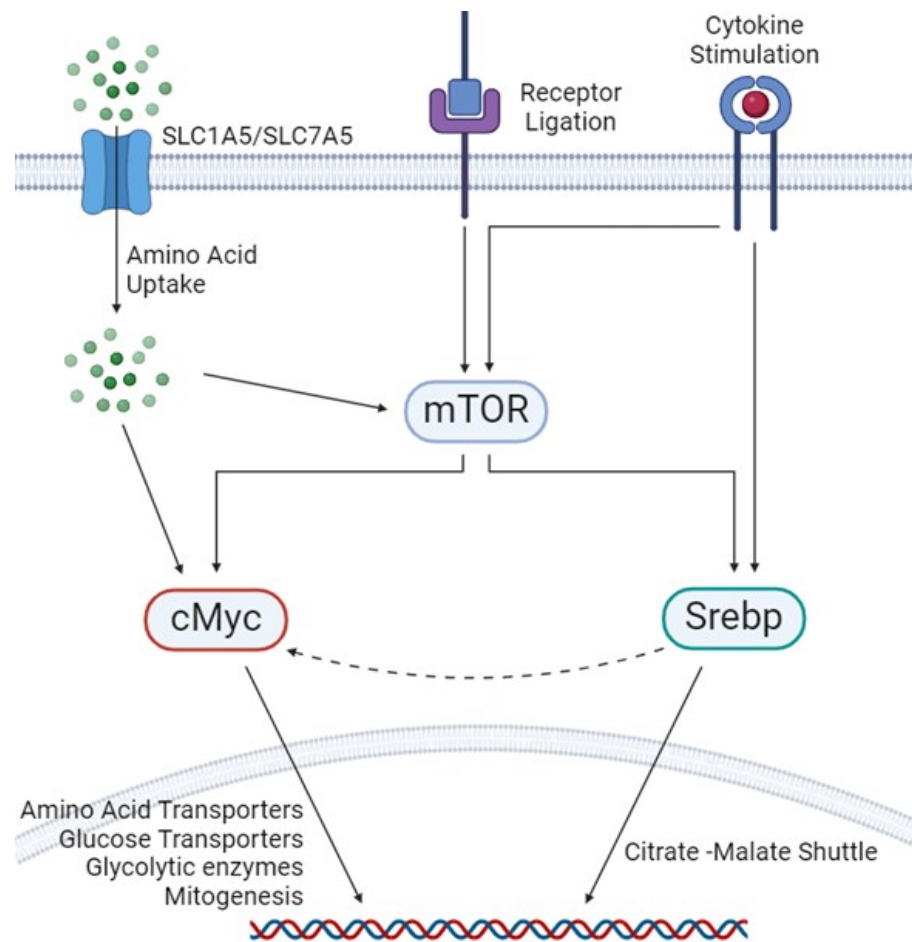


Figure 1.5 Integration of extrinsic signals to control intrinsic metabolic regulators in NK cells.

The activation of NK cells with cytokines or the engagement of NK cell activating receptors leads to the activation of mTORc1, a central regulator of metabolism. Amino acid uptake through SLC1A5 and SLC7A5 are essential to provide amino acids to support mTORc1 activation. mTORc1 signalling increases expression of the transcription factors cMyc and SREBP, which are essential for the expression of NK cell metabolic machinery. Although early activation of cMyc is mTORc1-dependent, sustained activation of cMyc is dependent on amino acid uptake to ensure the rate of cMyc synthesis is faster than the rate of proteasomal degradation. SREBP activation is downstream of mTORc1, though it also has another mTORc1-independent activation mechanism which is not yet understood. Furthermore, the mechanisms that link SREBP and cMyc activation (dotted line) are not yet understood. *Taken from Keane et al., 2025.*

to alterations in mTORc1 or cMyc signalling is not known (Mavilio *et al.*, 2005; Yang *et al.*, 2023).

It is likely that there are additional important intrinsic positive and negative regulators of metabolism that are important for the control of NK cells. SHP (SH2 domain-containing protein tyrosine phosphatase) 1 and 2 are phosphatases that have been shown to be essential for supporting mTORc1 signalling (Tajan *et al.*, 2015). In murine NK cells with deletion in SHP1 there was impaired NK cell development. Furthermore, these NK cells were functionally impaired (Viant *et al.*, 2014). SHP2 deletion was also shown to significantly alter NK cell development. These NK cells also showed significantly impaired rates of glycolysis and OxPhos which was associated with impaired mTORc1 activation, leading to increased susceptibility to MCMV (Niogret *et al.*, 2019).

Another negative regulator is regulatory factor X7 (RFX7), a transcription factor that is ubiquitously expressed but appears important for immune cell responses (Reith and Mach, 2001; Ludigs *et al.*, 2015; Castro *et al.*, 2018). In mice lacking RFX7 there was a decrease in the number of mature NK cells in the spleen (Castro *et al.*, 2018). The NK cells there were larger in size and had increased expression of nutrient transporters and had increased mTORc1 activity (Castro *et al.*, 2018). Despite the apparent increase in NK cell activation, these mice were more susceptible to MCMV infection, highlighting that controlled metabolism is essential to support NK cell homeostasis and efficient immunity following activation.

1.4.3 NK Cell Metabolism in Health and Disease

It is now well understood that metabolic changes are responsible for the impaired NK cell function observed in a number of diseases such as obesity, type II diabetes mellitus and cancer. The impairment in NK cell metabolism in these diseases is often associated with nutrient deprivation, hypoxia and an increase in the presence of inhibitory metabolites that ultimately impair NK cell functional responses. Therefore, in order to target NK cells in these diseases, metabolism should be considered for optimal responses.

Obesity

In murine models of obesity, such as ob/ob mice or mice fed a high fat diet there are impaired anti-viral and anti-tumour responses (Mori *et al.*, 2006; Smith *et al.*, 2007; Michelet *et al.*, 2018; Spielmann, Naujoks, *et al.*, 2020; Spielmann, Mattheis, *et al.*, 2020). This is mirrored in obesity in humans which is consistently associated with impaired NK cell responses, with reduced cytokine production and cytotoxicity (Perdu *et al.*, 2016; Tobin *et al.*, 2017; Viel *et al.*, 2017; Kedia-Mehta *et al.*, 2021; Michelet *et al.*, 2018; De Barra *et al.*, 2023). In NK cells isolated from the blood of obese individuals or mice fed a high fat diet, there was an increase in intracellular lipid levels due to increased fatty acid uptake as opposed to glucose uptake (Michelet *et al.*, 2018). This study showed that following culture in the presence of palmitic acid and oleic acid there is activation of peroxisome proliferator-activated receptor (PPAR) α/δ signalling which inhibits mTORc1 signalling. This resulted in impaired rates of glycolysis and OxPhos and could be mimicked using pharmacological agonists of PPAR α/δ (Michelet *et al.*, 2018). Lipid loaded NK cells in the context of obesity are also unable to kill target cells due to an inability to polarise the MTOC towards the immunological synapse (Michelet *et al.*, 2018).

Treatments for obesity such as bariatric surgery and weight loss through diet restriction and increased exercise has been shown to reverse some of the defects associated with NK cells in obesity. For example, 6 months post-bariatric surgery NK cells had a restoration of their *in vitro* cytotoxicity against tumour target cells (Moulin *et al.*, 2011). In human NK cells there were observed increases in both TRAIL and IFN γ expression following weight loss (Jahn *et al.*, 2015). The metabolic and functional impairments observed in individuals with obesity has been shown to be reversible following treatment with glucagon-like peptide (GLP)-1 (De Barra *et al.*, 2023). This hormone induces the release of insulin and induces satiety which has been used as an effective treatment for individuals with obesity. Matched samples from before and after treatment with GLP-1 showed restored NK cell production of IFN γ and granzyme B, and increased killing of target cells *in vitro* which was associated with rescued mTORc1 activity (Müller *et al.*, 2019; De Barra *et al.*, 2023). Interestingly, there were no significant associations between weight loss and rescue of NK cell

functions, indicating that these effects were occurring independently of weight loss and are in fact mediated by GLP-1 itself (De Barra *et al.*, 2023). This study also highlights that other humoral factors such as hormones may be acting on NK cells to alter metabolism and warrant investigation. Recently, studies have shown that NK cells have increased expression of glucocorticoid receptors which are elevated in fasting and can modulate NK cell metabolism and effector functions (Delconte *et al.*, 2024).

Cancer

NK cells have been shown to be dysfunctional in a wide variety of tumours and many of these changes were shown to be due to inhibition of metabolism in the TME. Nutrient restriction in the TME has consistently shown that TILs can become deprived of glucose and glutamine, leading to attenuated responses (Chang *et al.*, 2015; Palmer *et al.*, 2015; Sukumar *et al.*, 2015; Kolb *et al.*, 2020; Edwards *et al.*, 2021). It is reasonable to suggest that this is also the case for TINKs which have consistently been shown to have impaired metabolism in a number of different tumour models (Cong *et al.*, 2018; Kobayashi *et al.*, 2020). Hypoxia is another characteristic of the TME, which can greatly diminish NK cell functions through limiting oxidative metabolism. In human TINKs from liver tumours there was an enrichment of hypoxia-inducible genes such as *Hk2*, *Slc2a3* and *Kdm3a*. Furthermore, these NK cells had decreased rates of OxPhos and were shown to have smaller and more fragmented mitochondria (Zheng *et al.*, 2019). This was shown to be mediated by an increase in aberrant mTORc1 activity in TINKs which increases the activation of the mitochondrial fission protein: dynamin-related protein 1 (DRP1) (Zheng *et al.*, 2019). Although mTORc1 is essential to promote NK cell metabolism, it has been shown that excessive mTORc1 activation can result in an exhausted NK cell phenotype with decreased anti-tumour functions (Meixiang Yang *et al.*, 2016).

With high rates of glucose uptake and rates of aerobic glycolysis by tumour cells, there is an increase in extracellular lactate in the TME. This lactate, as well as the associated decreases in pH have been shown to be inhibitory to NK cell effector functions (Brand *et al.*, 2016). Comparable results were seen in liver-resident NK cells in colon carcinoma metastases. These metastases had

significantly increased concentrations of lactate in the liver which resulted in increased intracellular acidification of NK cells which was associated with increased cell death (Harmon *et al.*, 2019). These NK cells also showed mitochondrial impairments with an increase in mitochondrial reactive oxygen species (ROS) which is a strong indicator of disruptions to the ETC. In order to counteract the acidification of the TME, studies have also shown that systemic bicarbonate buffering can increase the pH of the TME which was associated with enhanced NK cell responses (Pötzl *et al.*, 2017).

A potent inhibitory metabolite in the TME is adenosine, which is associated with hypoxic environments. Many tumours are associated with increases in extracellular adenosine which facilitates tumour evasion from NK cells, T cells and myeloid cells when signalling through the A2A adenosine receptor (A2AR) (Vaupel and Mayer, 2016). In mice lacking A2AR there was an increase in the frequency of mature NK cells in the periphery that were more proliferative and showed increased tumour surveillance in an MCA-induced fibrosarcoma model (Young *et al.*, 2018). Furthermore, cytokine-stimulated human NK cells in the presence of adenosine showed significantly reduced production of ATP (Chambers *et al.*, 2018). The exact mechanism of inhibition used by adenosine in NK cells is not yet understood. Another oncometabolite, kynurenine is a product of tryptophan metabolism and is produced by the enzyme indoleamine 2,3 dioxygenase (IDO) 1. It has been shown to be an important oncometabolite for promoting cancer cell growth by signalling through the aryl hydrocarbon receptors as well as inhibiting the function of TILs (Gouasmi *et al.*, 2022). Human NK cells have decreased proliferation when exposed to kynurenine. Furthermore, increases in kynurenine concentrations in NK cell culture medium impaired expression of the NK cell receptors Nkp46 and NKG2D, suggesting a paracrine effect of tumour cells to decrease target cell recognition by TINKs in the TME (Frumento *et al.*, 2002; Chiesa *et al.*, 2006; Liu *et al.*, 2010). This is facilitated by the increase in SLC7A5 expression, which is essential for NK cell functional responses, but can allow for kynurenine uptake (Loftus *et al.*, 2018; Sinclair *et al.*, 2018).

Lipids can also be enriched in the TME and are associated with tumour aggressiveness (Vasseur and Guillaumond, 2022). Fatty acid accumulation in NK

cells is dependent on uptake by scavenging receptors such as CD36 and SCARB1, has been associated with impaired anti-tumour function in murine models and human studies (Niavarani *et al.*, 2019; Kobayashi *et al.*, 2020). It is known that TME cholesterol can inhibit CD8⁺ T cell responses (Ma *et al.*, 2019; Ma *et al.*, 2021). Though similar effects have yet to be described in relation to NK cells, oxidised cholesterol molecules such as 25- and 27-hydroxycholesterol are potent inhibitors of SREBP transcription factors and can therefore, impair NK cell metabolism (Assmann *et al.*, 2017; Choi, 2023).

1.5 Oxysterols

Cholesterol is an essential lipid required for cellular homeostasis. It is a critical regulator of cellular membranes, has roles in signal transduction and also is a precursor molecule for bile acids and steroid hormones (Schade *et al.*, 2020). Oxysterols are oxidised cholesterol derivatives that are known negative regulators of SREBP processing and therefore, have implications for NK cell functions (Assmann *et al.*, 2017; Choi, 2023). They are characterised by the addition of polar functional groups to cholesterol. These include hydroxyl, carbonyl and epoxy groups that can result in the production of a variety of structurally and functionally diverse metabolites.

The oxidation of cholesterol to produce oxysterols can occur through either enzymatic or non-enzymatic processes. Alternatively, they can be obtained in the diet, particularly in cholesterol-rich foods (Russell, 2000; Brown and Jessup, 2009). The auto-oxidation of cholesterol is associated with tissues that have abundant levels of ROS and primarily occurs on carbon 5, 6 and 7 of cholesterol which are found on the sterol ring. The two most common species of oxysterols formed by auto-oxidation are 7 β -hydroxycholesterol and 7-ketocholesterol (7KC) (Vejux and Lizard, 2009; Lordan *et al.*, 2009). They are commonly associated with both atherosclerotic plaques and the retinal drusen of the eye and are known for their pro-apoptotic properties (Brown and Jessup, 2009; Rodríguez and Larrayoz, 2010). The enzymatically produced oxysterols are formed as intermediates in what is classically known as the bile acid synthesis pathway. This pathway has two arms: the classical and alternative bile synthesis pathway. This has been characterised in the liver and has since been observed in other

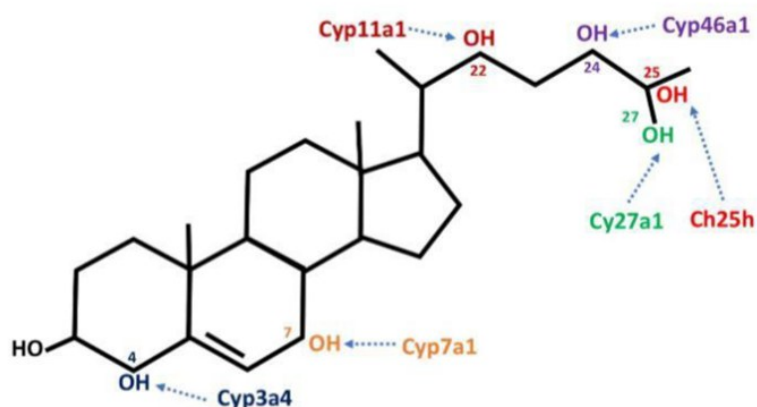


Figure 1.7 Enzymatic production of oxysterols

The 27-carbon structure of cholesterol is the substrate for oxysterol-synthesising enzymes. These enzymes include cytochrome p450 enzymes or CH25H. Depicted are the sites of oxidation on the cholesterol backbone and the enzymes involved. *Taken from Choi and Finlay, 2020.*

cell types. In the liver most bile acids are produced through the classical pathway whereas the alternative pathway only accounts for ~10% of bile acid production (Russell, 2003). The classical pathway begins with the enzyme CYP7A1 acting on cholesterol to produce 7 α -hydroxycholesterol and through many more enzyme catalysed reactions leads to the production of cholic and chenodeoxycholic acid (Russell, 2003). The alternative pathway produces a greater range of oxysterol species, termed the side chain oxysterols and has been described to occur in many other cell types outside of the liver. CYP46A1 acts to produce 24-hydroxycholesterol (24HC) and has been described in neurons, retina and microglia to remove excess cholesterol from those tissues (Lund *et al.*, 1999; Zhang and Liu, 2015). Another oxysterol species 27-hydroxycholesterol (27HC) is produced by CYP27A1 and is the most predominant oxysterol found in circulation. It is found in many tissues, including kidney, brain, muscle and is also produced by certain immune cells (Russell, 2000). Cholesterol 25 hydroxylase (CH25H) is another important oxysterol-synthesising enzyme that produces 25-hydroxycholesterol (25HC) which is associated with inflammation, and is produced predominantly by hepatocytes and myeloid cells (Park and Scott, 2010).

Oxysterols are transported in circulation in low-density lipoproteins (LDL) and oxidised LDL (oxLDL) due to their hydrophobic nature. They are found to a lesser extent in high density lipoproteins (HDL) and even less in very low density lipoproteins (VLDL) (Babiker and Diczfalusy, 1998). Another important serum transport protein: albumin has also been reported to transport oxysterols (Babiker and Diczfalusy, 1998). Intracellular transport of oxysterols is mediated by a family of lipid transport proteins called oxysterol-binding proteins (OSBPs) and OSBP-related proteins (ORPs) (Wenling Chen *et al.*, 2007). This facilitates their transport to organelles such as the Golgi and ER where they have roles in regulating gene expression. Their enzymatic degradation can be carried out by CYP7B1 which produces 7 α -hydroxylated derivatives of the side chain oxysterols. In certain immune cells this is important for the production of the chemokines 7 α -25/27-hydroxycholesterol (Yi *et al.*, 2012; Cyster *et al.*, 2014; Dang *et al.*, 2017). Alternative elimination of oxysterols can occur through sulfonation by the sulfonyl transferase family 2B member 1 (SULT2B1) (Wenling Chen *et al.*, 2007; Bensinger *et al.*, 2008). Cells can also remove intracellular oxysterols through ATP-binding cassette (ABC) transporters (Tarling *et al.*, 2013).

The role of oxysterols in the control of SREBP transcription factor activation has already been described. However, these oxysterols can also act as an agonist for another transcription factor called the liver X receptor (LXR) which is involved in inducing transcription of genes involved in cholesterol clearance (Calkin and Tontonoz, 2012). Both of these mechanisms act to maintain homeostatic lipid levels within cells. Like SREBP, LXR is also expressed in many immune cell types including macrophages, DCs, neutrophils, T cells and B cells where it has important roles in controlling their functions (Bensinger *et al.*, 2008; A-Gonzalez *et al.*, 2009; Bruckner *et al.*, 2012; Hong *et al.*, 2012; Ito *et al.*, 2015; Beceiro *et al.*, 2018).

1.5.1 25-Hydroxycholesterol

25HC is commonly associated with inflammation, due to its described role in controlling macrophage effector functions. One of the ways it regulates inflammation is through the inhibition of SREBP activation. *Nlrp1a* is a transcriptional target of SREBP1a, which links SREBP with NLRP1-

inflammasome-mediated production of IL1 β . In mice where there is no expression of SREBP1a there is a decreased polarisation of macrophages to pro-inflammatory phenotypes and these mice did not develop sepsis in response to LPS (Im *et al.*, 2011). Activation of macrophages through TLR4 is known to induce expression of 25HC which may act as a negative feedback mechanism to prevent excessive inflammasome activation. In fact, 25HC has also been shown to limit the activation of the AIM2-dependent inflammasome (Dang *et al.*, 2017). There are also important implications for 25HC altering membrane structure in pro-inflammatory macrophages. For example, a decrease in SREBP1a activation, which results in decreased fatty acid synthesis impairs phagocytosis due to reduced interactions between the cytoskeleton and lipid rafts (Lee *et al.*, 2018). Furthermore, the membrane of macrophages can be altered due to the interaction of 25HC with acyl CoA acyltransferase (ACAT) 2. ACAT2 is an important cholesterol-esterifying enzyme that uses 25HC as a co-factor. Increased 25HC-mediated activation of ACAT2 in pro-inflammatory macrophages was shown to make macrophages resistant to infection by *Listeria monocytogenes* (Abrams *et al.*, 2020). More recently this same observation was made in viral infection with SARS-CoV-2 (Zu *et al.*, 2020), and other viral infections such as lyssavirus and rabies virus (Liu *et al.*, 2013; Yuan *et al.*, 2019). TAMs have also been associated with expression of *Ch25h* and subsequent production of 25HC. This was shown to contribute to the cold immune environment in LLC tumours (Xiao *et al.*, 2024).

The regulation of lymphocyte effector functions by 25HC is less understood. The importance of SREBP for supporting NK cell glucose metabolism has already been discussed. There is also evidence that SREBP activation is important to support glucose metabolism in CTLs as deletion of SCAP led to impaired rates of glycolysis and OxPhos and was linked with impaired proliferation in lymphocytic choriomeningitis virus (LCMV) infection, though the mechanism for this impairment remains unclear (Kidani *et al.*, 2013). This suggests that 25HC-rich environments such as the TME may limit NK and T cell anti-tumour functions through restriction of glucose metabolism.

1.5.2 27-Hydroxycholesterol

27HC production is primarily associated with certain cancers, including ovarian cancer and breast cancer (Nelson *et al.*, 2013; Wu *et al.*, 2013; He *et al.*, 2019). 27HC can act as a selective oestrogen receptor modulator (SERM) and in doing so can promote tumour growth through autocrine signalling, which has been demonstrated in ER⁺ breast cancer. In fact, breast cancer patients had a clear correlation between progression of the tumour and the serum levels of 27HC (Nelson *et al.*, 2013; Wu *et al.*, 2013). Outside of the context of cancer, 27HC has been associated with activation of LXR signalling in DCs (Villablanca *et al.*, 2010; Bruckner *et al.*, 2012). Production of 27HC by tumours has been shown to impair CCR7 expression by DCs through an LXR-dependent mechanism, which decreased the migration of DCs into the tumour-draining lymph nodes (Villablanca *et al.*, 2010).

27HC has also been described as a potent inhibitor of SREBP activation, though its role in regulating lymphocyte functions through this mechanism is not fully understood. However, because of the importance of SREBP to support NK and T cell glucose metabolism, it could be reasoned that 27HC-rich environments in certain tumours could be a contributing factor to lymphocyte dysfunction. In fact, the inhibition of SREBP activation in NK cells by 27HC could be a contributing reason for the dysfunction seen in circulating NK cells in breast cancer (Slattery *et al.*, 2021).

1.6 Thesis Aims

Communication axes between immune cells are essential to coordinate the development of the immune response or promote resolution of inflammation. In certain conditions such as cancer, these communication axes are manipulated to promote the growth of the tumour. For example, it is now appreciated that metabolites participate in communication between immune cells, which can alter their activation through both autocrine and paracrine signalling. This thesis aims to **understand how metabolic processes in macrophages can communicate with local NK cells through mechanisms that might include soluble metabolites or cell-cell interactions, to fine-tune NK cell responses.**

Specific Objectives

1. To investigate the impact of macrophage activation on local NK cell metabolism and function.
2. To determine whether macrophage-derived 25HC can alter local NK cell metabolism and function.
3. To explore if other soluble and non-soluble macrophage-derived factors affect NK cell responses.
4. To investigate the relationship between macrophage and NK cell lipid metabolism.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Human IL15 was obtained from NIH. Murine IL15 was obtained from Peprotech. IL2 was obtained from NCI clinical repository. IL12 was purchased from Miltenyi Biotech. IL18 was obtained from GlaxoSmithKline (GSK). Murine CCL2 was purchased from Biotechne. RPMI 1640, Iscove's modified Dulbecco medium (IMDM), Dulbecco's modified Eagle medium (DMEM), Dulbecco's phosphate buffered saline (PBS), Hank's balanced salt solution (HBSS) and L-glutamine (100X) were purchased from Gibco. Calcein AM, golgi plug, Cytofix/Cytoperm and Perm/Wash (10X) were purchased from BD Biosciences. Penicillin/streptomycin was purchased from Labtech International. β -mercaptoethanol, 25-hydroxycholesterol (25HC), 27-hydroxycholesterol (27HC), GW3965, PF429242, latrunculin A, dimethyl-sulfoxide (DMSO), sodium oleate, kynurenine, sodium L-ascorbate, holotransferrin, sodium azide, research grade ethanol, research grade isopropanol, poly-L-lysine (0.1% w/v), paraformaldehyde (PFA), PBS tablets, sodium bicarbonate, rotenone, antimycin-A, oligomycin, BAM15, fluoro-carbonyl cyanide phenylhydrazone (FCCP), saponin, copper (II) sulfate pentahydrate, agarose, 4'6'-diamidine-2'-phenylindole dihydrochloride (DAPI), Mowiol® 4-88, Tris-hydrochloride, sodium deoxycholate, sodium dodecyl sulfate, ethylene diamine tetraacetic acid (EDTA), bovine serum albumin (BSA) and Triton-X were purchased from Merck. Sodium chloride was purchased from Tesco Ireland. 7-ketocholesterol was purchased from Avanti Polar Lipids. MojoSort™ mouse NK cell isolation kit and custom Legendplex™ cytokine bead array were purchased from Biolegend. Aminoguanidine hydrochloride was purchased from Cayman Chemical. Tris-hydroxypropyltriazolylmethylamine was purchased from Click Chemistry Tools. O-propargyl puromycin and AlexaFluor™ 647 Azide were purchased from Jena Bioscience. Oleic acid alkyne was donated by Prof. Sander van Karsteren, Leiden Institute of Chemistry, Leiden University, The Netherlands. Transferrin from human serum, AlexaFluor™ 647 conjugate and UltraPure™ DNase/RNase-free distilled water were purchased from

Invitrogen. 4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid (C75) and 5-tetradecyloxy-2-furoic acid (TOFA) were purchased from Cambridge Biosciences. Rapamycin, GW6471, GSK3787, trypan blue solution (0.4% w/v), Pierce™ BCA protein assay kit and DEPC-treated water were purchased from Fisher Scientific. iQ SYBR Green, microscope slides and coverslips were purchased from VWR. LPS from *E. coli* O111:B4 was purchased from Enzo Scientific or Cayman Chemical. Zymosan, low molecular weight polyI:C, ODN1826 (CpG) and R848 were purchased from Invivogen. RNeasy RNA purification mini kit, RNase-free DNase kit and gel extraction kit were purchased from Qiagen. Qscript cDNA supermix was purchased from Quanta Biosciences. Cell-Tak™ was purchased from Corning. Seahorse XF media and calibration buffer were purchased from Agilent Technologies. Glucose colourimetric assay kit was purchased from Assay Genie. Absolute ethanol was purchased from school stores.

2.1.2 Equipment

Tissue culture dishes (6,12,24,48 and 96 well), tissue culture flasks (25 cm², 75 cm² and 175 cm²) falcon tubes (15 mL, 50 mL), Eppendorf's (0.5 mL, 1.5 mL and 2 mL), pipette tips, transfer pipettes, filtered pipette tips, syringes (1 mL, 2 mL, 50 mL), syringe-assisted filters (20 µm), cell strainers (70 µm) cryovials, absolute qPCR seals and AB-1900 low profile PCR plates were purchased from Cruinn Diagnostics. P1000, P200, P20, P10 and P2.5 pipettes were purchased from StarLab. 96 well flat black polystyrene plates were purchased from Costar. 24 well plates with trans-well inserts (0.4 µm and 5 µm membrane pores) and 1.8 cm blade cell scrapers were purchased from Corning. Petri dishes (100 × 200 mm) were purchased from CellStar. CountBright™ absolute counting beads were purchased from Invitrogen. Haemocytometers were purchased from Hausser Scientific. EasySep™ magnets for column-free separation were obtained from Miltenyi Biotech. Nanodrop spectrophotometer (ND-100) was obtained from Nanodrop® Technologies. NovoCyte flow cytometer was loaned by Agilent Technologies. µ-dishes for live cell imaging were purchased from Ibidi. Steri-Cycle CO₂ incubator is from Thermo Forma. Bioair topsafe fumehood was from Crowthorne HiTEC Services. 1500 standard fumehood was from Phoenix Controls. Heraeus pico 17 centrifuge was from Thermo Scientific. 5810R41

centrifuge was from Eppendorf. Legend RT centrifuge was from Sorvall. FLUOstar OPTIMA microplate reader was from BMG LabTech. FACS Canto, LSR Fortessa, 5 mL polystyrene FACS tubes, 5 mL polypropylene tubes and needles (25 G, 27 G) were purchased from Becton Dickinson (BD). ABI 7900HT Fast RT-qPCR machine and QuantStudio 3 Real-Time PCR system were from Applied Biosciences. TCS SP8 STED Nanoscope was from Leica Microsystems. IncuCyte Live Cell Analysis System was from Sartorius. FlowJo™ software was purchased from TreeStar. ImageJ software was obtained from NIH. GraphPad Prism was purchased from GraphPad Software.

2.1.3 Mice

C57BL/6J mice were purchased from Harlan (Bicester, U.K.) or Charles River or were bred in house. CD36^{KO} (B6.129S1-*Cd36*^{tm1Mfe}/J) mice were purchased from Jackson Laboratories. All mice were maintained in compliance with project license, EU, and Health Products Regulatory Authority regulations, with approval of the University of Dublin's ethical review board. Animals were culled by CO₂ inhalation or cervical dislocation. Tissues were harvested in the comparative medicine unit (CMU), Trinity College Dublin, and carcasses were disposed of in accordance with the guidelines described by the CMU.

2.1.4 Cell Lines

K562, a human chronic myelogenous leukaemia cell line was donated by Prof. Clair Gardiner, School of Biochemistry and Immunology, Trinity College Dublin. RMA/S, a murine Rauscher virus-induced T cell lymphoma cell line was donated by Jun.-Prof. Elisabeth Littwitz-Salomon, Institute for The Research on HIV and AIDS-Associated Diseases, University Hospital Essen. L929, a murine fibroblast cell line was donated by Prof. Luke O'Neill, School of Biochemistry and Immunology, Trinity College Dublin.

2.1.5 Flow Cytometry and Confocal Microscopy Antibodies and Stains

Sources of antibodies and stains used for flow cytometry and confocal microscopy are listed in table 2.1.

2.1.6 Buffers and Solutions

The composition of the various buffers and solutions used are outlined in table 2.2.

Table 2.1 Antibodies and stains used for flow cytometry and confocal microscopy.

Target/Stain	Fluorophore	Clone	Source	Dilution
Fc block	-	2.4G2	BD Bioscience	1/100
NK1.1	PECy5	PK136	Biolegend	1/200
NK1.1	BV421	PK136	Biolegend	1/200
NKp46	PerCP	29A1.4	eBioscience	1/200
NKp46	PE	29A1.4	eBioscience	1/200
CD3	FITC	145-2C11	eBioscience	1/200
CD3	APC	145-2C11	Biolegend	1/200
CD3	BV510	17A2	Biolegend	1/200
CD3	BV650	17A2	Biolegend	1/200
B220	FITC	RA3-6B2	BD Bioscience	1/200
CD19	APC	1D3	BD Bioscience	1/200
CD19	BV650	1D3	BD Bioscience	1/200
CD40	FITC	3/23	Biolegend	1/200
CD49b	BV421	DX5	BD Bioscience	1/200
CD49b	PE	DX5	BD Bioscience	1/200
CD11b	FITC	M1/70	Biolegend	1/1000
CD11b	PECy7	M1/70	BD Bioscience	1/1000
F4/80	BV510	BM8	Biolegend	1/1000
IFN γ	BV421	XMG1.2	BD Bioscience	1/100
Perforin	APC	S16009B	Biolegend	1/800
Granzyme B	PECy7	NGZM	eBioscience	1/100
IL12p40/p70	BV421	C15.6	BD Bioscience	1/100
IL6	APC	MP5-20F3	Biolegend	1/100
TNF α	PECy7	MP6-XT22	eBioscience	1/100
CD36	PE	CRF D-2717	BD Bioscience	1/200
CD71	BV510	C2	BD Bioscience	1/200
CD25	BV786	PC61	Biolegend	1/200

Di-4-ANEPPDHQ	-	-	Invitrogen	0.5/5 μ M
Azide	AF488	-	Jena Bioscience	5 μ M
MitoTracker™ Green	-	-	Invitrogen	100 nM
Acridine Orange	-	-	Invitrogen	100 nM
TMRM	-	-	Invitrogen	100 nM
Transferrin	AF647	-	Invitrogen	5 μ g/mL
LipidTOX™ Green	-	-	Invitrogen	1X
LipidTOX™ Deep Red	-	-	Invitrogen	1X
DAPI	-	-	Merck	1 μ g/mL
CFSE	-	-	Invitrogen	1 μ M
CellTracker™ Deep Red	-	-	Invitrogen	1 μ M
Zombie™ NIR	-	-	Invitrogen	1/400
Zombie™ Yellow	-	-	Invitrogen	1/400
Zombie™ Aqua	-	-	Invitrogen	1/400

Table 2.2 Buffers' and solutions' recipes.

Media/Buffer/Solution	Components
cRPMI	RPMI 1640, L-glutamine (1X), FCS (10% v/v), penicillin/streptomycin (1% v/v), β -ME (10 μ M)
NK cell culture medium (IL15 expansions)	RPMI 1640, L-glutamine (1X), FCS (10% v/v), penicillin/streptomycin (1% v/v), β -ME (10 μ M), human IL15 (10 ng/mL)
NK cell culture medium (IL2 expansions)	RPMI 1640, L-glutamine (1X), FCS (10% v/v), penicillin/streptomycin (1% v/v), β -ME (10 μ M), IL2 (100 ng/mL)
BMDM culture medium	RPMI 1640, L-glutamine (1X), FCS (10% v/v), penicillin/streptomycin (1% v/v), β -ME (10 μ M), L929 conditioned medium (10% v/v)
K562 culture medium	IMDM, L-glutamine (1X), FCS (10% v/v), penicillin/streptomycin (1% v/v)
RMA/S culture medium	RPMI 1640, L-glutamine (1X), FCS (10% v/v), penicillin/streptomycin (1%)

L929 culture medium	DMEM, L-glutamine (1X), FCS (10%), penicillin/streptomycin (1%), β -ME (10 μ M)
MojoSort™ buffer	Dulbecco's PBS, BSA (0.5% w/v), EDTA (2 μ M)
RNA lysis buffer	RLT buffer (Qiagen), β -ME (1%)
Killing assay wash buffer	Dulbecco's PBS, BSA (20% w/v)
FACS buffer	Dulbecco's PBS, BSA (0.1% w/v), sodium azide (0.02% w/v)
Transferrin uptake buffer	RPMI 1640, BSA (5% w/v)
Click chemistry permeabilisation buffer	Dulbecco's PBS, BSA (1% w/v), saponin (0.01% w/v)
Acid wash	Dulbecco's PBS, NaCl (150 mM), citric acid (20 mM), pH5
Cell-Tak™ for cell adhesion	9.25 μ L Cell-Tak™ (Corning), sodium bicarbonate (0.1 μ M), NaOH (1M)
RIPA Buffer	dH ₂ O, Tris-hydrochloride (10 mM), EDTA (1 mM) (Triton-X-100 (1%), sodium deoxycholate (0.1%), sodium dodecyl sulfate (0.1%), sodium chloride (140 mM)

2.2 Methods

2.2.1 NK Cell Culture

IL15 NK Cell Expansion

Mice were sacrificed and spleens were harvested. Spleens were passed through a 70 μ m strainer into a 50 mL falcon tube in a laminar flow hood. The resulting cell suspension was then centrifuged at 250 g for 4 min at room temperature (RT). The supernatant was discarded before red blood cells were lysed by osmotic lysis. Cells were resuspended in ddH₂O for 3 s before 9 mL cRPMI was added to the tube. The cell suspension was again centrifuged at 250 g for 4 min and the cells were resuspended to give a cell suspension of 2.3×10^6 cells/mL in cRPMI. The suspension was then plated at 5 mL/well in 6 well plates. The cells were cultured for 6 days in the presence of human IL15 (10 ng/mL, NIH) in an incubator at 37°C at 5% CO₂. IL15 was given at this concentration on day 0 and day 4.

IL2 NK Cell Expansion

Mice were sacrificed and spleens were harvested. Spleens were passed through a 70 µm strainer into a 50 mL falcon tube in a laminar flow hood. The resulting cell suspension was then centrifuged at 250 g for 4 min at RT. The supernatant was discarded before red blood cells were lysed by osmotic lysis. Cells were resuspended in ddH₂O for 3 s before 9 mL cRPMI was added to the tube. The cell suspension was again centrifuged at 250 g for 4 min and the cells were resuspended to give a cell suspension of 100×10^6 cells/mL in MojoSort™ buffer. NK cells were purified by magnetic activated cell sorting (MACS) using the MojoSort™ mouse NK cell isolation kit according to manufacturer's instructions. The purified NK cells were centrifuged at 250 g for 4 mins and the supernatant was discarded before all the NK cells were resuspended in 5 mL cRPMI in a well of a 6 well plate. The cells were cultured for 6 days in the presence of IL2 (100 ng/mL, NCI clinical repository) in an incubator at 37°C and 5% CO₂. IL2 was given at this concentration on day 0 and day 4.

NK Cell Stimulation

The following method of stimulating cultured NK cells was used for both IL15- and IL2-expanded NK cells. On day 6 all NK cells from the culture were pooled and counted. For analyses where a pure population of NK cells was required, NK cells were purified by MACS using the MojoSort™ mouse NK cell isolation kit according to manufacturer's instructions. The suspension was then centrifuged at 250 g for 4 mins and the supernatant discarded before cells were resuspended at $1-2 \times 10^6$ cells/mL. Cells were stimulated with the cytokines IL2 (20 ng/mL, NCI clinical repository) and IL12 (10 ng/mL, Miltenyi Biotech). Unstimulated NK cells from IL15-expanded cultures were treated with a low dose IL15 (2 ng/mL, NIH). Unstimulated NK cells from IL2-expanded cultures were cultured in the absence of cytokine. Stimulations were carried out in the presence or absence of a number of different cell treatments or vehicle controls (detailed in table 2.3).

2.2.2 Bone Marrow-Derived Macrophage (BMDM) Culture

BMDM Differentiation

Mice were sacrificed and femurs and tibiae were harvested. Tissue was removed from around these bones in a laminar flow hood. A small amount of bone was cut

from each end of the bones. The bone marrow was flushed from the bones using 5 mL of cRPMI in a syringe with a 25 G needle until the bone appeared white and was collected in a 50 mL falcon. The cell suspension was then passed through a 70 μ m strainer to remove any muscle or bone fragments. The suspension was centrifuged at 250 g for 4 mins at RT. The supernatant was discarded. Red blood cells were lysed by osmotic lysis. Cells were resuspended in ddH₂O for 3 s before 9 mL cRPMI was added to the tube. The cell suspension was again centrifuged at 250 g for 4 min and the cells were resuspended in cRPMI giving a cell suspension of 1×10^6 cells/mL. The medium was supplemented with 10% v/v L929-conditioned medium. The cell suspension was then plated at 10 mL/dish in petri dishes (100 $\times$ 20 mm) and cultured in an incubator at 37°C and 5% CO₂. On day 4 the medium was removed and replaced with 10 mL cRPMI supplemented with 10% w/v L929-conditioned medium. On day 5 or 6, the cells were examined under the light microscope to ensure elongated morphology and confluency. The BMDM culture medium was removed, and cells were washed with PBS before ice-cold PBS, supplemented with 10 mM EDTA (Merck) was added. The cells were placed on ice for 30 mins to cold lift the BMDMs. Differentiated BMDMs were then gently scraped from the petri dishes and the cell suspension was transferred to a 50 mL falcon. Cells were then centrifuged at 250 g for 4 mins and seeded at 1×10^6 cells/mL/well in BMDM culture medium in a 24 well plate, before being returned to an incubator at 37°C and 5% CO₂.

BMDM Stimulation

On day 6 or 7, seeded BMDMs were ensured to be correctly seeded by examination with a light microscope ensuring elongated morphology and confluency. Cells were either unstimulated or were stimulated with various TLR agonists in cRPMI. Details of the treatments can be found in table 2.3.

2.2.3 IL2-expanded NK Cell-BMDM Co-Culture

BMDMs were differentiated and 1×10^6 cells were seeded in a 24 well plate on day 5 of the culture. On day 6 BMDM culture medium was removed, and cells were left unstimulated or stimulated with TLR agonists in 1 mL cRPMI for 6 h. In this time, IL2-expanded NK cells from a parallel culture were harvested and counted. For analyses where a pure population of NK cells was required, NK cells

were purified by MACS using the MojoSort™ mouse NK cell isolation kit according to manufacturer's instructions. The cell suspension was then centrifuged at 250 g for 4 mins at RT and the supernatant was discarded before the cells were resuspended at 2×10^6 cells/mL in cRPMI. 0.5 mL of the NK cell suspension was either directly added on top of the 1 mL BMDM culture or was added in a trans-well (5 μ m membrane pore). This gives a ~1:1 BMDM:NK cell ratio. Cells were co-cultured in an incubator at 37°C and 5% CO₂.

2.2.4 Splenic NK Cell-Peritoneal Macrophage Co-Culture

Isolation of Peritoneal Macrophages

Mice were sacrificed and 4 mL of PBS was injected intraperitoneally (i.p.) using a 25 G needle. The abdomen was massaged for 30 s to wash PBS in the peritoneal cavity. The peritoneum of the mouse was then opened over a petri dish to collect the cell suspension. The lavage was visually inspected for signs of blood or faecal contamination. If there was significant blood or faecal contamination, the lavage was disposed of. The lavage was then removed from the petri dish using a 5 mL syringe and transferred to a 15 mL falcon in a laminar flow hood. The lavage was passed through a 70 μ m strainer to give a single cell suspension before being centrifuged at 250 g for 4 mins at RT. The supernatant was discarded, and peritoneal cells were resuspended at 15×10^6 cells/mL in cRPMI. The suspension was then plated at 100 μ L/well in a flat bottom 96 well plate. Cells were allowed to adhere for 4 h in an incubator at 37°C and 5% CO₂. Following the 4 h incubation the medium and any non-adhered cells were removed and discarded. The cells were washed with PBS before visual inspection that there were adhered macrophages present at no less than 50% confluency. If peritoneal macrophages were to be pre-treated prior to co-culture with NK cells this was done for 2 h. Details of cell treatments can be found in table 2.3.

Enrichment of Splenic NK cells and Co-Culture with Peritoneal Macrophages

Spleens were harvested from mice that had been sacrificed for peritoneal lavage. Spleens were passed through a 70 μ m strainer into a 50 mL falcon tube in a laminar flow hood. The resulting cell suspension was then centrifuged at 250 g

for 4 min at RT. The supernatant was discarded before red blood cells were lysed by osmotic lysis. Cells were resuspended in ddH₂O for 3 s before 9 mL cRPMI was added to the tube. The cell suspension was again centrifuged at 250 g for 4 min and the cells were resuspended to give a cell suspension of 100×10^6 cells/mL in MojoSort™ buffer. NK cells were purified by magnetic activated cell sorting (MACS) using the MojoSort™ mouse NK cell isolation kit according to manufacturer's instructions. The suspension was then centrifuged at 250 g for 4 mins and the supernatant discarded before cells were resuspended at 1.5×10^6 cells/mL in cRPMI. Cells were stimulated with IL12 (10 ng/mL, Miltenyi Biotech), IL15 (10 ng/mL, Peprotech) and IL18 (50 ng/mL, GSK). Unstimulated NK cells were treated with low dose murine IL15 (2 ng/mL). 200 µL of the enriched NK cell suspension was plated in the presence or absence of the previously isolated peritoneal macrophages. Co-cultures were carried out in the presence or absence of a number of different cell treatments or vehicle controls before culture in an incubator at 37°C and 5% CO₂.

Table 2.3 Cell Treatments

Compound	Stock Concentration	Final Dilution	Vehicle
Human IL15 (culturing dose)	0.51 mg/mL	10 ng/mL	-
Human IL15 (unstim. dose)	0.51 mg/mL	5 ng/mL	-
Murine IL15 (activating dose)	500 µg/mL	10 ng/mL	-
Murine IL15 (unstim. dose)	500 µg/mL	2 ng/mL	-
IL2 (culturing dose)	20 µg/mL	100 ng/mL	-
IL2 (activating dose)	20 µg/mL	20 ng/mL	-
IL12	50 µg/mL	10 ng/mL	-
IL18	100 µg/mL	50 ng/mL	-
Zymosan	10 mg/mL	100 µg/mL	-
PolyI:C	20 mg/mL	10 µg/mL	-
LPS	1 µg/mL	10 ng/mL	-
R848	1 mg/mL	1 µg/mL	-
CpG	3.2 mg/mL	4 µg/mL	-
25HC	4.5 mM	2.5 µM	DMSO
27HC	5 mM	5µM	DMSO

PF429242	100 mM	100 μ M	DMSO
7KC	5 mM	2.5/5/10 μ M	EtOH
Rapamycin	2.5 mM	50 nM	-
2DG	1 M	30 mM	-
Oligomycin	2 mM	2 μ M	-
FCCP	1 mM	1 μ M	-
BAM15	2.5 mM	2.5 μ M	-
Latrunculin A	1 mM	10 μ M	DMSO
TOFA	31 mM	15 μ M	DMSO
Zaragozic Acid	10 mM	10 μ M	DMSO
C75	50 mM	40 μ M	DMSO
Etomoxir	10 mM	5 μ M	-
GSK3787	10 mM	10 μ M	DMSO
GW6471	10 mM	10 μ M	DMSO

2.2.5 LPS-Induced Peritonitis Model

A mouse model of LPS-induced peritonitis was used in this study to mimic the effects of bacterial infections and sepsis on NK cell functions. Mice were administered LPS from *E. coli* O111:B4 (Cayman Chemical) in a volume of 100 μ L PBS, using a 27 G needle, by intraperitoneal injection at indicated doses, based on the weight of the mice. A dose of 2.4 mg LPS/kg was used to mimic sepsis. Control mice were administered 100 μ L PBS, using a 27 G needle, by intraperitoneal injection. The treatment was carried out for 24 h before mice were sacrificed and spleens and peritoneal cells were harvested for further analysis. This procedure was carried out in compliance with project license, EU, and Health Products Regulatory Authority regulations, with approval of the University of Dublin's ethical review board.

2.2.6 Culture of Cell Lines

K562

K562 human chronic myelogenous leukaemia cells were cultured in IMDM supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin. As they are highly proliferative cells, they were resuspended at 0.5×10^6 cells/mL every 2-3 days.

RMA/S

RMA/S murine Rauscher virus-induced T cell lymphoma cells were cultured in RPMI supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin. As they are highly proliferative cells, they were resuspended at 0.2×10^6 cells/mL every 2-3 days.

L929

L929 murine fibroblast cells were cultured in DMEM supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin. L929 cells were cultured until confluent in a 175 cm² tissue culture flask and then cultured for a further 8 days before supernatants were harvested and filter sterilised. This was stored at -80°C and was used to generate L929-conditioned medium for BMDM culture.

2.2.7 RNA Analysis

RNA Isolation

RNA lysates were obtained from purified NK cells or BMDMs. Cells were transferred to Eppendorf tubes and washed with PBS. The supernatant was discarded, and cell pellets were lysed in 350 µL RLT buffer containing 1% βME. RNA was isolated using the RNeasy mini kit (Qiagen) according to manufacturer's instructions. When intronless genes were measured, genomic DNA was removed using the RNase-free DNase kit (Qiagen) according to manufacturer's instructions. RNA samples were assessed for purity and RNA concentration using a Nanodrop spectrophotometer (ND-100, Nanodrop® Technologies). RNA lysates were stored at -80°C prior to cDNA synthesis.

cDNA Synthesis

20 µL of cDNA was synthesised from 1.6 µg of isolated RNA. RNA was diluted with DEPC water and cDNA was generated using 4 µL of q-Script Supermix (Quanta Biosciences) according to manufacturer's instructions. The reverse transcription was carried out using a ³Prime Thermal Cycler (Techne) according to temperature cycles listed in table 2.4.

Primer Design

Primers for RT-qPCR were designed using BLAST software located on the NCBI website (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>). The transcript ID for

the gene of interest was entered into the Primer Blast and parameters for primer design were set as follows:

- GC content of between 40-60%
- GC clamp at one end
- spanning an exon-exon junction (unless designing primers for intronless genes)
- have at least three mismatches to other sequences in the gene of interest
- to have less than 1°C between melting temperatures
- forward and reverse primers are similar length

Primer sequences were verified using the Ensembl genome browser (<http://www.ensembl.org/index.html>). Sequences of primers used for RT-qPCR are listed in table 2.5.

Table 2.4 Reverse Transcription Temperature Cycles

Duration	Temperature
5 min	25°C
30 min	42°C
5 min	85°C
Hold	4°C

Table 2.5 RT-qPCR Primer Sequences

Primer/Gene Name	Primer Sequence
<i>Gapdh</i> Forward	5'-CATGGCCTTCCGTGTTCTTA-3'
<i>Gapdh</i> Reverse	5'-CCTGCTTCACCACTTCTTGAT-3'
<i>Rplp0</i> Forward	5'-CATGTCGCTCCGAGGGAAG-3'
<i>Rplp0</i> Reverse	5'-CAGCAGCTGGCACCTTATTG-3'
<i>Hprt</i> Forward	5'-TGATCAGTCAACGGGGGACA-3'
<i>Hprt</i> Reverse	5'-TTCGAGAGGTCCTTTTCACCA-3'
<i>Cyp7a1</i> Forward	5'-ACTTCTGCGAAGGCATTTGG-3'
<i>Cyp7a1</i> Reverse	5'-CATAATCCCTTCCGTGACCC-3'
<i>Ch25h</i> Forward	5'-GCTCGTCCAGCTCCT-3'
<i>Ch25h</i> Reverse	5'-AGCACGTCTGAACAAG-3'
<i>Cyp27a1</i> Forward	5'-AGAGCCAAGGCCACGATCC-3'

<i>Cyp27a1</i> Reverse	5'-TGGTCTTGTTTCAGCACCTGGAG-3'
<i>Cyp7b1</i> Forward	5'-TTGGTCTGCCTGGAAAGCAC-3'
<i>Cyp7b1</i> Reverse	5'-CCTTTGGAGCATCGAAGATTTC-3'
<i>Hsd3b7</i> Forward	5'-CCTGGGACTTACTACAGGTGGC-3'
<i>Hsd3b7</i> Reverse	5'-CACCTCATGGGCCTGAGTCAC-3'
<i>Fasn</i> Forward	5'-GGTATAGACGACGGGCACAG-3'
<i>Fasn</i> Reverse	5'-GCCATTCCAGGTAAATGGGC-3'
<i>Acat2</i> Forward	5'-CCAGAGCGACAAGATGAATGC-3'
<i>Acat2</i> Reverse	5'-CCACAACCTGCCGTCAAGA-3'
<i>Hmgcs1</i> Forward	5'-CATTGGGCGGCTAGAAGTTG-3'
<i>Hmgcs1</i> Reverse	5'-ACCAGAGCATATCGTCCATCC-3'
<i>Acly</i> Forward	5'-CCCCAAGATTTCAGTCCCAAGTC-3'
<i>Acly</i> Reverse	5'-GATCCCCAGTGAAAGGGTAGAC-3'
<i>Abca1</i> Forward	5'-GGAAGTTGGCAAGGTTGGTG-3'
<i>Abca1</i> Reverse	5'-TTCCACAAGAATCTCCGGGC-3'
<i>Abcg1</i> Forward	5'-GACCTTTCCTACTCTGTACCCG-3'
<i>Abcg1</i> Reverse	5'-TTTCATGCCAGTCTCCCTGT-3'
<i>Tfrc</i> Forward	5'-TAGAGGCGCTTCCTA-3'
<i>Tfrc</i> Reverse	5'-CACTAGCCTTCATGTTATTGTCGG-3'
<i>Slc40a1</i> Forward	5'-GTG ACGTCTGGGCCACTTTA-3'
<i>Slc40a1</i> Reverse	5'- TGGCCACTCTCTCTCCACTT-3'
<i>Slc2a1</i> Forward	5'-GGAATCGTCGTTGGCATCCT-3'
<i>Slc2a1</i> Reverse	5'-CGAAGCTTCTTCAGCACACTC-3'
<i>Hk2</i> Forward	5'-TCGCCTGCTTATTCACGGAG-3'
<i>Hk2</i> Reverse	5'-TCGCCTGCTTATTCACGGAG -3'
<i>Ldha</i> Forward	5'-CTGGGAGAACATGGCGACTC-3'
<i>Ldha</i> Reverse	5'-ATGGCCCAGGATGTGTAACC-3'
<i>Cd36</i> Forward	5'-GGACATTGAGATTCTTTTCCTCTG-3'
<i>CD36</i> Reverse	5'-GCAAAGGCATTGGCTGGAAGAAC -3'
<i>Scarb1</i> Forward	5'- ACACCCGAATCCTCGCTGGAAT -3'
<i>Scarb1</i> Reverse	5'- CCGTTGGCAAACAGAGTATCGG -3'
<i>Scarb2</i> Forward	5'- TAGCCAACACCTCCGAAAACGC -3'
<i>Scarb2</i> Reverse	5'- CGAACTTCTCGTCGGCTTGGTA -3'
<i>Cpt1a</i> Forward	5'-GACTCCGCTCGCTCATTCC-3'

<i>Cpt1a</i> Reverse	5'-GCCATTCTTGAATCGGATGAACT-3'
<i>Fabp5</i> Forward	5'-GACGACTGTGTTCTCTTGTAACC-3'
<i>Fabp5</i> Reverse	5'- TGTTATCGTGCTCTCCTTCCCG-3'
<i>Plin2</i> Forward	5'-CAGCCAACGTCCGAGATTG-3'
<i>Plin2</i> Reverse	5'-CACATCCTTCGCCCCAGTT-3'

RT-qPCR

Real time quantitative polymerase chain reaction (RT-qPCR) was performed in triplicate in a 384-well plate using iQ SYBR Green (VWR) based detection on a QuantStudio 3 RT-qPCR System (Applied Biosystems). Each 10 μ L PCR reaction consisted of 0.5 μ L cDNA, 5 μ L SYBR Green supermix, 3.7 μ L DEPC water and 0.4 μ L each of sense and anti-sense primers. Plates were sealed with a qPCR seal and run under cycle conditions listed in table 2.6 Gene expression was measured by the ΔC_t method:

ΔC_t : $2^{-(C_t \text{ of endogenous control gene}) - (C_t \text{ of gene of interest})}$

Relative changes in mRNA content were calculated by the $\Delta\Delta C_t$ method:

$\Delta\Delta C_t$: $2^{-(C_t \text{ of target gene target sample} - C_t \text{ of reference gene target sample}) - (C_t \text{ of target gene reference sample} - C_t \text{ reference gene reference sample})}$

Table 2.6 RT-qPCR Temperature Cycle

Stage	Temperature	Time	Cycles
1	95°C	60 s	-
2	95°C	1 s	32
2	57°C	20 s	32
3	95°C	15 s	-
3	55°C	30 s	-
3	95°C	15 s	-

2.2.8 Fluorescence Release Cytotoxicity Assay

K562 or RMA/S target cells were washed with PBS and stained with calcein-AM (10 μ M, BD Biosciences), at 37°C for 30 min. The cells were then washed with wash buffer and resuspended at 0.3×10^6 cells/mL in NK cell culture media. NK cells were counted and resuspended at 1.5×10^6 cells/mL. 0.03×10^6 K562 or

RMA/S target cells were added to a V bottom 96 well plate, corresponding to 100 μ L of the cell suspension, and NK cells were added in NK cell to target cell ratios of 5:1, 2:1 and 1:1, or otherwise specified. For measurements of spontaneous and maximum cell death, only target cells were added to the well and they were left untreated or treated with 0.2% Triton-X-100 lysis buffer. Cells were incubated for 3 h at 37°C and 5% CO₂. Following incubation, the 96 well plate was centrifuged at 200 g for 5 min. 75 μ L of the supernatant was transferred to a black flat bottom 96 well plate. Fluorescence was measured on a FLUOstar OPTIMA microplate reader (BMG LabTech), with an excitation wavelength of 495 nm and an emission wavelength of 515 nm. All samples were measured in triplicate and the mean was used for further analysis. The experiment was only considered valid if the spontaneous release was <30%. Specific lysis was calculated using the following formula:

$$\% \text{Specific Lysis} = ((\text{mean}_{\text{exp}} - \text{mean}_{\text{spons}}) / (\text{mean}_{\text{max}} - \text{mean}_{\text{spons}})) \times 100$$

where mean_{exp} is the mean fluorescence of the condition of interest, $\text{mean}_{\text{spon}}$ is the mean fluorescence of the spontaneous death wells and mean_{max} is the mean fluorescence of the maximum death wells.

2.2.9 Flow Cytometry

Surface Staining

Following incubation, cells were washed with FACS buffer to remove any remaining culture medium. Cells were incubated with Fc blocking antibody for 5 min at 4°C to prevent non-specific binding of FACS antibodies to the cells. Zombie™ viability dyes (Invitrogen) were used as viability stains and added to the cell suspension. Cells were then stained with saturating concentrations of antibodies specific for surface markers and incubated for 20 min at 4°C in the dark. The stained sample was then washed with FACS buffer and resuspended in FACS buffer for analysis. Samples were analysed using FACS Canto II (BD) or LSR Fortessa (BD) using FACS Diva software and data was analysed using FlowJo™ (TreeStar). Gating strategies are described in figures 2.1-4.

Intracellular Staining

Where intracellular staining was required 100 μ L of cell suspension was taken 4 h before the end of the incubation and transferred to a U bottom 96 well plate.

When samples were obtained directly *ex vivo* the cell suspension was resuspended in 100 μ L cRPMI and transferred to a U bottom 96 well plate. GolgiPlug™ (0.2 μ g/mL, BD Biosciences) was added to the sample and incubated for 4 h in an incubator at 37°C and 5% CO₂. Cells were surface stained, followed by fixation and permeabilisation using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Intracellular antibodies were added to the permeabilised cells at saturating concentrations for a further 30 mins at 4°C in the dark before the cells were washed with FACS buffer and analysed.

Di-4-ANEPPDHQ Staining

Cells were washed from stimulation medium and added to a U bottom 96 well plate. Cells suspensions were then washed with FACS buffer and resuspended in FACS buffer containing Di-4-ANEPPDHQ (0.5 μ M, Invitrogen) and the required antibodies for surface staining, for 20 mins at 4°C in the dark. The stained samples were then washed with FACS buffer and resuspended in FACS buffer for analysis. Samples were measured immediately to reduce internalisation of Di-4-ANEPPDHQ from the plasma membrane. Samples were measured using FACS Canto II (BD, 488 nm laser, 530/30, 585/45, 670-735, 780/60 BP filter for Di-4-ANEPPDHQ detection). Data was analysed using FlowJo™ (TreeStar) to calculate the ratiometric general polarisation (rGP) of Di-4-ANEPPDHQ which is an indicator of the order of the plasma membrane according to the following formula:

$$\text{rGP} = (\text{MFI}_{585} - \text{MFI}_{670}) / (\text{MFI}_{585} + \text{MFI}_{670})$$

where MFI₅₈₅ indicates the geometric mean fluorescence intensity of Di-4-ANEPPDHQ detected in the 585/45 BP filter (ordered phase) and the MFI₆₇₀ indicates the geometric mean fluorescence intensity of Di-4-ANEPPDHQ in the 670-735 BP filter (disordered phase).

Puromycin Click Chemistry

Puromycin incorporation was used as a measure of protein translation. Cells were washed from stimulation medium or directly taken *ex vivo* and transferred to a U bottom 96 well plate. Cells were surface stained before being washed with pre-warmed HBSS. Samples were then incubated with O-propargyl puromycin

(20 μ M, Jena Bioscience) in HBSS for 30 mins at 37°C. If this was performed in the presence of various cell treatments, cells were pre-treated with these treatments for 10 mins at 37°C prior to addition of O-propargyl puromycin. After incubation, incorporation was stopped by fixing cells with PFA (1%, Merck) for 15 mins at RT. Cells were washed with PBS before being permeabilised with click chemistry permeabilisation buffer for 15 mins at RT. Cells were then treated with a click chemistry mix containing copper (II) sulfate (1 mM, Merck), sodium-L-ascorbate (10 mM, Merck), tris-hydroxypropyltriazolylmethylamine (1 mM, Click Chemistry Tools), aminoguanidine hydrochloride (10 mM, Cayman Chemical) and azide fluorophore (5 μ M, Jena Bioscience) for 1 h at RT. After staining, cells were washed three times with PBS and samples were analysed on FACS Canto II (BD).

MitoTracker™ Green and Acridine Orange Staining

MitoTracker™ Green or Acridine Orange were used as indicators of mitochondrial mass. Cells were washed from stimulation medium and added to a U bottom 96 well plate. Cell suspensions were then resuspended in RPMI containing MitoTracker™ Green (100 nM, Invitrogen) or Acridine Orange (100 nM, Invitrogen) for 30 mins prior to surface staining and analysis. Analysis was carried out using LSR Fortessa (BD, 488 nm laser, 530/30 BP filter for detection of MitoTracker™ Green and Acridine Orange).

TMRM Staining

Tetramethyl rhodamine (TMRM) was used as an indicator of mitochondrial inner membrane polarisation. Cells were washed from stimulation medium or directly taken *ex vivo* and added to a U bottom 96 well plate. Cell suspensions were then resuspended in RPMI containing TMRM (100 nM, Invitrogen) for 30 mins prior to surface staining and analysis. Oligomycin (2 μ M, Merck) was used as a control to induce hyperpolarisation. FCCP (20 μ M, Merck) or BAM15 (2.5 μ M, Merck) were used as controls to induce hypopolarisation. Analysis was carried out using LSR Fortessa (BD, 561 nm laser, 582/15 BP filter for detection of TMRM).

Transferrin Uptake Assay

Fluorescently labelled holotransferrin (AF647-holotransferrin) was used to assess capacity for transferrin uptake by cells. Cells were washed from

stimulation medium or taken directly *ex vivo* with transferrin uptake buffer. Cells were then incubated with transferrin uptake buffer at 37°C and 5% CO₂. Cells were washed with transferrin uptake buffer and resuspended in transferrin uptake buffer containing AlexaFluor647-conjugated holotransferrin (5 µg/mL, Invitrogen) for 10 mins at 37°C. Uptake was stopped by placing cells on ice and washing twice with acid wash prior to surface staining and analysis. Unlabelled holotransferrin (500 µg/mL, Merck) was used as a competition control. Samples were analysed on FACS Canto II (BD).

LipidTOX™ Green Staining

LipidTOX™ Green was used as an indicator of neutral lipid content of cells. Cells were washed from stimulation medium or directly taken *ex vivo* and added to a U bottom 96 well plate. Cells were washed from stimulation media and surface stained in the presence of LipidTOX™ Green (1X, Invitrogen) before being washed with FACS buffer and analysed using FACS Canto II (BD, 488 nm laser, 530/30 BP filter for detection of LipidTOX™ Green).

Oleic Acid Click Chemistry

Oleic acid alkyne was used as a measure of oleic acid uptake using click chemistry. Cells were washed from stimulation medium or directly taken *ex vivo* and transferred to a U bottom 96 well plate. Cells were washed with pre-warmed RPMI before being resuspended in RPMI containing oleic acid alkyne (6 µM, Leiden Institute of Chemistry, Leiden University, The Netherlands). Cells were incubated at 37°C for 10 mins. Sodium oleate (60 µM, Merck) was used as a competition control. Uptake was stopped by placing cells on ice and washing twice with acid wash prior to fixing with PFA (1%, Merck). Cells were washed with PBS before being permeabilised with click chemistry perm buffer for 15 mins at RT. Cells were then treated with a click chemistry mix containing copper (II) sulfate (1 mM, Merck), sodium-L-ascorbate (10 mM, Merck), tris-hydroxypropyltriazolylmethylamine (1 mM, Click Chemistry Tools), aminoguanidine hydrochloride (10 mM, Cayman Chemical) and azide fluorophore (5 µM, Jena Bioscience) for 1 h at RT. After staining, cells were washed three times with PBS and samples were analysed on FACS Canto II (BD).

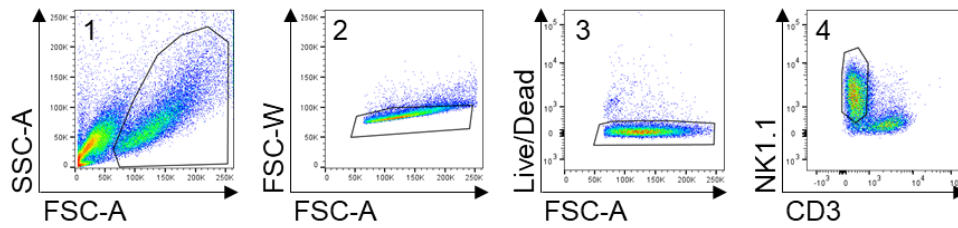


Figure 2.1 Gating strategy for cultured NK cells

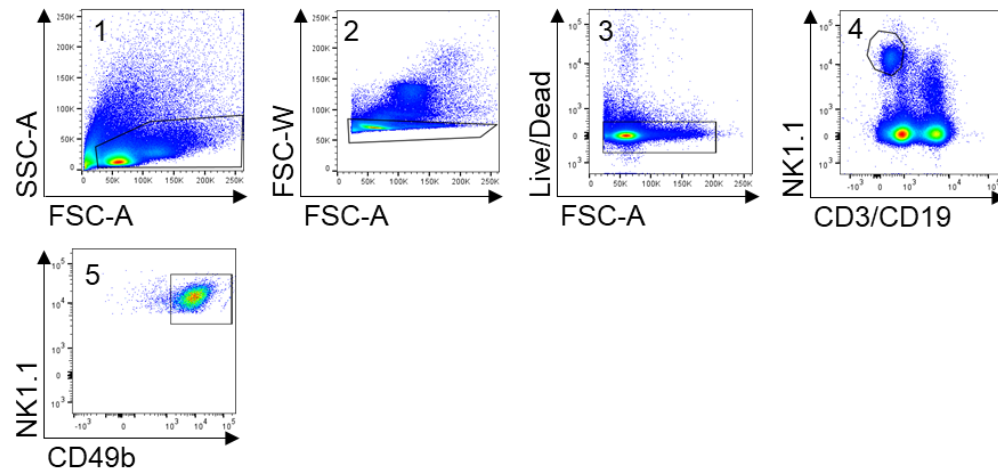


Figure 2.2 Gating strategy for NK cells *ex vivo*

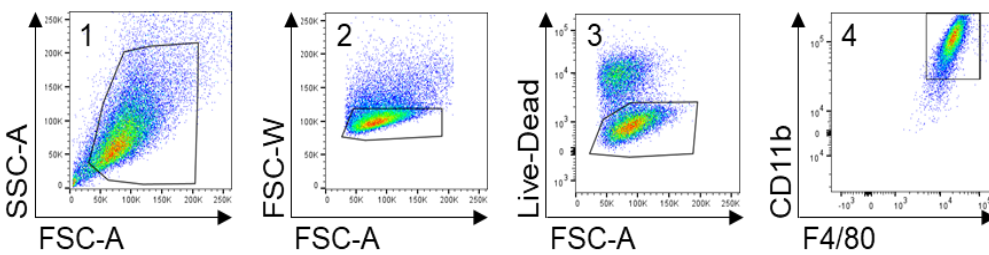


Figure 2.3 Gating strategy for cultured macrophages

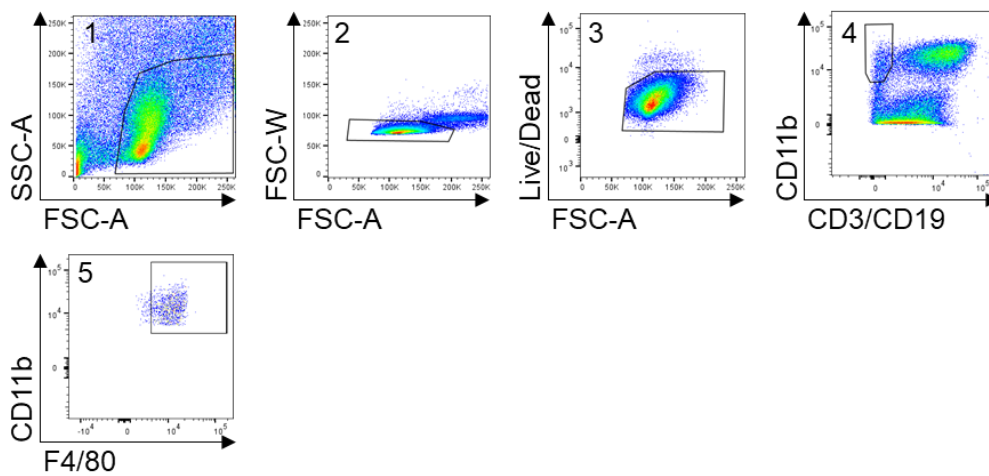


Figure 2.4 Gating strategy for small peritoneal macrophages *ex vivo*

2.2.10 Confocal Microscopy

Di-4-ANEPPDHQ Staining

Di-4-ANEPPDHQ membrane stain was visualised by confocal microscopy to measure plasma membrane order of NK cells. μ -dishes (Ibidi) were coated with 0.01% poly-L-lysine (Merck) at RT and allowed to dry overnight. Purified NK cells were washed from stimulation medium and added to a 1.5 mL Eppendorfs. 0.2×10^6 NK cells were taken and resuspended in cRPMI containing Di-4-ANEPPDHQ (2 μ M, Invitrogen) in poly-L-lysine-coated μ dishes and placed at 37°C for 30 mins. Cells were imaged using a Leica TCS SP8 STED (Leica Microsystems) using LASX software. Di-4-ANEPPDHQ was excited at 488 nm and the emission was detected at 500-580 nm (ordered phase) and 620-750 nm (disordered phase). At least 20 images were obtained per sample. Images were analysed using ImageJ software (NIH). The macro for the generation of rGP images was developed by Owen *et al.*, 2012. Plasma membrane regions of interest (ROI) were drawn and the rGP of each pixel was recorded for further analysis.

Measuring NK Cell Circularity and Roundness

The plasma membrane of the cell was identified based on Di-4-ANEPPDHQ staining, and the resulting shape was analysed using ImageJ software (NIH).

Circularity was used as a measure of NK cell plasma membrane ruffling as it is calculated using the perimeter of the plasma membrane according to the following formula:

$$\text{Circularity} = 4\pi(\text{Area}/\text{Perimeter}^2)$$

Roundness was used as a measure of NK cell polarisation as it is calculated using the maximum Feret's diameter (the longest distance between any two points on the shape) of the plasma membrane according to the following formula:

$$\text{Roundness} = (4 \times \text{Area})/(\pi \times \text{Feret's diameter}^2)$$

In both instances a perfect circle will give a value of 1, with values approaching zero indicating less circularity and roundness.

LipidTOX™ Deep Red Staining

LipidTOX™ Deep Red was used to visualise neutral lipid dispersion and lipid droplet content of NK cells by confocal microscopy. 1×10^6 purified NK cells were washed from stimulation medium and added to a V bottom 96 well plate before being fixed with PFA (1%, Merck) for 15 mins at RT. Cells were washed before being stained with LipidTOX™ Deep Red (1X, Invitrogen) and DAPI (1 $\mu\text{g/mL}$, Merck) for 1 h at 4°C in the dark. Cells were washed with PBS and the pellet was ensured to be completely dry before being resuspended in Mowiol® 4-88 (Merck) mounting medium to embed the cells on a glass microscope slide (VWR). Cells were imaged using a Leica TCS SP8 STED (Leica Microsystems) using LASX software. LipidTOX™ Deep Red was excited at 630 nm and the emission was detected at 650 nm. DAPI was excited at 360 nm and the emission was detected at 460 nm. At least 20 images were obtained per sample. Images were analysed using particle analysis on ImageJ software to quantify the number of lipid droplets/cell. A lipid droplet was defined as punctate LipidTOX™ Deep Red staining with a diameter of >100 nm.

2.2.11 Cytokine Bead Array

For measurements of cytokine and chemokine levels in the peritoneal cavity, mice were sacrificed, and a peritoneal lavage was carried out using 4 mL PBS. PBS was harvested from the peritoneum and centrifuged at 250 g for 4 mins. Pellets were inspected for blood contamination and any pellets that showed any visible red blood cell contamination were not included for further analysis. Samples were stored at -80°C before further analysis. For measurements of cytokine and chemokine levels in cell culture supernatant of peritoneal macrophages, cell culture medium was collected and centrifuged at 250 g for 4 mins. The supernatants were then removed and stored at -80°C before further analysis. A custom Legendplex™ cytokine bead array (Biolegend) was then used to measure levels of cytokines and chemokines in these samples, according to the manufacturer's instructions and analysed by flow cytometry using NovoCyte (Agilent). The beads for each analyte were detected using the 640 nm laser, 675/30 BP filter. The mean fluorescence intensity of each analyte was detected using the 488 nm laser, 572/28 BP filter. A control solution, provided by the manufacturer, was used to generate standard curves for each analyte using

GraphPad Prism (10.4.1) (GraphPad Software). These standard curves were then used to interpolate the concentration of each analyte in the samples.

2.2.12 IncuCyte Live Cell Imaging

Peritoneal macrophages were enriched from peritoneal lavage. Following the lavage, peritoneal cells were resuspended at 15×10^6 cells/mL in cRPMI. The suspension was then plated at 200 μ L/well in a flat bottom 96 well plate. Cells were allowed to adhere for 4 h in an incubator at 37°C and 5% CO₂. Following the 4 h incubation the medium and any non-adhered cells were removed and discarded. The cells were washed with PBS before visual inspection that there were adhered macrophages present at no less than 90% confluency. Macrophages were then left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL) in 200 μ L cRPMI for 24 h in an incubator at 37°C and 5% CO₂. Following the stimulation macrophages were washed with PBS and stained with CellTracker™ Deep Red (1 μ M, Invitrogen) at 37°C for 30 mins. Following staining macrophages were washed with PBS and co-cultured with splenic NK cells. NK cells were purified from the spleen using MACS and stained with CFSE (1 μ M, Invitrogen) at 37°C for 30 mins. Following staining NK cells were washed with PBS and resuspended in cRPMI at 0.1×10^6 cells/mL. 100 μ L of this cell suspension was co-cultured with macrophages. The 96 well plate was then spun at 400 g for 1 minute to settle the cells to the bottom. The plate was then inserted into an Incucyte Cell Imager (Sartorius) at 37°C and 5% CO₂. The cell imager was programmed to take photos of the co-culture every 5 mins for 4 h at 10X magnification. CFSE was used to identify NK cells in the green channel and was excited at 441-481 nm and detected at 503-544 nm. CellTracker™ Deep Red was used to identify macrophages in the red channel and was excited at 567-607 nm and detected from 622-704 nm. Images were analysed using particle analysis on ImageJ software to quantify the number of points that were stained for both CFSE and CellTracker™ Deep Red, which indicated direct cell-cell contacts.

2.2.13 Interrogation of Publicly Available RNA-seq Datasets

A publicly available dataset was used to assess gene expression of peritoneal macrophages stimulated with LPS (GSE239696) (RNA-seq) and was identified using Gene Expression Omnibus on the NCBI website

(<https://www.ncbi.nlm.nih.gov/geo/>). Differential expression analysis was performed on log₂ transformed relative expression values/cell using LIMMA (Bioconductor). Volcano plots and heatmaps were made using GraphPad Prism (10.4.1) (GraphPad Software).

2.2.14 Total Protein Measurements

Total protein was measured using a bicinchoninic acid (BCA) assay. Cells were washed from stimulation medium and resuspended in RIPA buffer (1X) in a 1.5 mL Eppendorf tube, before being placed on a heat block at 90°C for 7 mins. Samples were stored at -20°C before further analysis. Total protein content in cell lysates was carried out using a Pierce™ BCA protein assay kit (Fisher Scientific) according to manufacturer's instructions. Absorbance was measured on a FLUOstar OPTIMA microplate reader (BMG LabTech), at 562 nm. All samples were measured in triplicate and the mean was used for further analysis. A standard curve was generated using BSA standards provided in the Pierce™ BCA protein assay kit (Fisher Scientific). Total protein concentrations were interpolated from absorbance values using GraphPad Prism (10.4.1) (GraphPad Software).

2.2.15 Oxysterol Measurements by LC/MS

25-hydroxycholesterol and 27-hydroxycholesterol measurements by LC/MS were generated in collaboration with the Metabolomics Core Facility, European Molecular Biology Laboratory (EMBL), Heidelberg and were performed by Dr Bernhard Drotleff. BMDM culture supernatants were harvested and added to 1.5 mL Eppendorf tubes and centrifuged at 250 g for 4 mins at 4°C. The supernatants were then transferred to a 1.5 mL Eppendorf and snap frozen in liquid nitrogen before storage at -80°C before further analysis. Oxysterol measurements were then carried out at EMBL, Heidelberg. BMDMs were then washed with PBS and lysed using RIPA buffer (1X) and total protein measurements were performed for normalisation (see 2.2.15).

2.2.16 Co-Culture Supernatant Metabolomics Dataset

Metabolomics datasets were generated in collaboration with Prof. Lorraine Brennan, School of Agriculture and Food Science, University College Dublin.

BMDMs and purified NK cells were stimulated for 18h in before supernatants were removed and the cells were co-cultured for a further 24 hours in RPMI containing penicillin/streptomycin (1% v/v) and β -ME (10 μ M). Notably FCS was not present in these co-cultures to prevent any detection of its' metabolites. Cell culture supernatants were harvested and transferred to 1.5 mL Eppendorf tubes and were centrifuged at 250 g for 4 mins at 4°C. Supernatants were then transferred to 1.5 mL Eppendorf's and snap frozen in liquid nitrogen before storage at -80°C before further analysis. Samples of co-culture medium without any cells were also stored as controls. Quantification of metabolites was performed at the School of Agriculture and Food Science, University College Dublin by LC/MS and FIA/MS using the MxP® Quant 500 XL kit (Biocrates). Differential expression analysis was performed on cube root transformed metabolite concentrations that were subtracted from RPMI controls using Excel (Microsoft). This showed the change in concentration in co-culture supernatants compared to medium alone. Volcano plots and heatmaps were generated using GraphPad Prism (10.4.1) (GraphPad Software).

2.2.17 Iron Measurement by Graphite Furnace Atomic Absorption (GFAAS)

Iron measurements by GFAAS were generated in collaboration with Dr Suzanne Cloonan, School of Medicine, Trinity College Dublin and were performed by Dr Sarah Kenny. Cells were washed from stimulation medium before 0.3×10^6 cells were counted and resuspended in PBS. The sample was divided in 2 and 0.15×10^6 cells were resuspended in PBS containing 1% Triton-X-100 (Merck) and stored at -20°C until further analysis. PBS containing 1% Triton-X-100 was also stored as a background control. Samples were provided to Dr Sarah Kenny, Cloonan Lab, Trinity College Dublin, for further digestion and measurement of iron by GFAAS.

2.2.18 Statistical Analysis

GraphPad Prism (10.4.1) (GraphPad Software) was used for statistical analysis. When sample sizes of at least 8 observations were analysed in each group, D'Agostino-Pearson normality tests were performed for Gaussian distribution. A two-tailed Student's t-test was used when there were only two normally

distributed groups with equal variance for analysis. A Welch's t-test was used when there were two normally distributed groups with unequal variance for analysis. A two-tailed Mann-Whitney U test was used when there were only two groups for analysis that did not have normal distribution. A one-way ANOVA was used for the comparison of more than two groups with normal distribution throughout, with Tukey's post-test for multiple comparisons. A Kruskal-Wallis test was used for the comparison of more than two groups that were not normally distributed, with Dunn's post-test for multiple comparisons. Analysis of data with more than two groups and two independent variables was carried out using a two-way ANOVA. When data with two independent variables required multiple comparisons within groups, Šidák's post-test was performed. When data with two independent variables required multiple comparisons across groups, Tukey's post-test was performed. Correlation analysis was performed to obtain the coefficient of determination (R^2 value). A p value <0.05 was considered statistically significant. Throughout this report, $*p<0.05$, $**p<0.01$, $***p<0.001$.

Chapter 3

Macrophages alter NK cell effector function through oxysterol-independent mechanisms.

3.1 Introduction

Oxysterols are immunosuppressive metabolites that can impair the activation of NK cells due to inhibiting SREBP-dependent metabolic reprogramming (Assmann *et al.*, 2017; O'Brien *et al.*, 2021). 25HC and 27HC are physiological inhibitors of SREBP activation that are involved in maintaining lipid homeostasis in many cells. However, because of NK cell's requirement for SREBP activation to support glucose metabolism, the presence of these molecules prevents NK cell activation, through inhibition of this metabolic reprogramming. 25HC has already been described in this context (Assmann *et al.*, 2017; O'Brien *et al.*, 2021). 27HC has recently been shown to exert the same effects on NK cell activation (Choi, 2023). However, oxysterols can also elicit other SREBP-independent effects within many cell types. They are structurally similar to cholesterol, meaning they can insert into membranes and can allosterically alter the activity of certain enzymes (Olsen *et al.*, 2009; Chang *et al.*, 2009). These SREBP-independent effects of oxysterols on NK cell functions have not been previously investigated.

There are numerous physiological settings that have been associated with increased oxysterol synthesis. 25HC and 27HC are synthesised from cholesterol through the activity of the enzymes CH25H and CYP27A1 respectively. Serum 25HC production by pro-inflammatory macrophages has been shown following injection of LPS into healthy individuals (Diczfalusy *et al.*, 2009), and in SARSCoV2 infection (Asano *et al.*, 2023). Furthermore, TAMs are responsible for 25HC accumulation in tumours and have been shown to dampen CD8⁺ T cell responses (Fletcher *et al.*, 2015; Xiao *et al.*, 2024). 27HC is more associated with cancer and is produced by ER⁺ breast cancer cells (Nelson *et al.*, 2013; Wu *et al.*, 2013). It too has been linked with defective CD8⁺ T cell responses (Yan *et al.*, 2023). Given the interest in developing NK cell-based cancer immunotherapies, it is important to understand the impact that oxysterols in the TME might have on

NK cell therapeutic products. It is known that oxysterols are inhibitory to NK cell activation, but the effects of oxysterols on previously activated NK cells, that have already reprogrammed their metabolism, has not been investigated. This study investigated what might occur when a cytokine-activated NK cell migrates into an oxysterol-rich environment such as the site of inflammation or the TME.

3.2 Oxysterols alter NK cell plasma membrane order and polarisation.

To investigate whether oxysterols could inhibit cytotoxicity of previously activated NK cells, IL15-expanded NK cells were stimulated with IL2 and IL12 for 24 h, giving a robust activation, as previously shown (Donnelly *et al.*, 2014; Assmann *et al.*, 2017). These activated NK cells were then exposed to oxysterols for a further 4 h or 18 h. These experiments provide information on whether oxysterols have different effects on NK cells during acute or prolonged exposure.

Firstly, cytotoxicity was assessed, revealing that exposure to oxysterols for 4 h can cause a significant impairment in NK cell cytotoxicity (Figure 3.1a), with a much more pronounced impairment following 18 h exposure (Figure 3.1b). Expression of the NK cell effector molecules IFN γ , perforin and granzyme B were also assessed, revealing that following 4 h exposure to oxysterols there is no change in expression of these proteins (Figure 3.2a,b, 3.3a). Following 18 h exposure to oxysterols there are significant decreases in expression of IFN γ and perforin, with no changes in expression of granzyme B (Figure 3.2c,d, 3.3b). In addition, there were no metabolic changes in the NK cells exposed to oxysterols for 4 h, but inhibition of metabolic processes was detected after 18 h exposure (Choi, 2023). The fact that cytotoxicity is impaired following 4 h exposure to oxysterols, even though the NK cells are expressing their cytolytic effector molecules, indicates that the oxysterols may be altering other important processes required for cytotoxicity such as NK cell polarisation, movement, or recognition of targets. As the structure of the NK cell plasma membrane is essential for each of these processes, whether 25HC and 27HC could alter the order of the plasma membrane was investigated next. Oxysterols have been described to impact the order of the plasma membrane using various *in vitro* systems (Holmes and Yoss, 1984; Kauffman *et al.*, 2000; Olsen *et al.*, 2009). The

findings of these studies regarding how oxysterols can insert into the plasma membrane are depicted in a simplified manner in Figure 3.4.

To demonstrate the potential for oxysterol-mediated changes to plasma membrane order in NK cells, 7KC was initially used. This oxysterol is known to strongly affect plasma membrane order while weakly altering the activities of SREBP and LXR (Crestani *et al.*, 2004; Radhakrishnan *et al.*, 2007). Many studies describe 7KC as a potent inducer of plasma membrane disorder in lymphocytes making it a useful tool to validate the assay for measurements of NK cell plasma membrane order (Rentero *et al.*, 2008; Schieffer *et al.*, 2014; Li and Orange, 2021). Di-4-ANEPPDHQ is a membrane-specific, potentiometric dye that will emit fluorescence at two different wavelengths depending on the electron density in the local environment (Dinic *et al.*, 2011). Dense packaging of phospholipids will increase the electron density in the local environment, meaning di-4-ANEPPDHQ will have an emission maximum at 560 nm. This state is designated as one of increased plasma membrane order throughout this thesis. Insertion of oxysterols will promote fluidity of the plasma membrane because of the ways that they insert into the membrane, decreasing phospholipid packaging and reducing electron density (Figure 3.4). In this environment di-4-ANEPPDHQ will have an emission maximum at 610 nm. This state is designated as one of increased plasma membrane disorder throughout this thesis. Treatment of previously activated NK cells with 7KC induced plasma membrane disorder in NK cells by both flow cytometry and confocal microscopy (Figure 3.5). Although the changes were not as apparent by flow cytometry they were still comparable with the changes to plasma membrane order visualised by confocal microscopy. Measurement of plasma membrane order by flow cytometry has the added complexity of cells being maintained at 4°C to prevent internalisation of the dye, and subsequent staining of intracellular membranes. This change in temperature is likely to cause some changes to the structure of the plasma membrane and somewhat complicate analysis (Los and Murata, 2004). This was not an issue using confocal microscopy as the plasma membrane could be specifically analysed in each image without any signal from other cellular membranes. Nevertheless, taken together the data from both approaches clearly show increased plasma membrane disorder when exposed to 7KC.

Next these assays were applied to 25HC and 27HC. Exposure of IL2/12-stimulated NK cells to oxysterols for 4 h significantly increased membrane disorder (Figure 3.6). The effect was even more pronounced after 18 h exposure (Figure 3.7). The confocal microscopy images revealed another difference regarding the morphology of oxysterol-treated NK cells. Oxysterols appeared to reduced NK cell polarisation and membrane ruffling, resulting in a more rounded shape. This was particularly evident following 18 h exposure. Two formulas were used to quantify the morphology of the NK cells. Firstly, circularity of the cells was used as a surrogate measurement for the ability of the NK cells to ruffle their plasma membranes. Secondly, roundness was used as a surrogate measurement for the ability of the NK cells to polarise their plasma membranes (Olofsson *et al.*, 2014). In line with the measurements of plasma membrane order following 4 h exposure to 25HC there was a significant increase in NK cell circularity and roundness (Figure 3.8a,b). As predicted the changes were more striking after 18 h oxysterol exposure, and both 25HC and 27HC significantly increased the circularity and roundness of the NK cells (Figure 3.8c,d).

Data presented in this chapter indicates that the impaired cytotoxicity of NK cells following exposure to 25HC and 27HC for 4 h may be due to disruptions to the plasma membrane. However, although there was no change in expression of effector molecules at this timepoint, it has been shown that exposure of NK cells to 25HC and 27HC for 4 h can significantly reduce expression of SREBP target genes (Choi, 2023). This means that any impact of these oxysterols on NK cell metabolism cannot be excluded. In order to address this, complimentary experiments were performed using 7KC, an oxysterol that has weak inhibition of SREBP signalling (Radhakrishnan *et al.*, 2007), but can significantly alter plasma membrane order (Figure 3.5) As 7KC does not inhibit SREBP activation in the way that 25HC and 27HC do, it was used to assess whether changes to plasma membrane order during NK cell activation were sufficient to impair NK cell cytotoxicity.

To measure the extent of inhibition of SREBP signalling by 7KC, RT-qPCR was performed to analyse SREBP target genes during stimulation with IL2/12 in the presence of 7KC (5 μ M) or 27HC (5 μ M). The pharmacological inhibitor, PF429242 (10 μ M) was also used as a positive control for SREBP inhibition. This

pharmacological inhibitor of SREBP activation inhibits the activity of S1P, an important protein for the processing of active SREBP. While 27HC was effective at significantly reducing expression of SREBP target gene mRNA, the impact of 7KC was not significant, or was less than that of 27HC (Figure 3.9), demonstrating its poor capacity to inhibit SREBP activity. The only gene with significantly reduced expression was *Hmgcs1* in the cholesterol synthesis pathway (Figure 3.9c), which has been shown to be dispensable for NK cell activation (Assmann *et al.*, 2017). Furthermore, at low doses (2.5 μ M, 5 μ M) 7KC did not impact any functional or metabolic parameters measured in NK cells except for endocytosis of holotransferrin (Figure 3.10). This is likely due to the impact 7KC has on the plasma membrane, as expression of the holotransferrin receptor (CD71) remained unchanged (Figure 3.10). At the highest dose of 7KC used (10 μ M) there was evidence of mitochondrial dysfunction, indicated by the significant increase in mitochondrial mass (Figure 3.10). Having shown that 7KC is a weak inhibitor of SREBP activation and at lower doses does not impair NK cell metabolism or expression of effector molecules, its effects on NK cell cytotoxicity were assessed. For both K562 and RMA/S target cells, NK cells had impaired cytotoxicity following activation in the presence of 7KC in a dose-dependent manner (Figure 3.11). The use of 7KC shows that alterations to the plasma membrane may be sufficient to impair NK cell cytotoxicity, independent of any significant changes to metabolism.

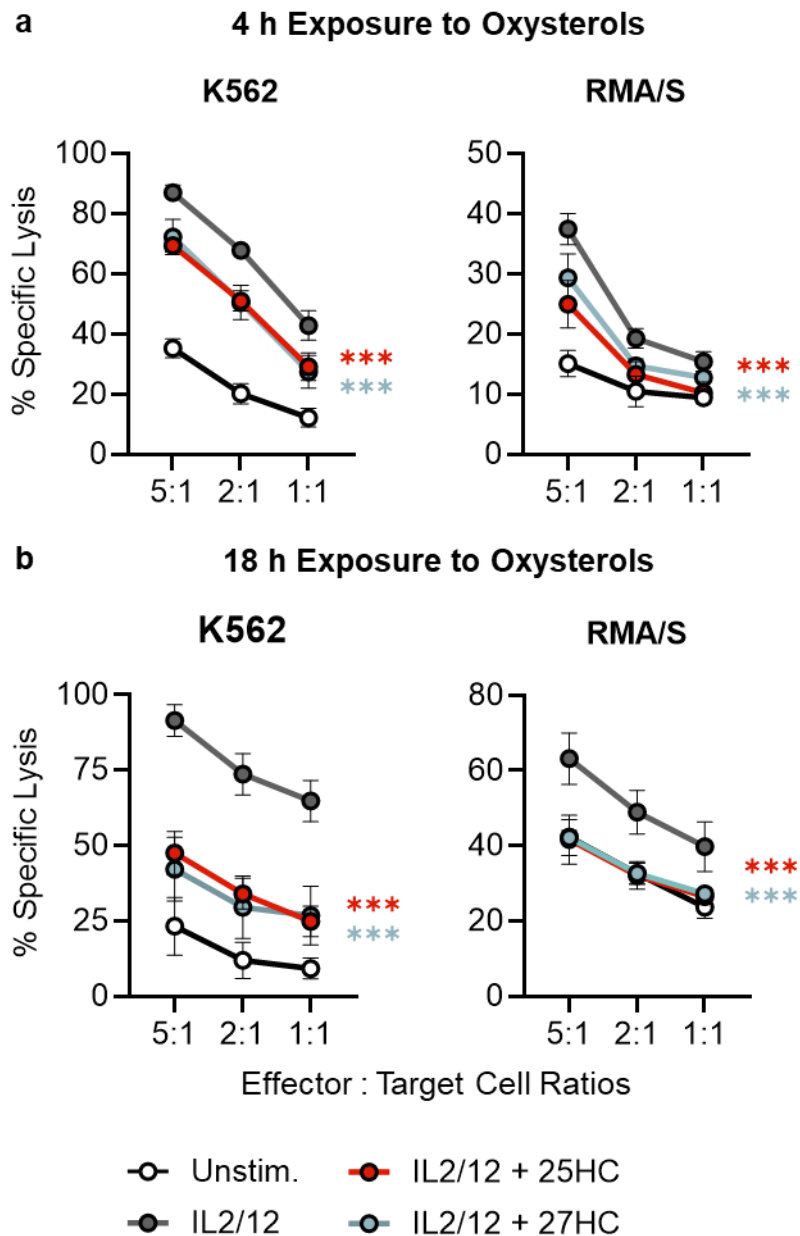


Figure 3.1 Oxysterols impair cytotoxicity of previously activated NK cells.

IL15-expanded NK cells were treated with low-dose human IL15 (5 ng/mL) or were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 24 h before culture for a further 4 h (a) or 18 h (b) in the presence or absence of 25HC (2.5 μ M) or 27HC (5 μ M). Cytotoxicity against K562 and RMA/S target cells was measured by fluorescence release cytotoxicity assay. Data is mean $\pm$ SEM of 4 biological replicates across 2 independent experiments. Statistical analysis was performed using a two-way ANOVA (** $p < 0.01$, *** $p < 0.001$). *Some replicates were performed by Dr Chloe Choi, Finlay Laboratory, Trinity College Dublin.*

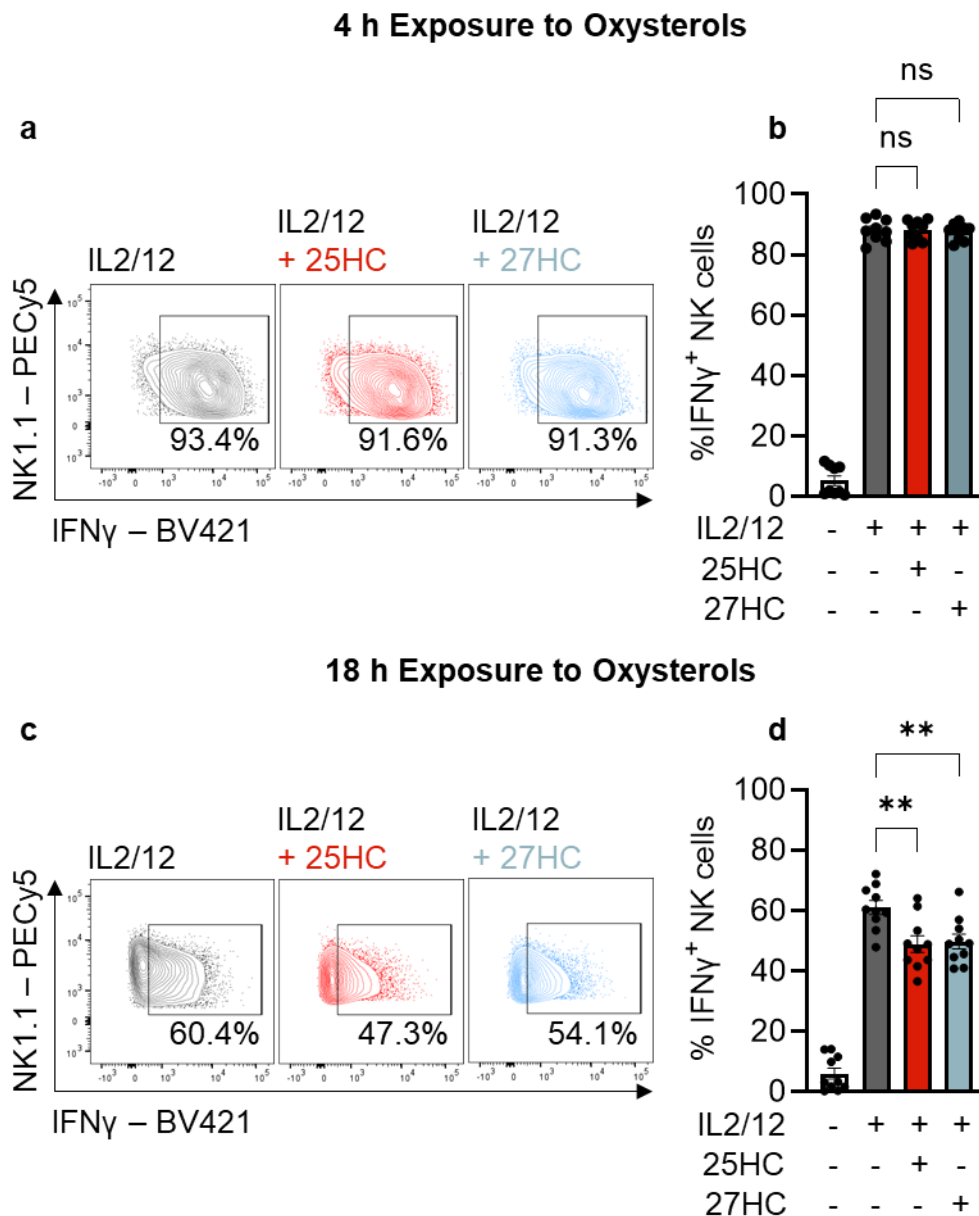


Figure 3.2 18 h exposure to oxysterols impairs IFN γ expression by previously activated NK cells.

IL15-expanded NK cells were treated with low-dose human IL15 (5 ng/mL) or were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 24 h before culture for a further 4 h (a,b) or 18 h (c,d) in the presence or absence of 25HC (2.5 μ M, red) or 27HC (5 μ M, blue). IFN γ expression was measured by flow cytometry. Data is representative (a,c) or mean $\pm$ SEM of at least 9 biological replicates across 3 independent experiments (b,d). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (** $p < 0.01$, ns=non-significant).

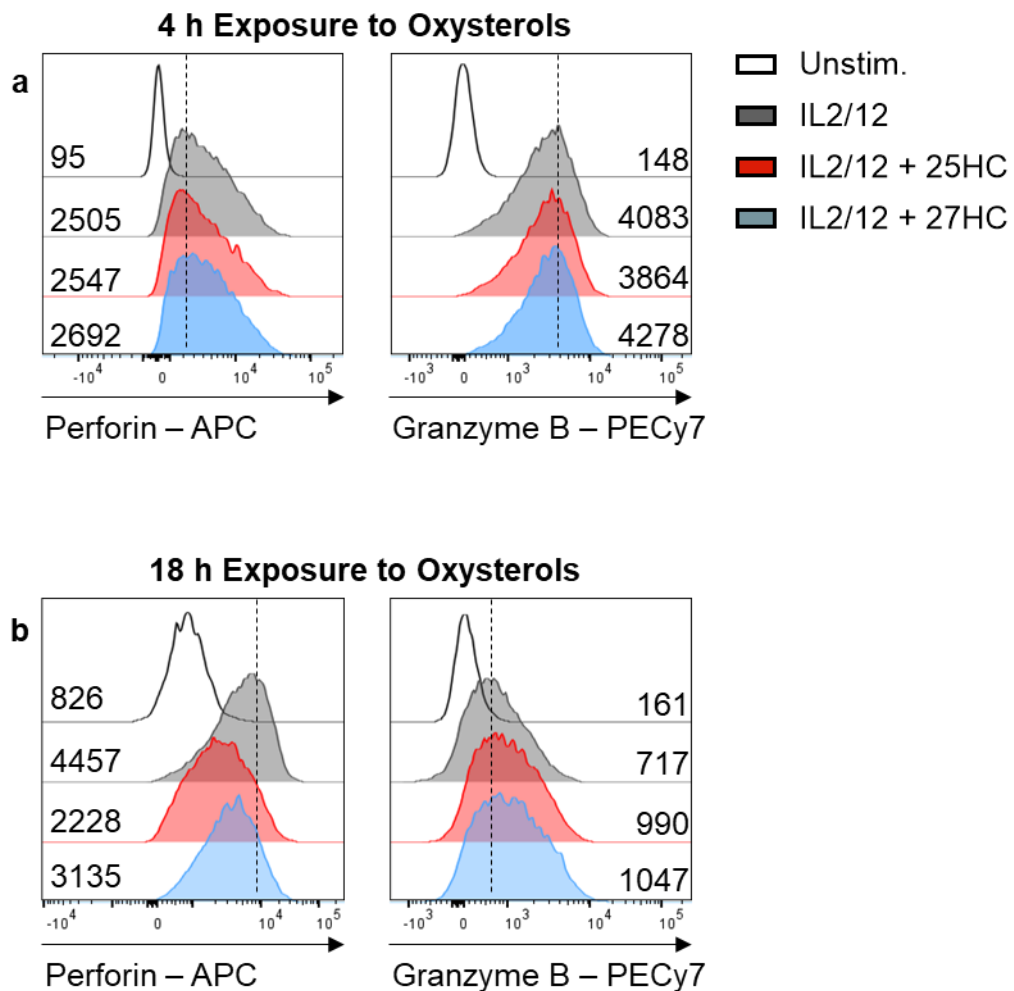


Figure 3.3 18 h exposure to oxysterols impairs perforin expression by previously activated NK cells.

IL15-expanded NK cells were treated with low-dose human IL15 (5 ng/mL) or were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 24 h before culture for a further 4 h (a) or 18 h (b) in the presence or absence of 25HC (2.5 μ M, red) or 27HC (5 μ M, blue). Perforin and granzyme B expression were measured by flow cytometry. Data is representative of at least 9 biological replicates across 3 independent experiments.

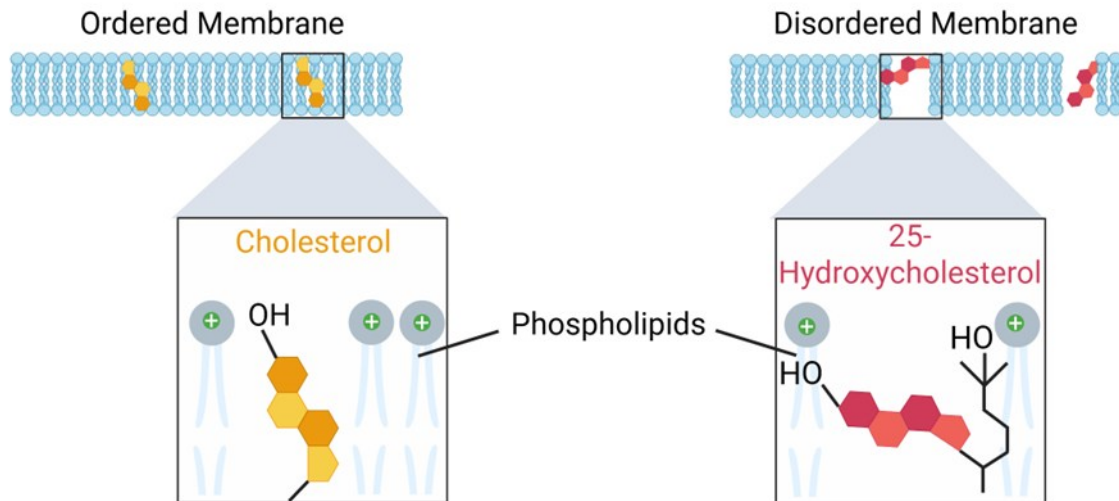


Figure 3.4 Oxysterols can insert into plasma membranes.

Cellular membranes in homeostasis consist of tightly packed phospholipid bilayers with interspersed cholesterol molecules to maintain membrane fluidity, allowing for cellular movement, endocytosis and protein trafficking. The cholesterol backbone and side chain interact with the hydrophobic tail regions of the phospholipids and the hydroxyl group's lone-pair electrons can form interactions with the positively charged phospholipid head groups. However, oxysterols, have other areas of electron density because of their additional hydrophilic functional groups. 25HC has a hydroxyl group on carbon-25 on the sterol side chain. This means that the sterol backbone and side chain change their orientation to facilitate the additional interaction between the carbon-25 hydroxyl groups lone pair electrons and the phospholipid head group. This leads to decreased packing of the phospholipid bi-layer and induces further fluidity leading to plasma membrane disorder. *This figure was created with Biorender.com.*

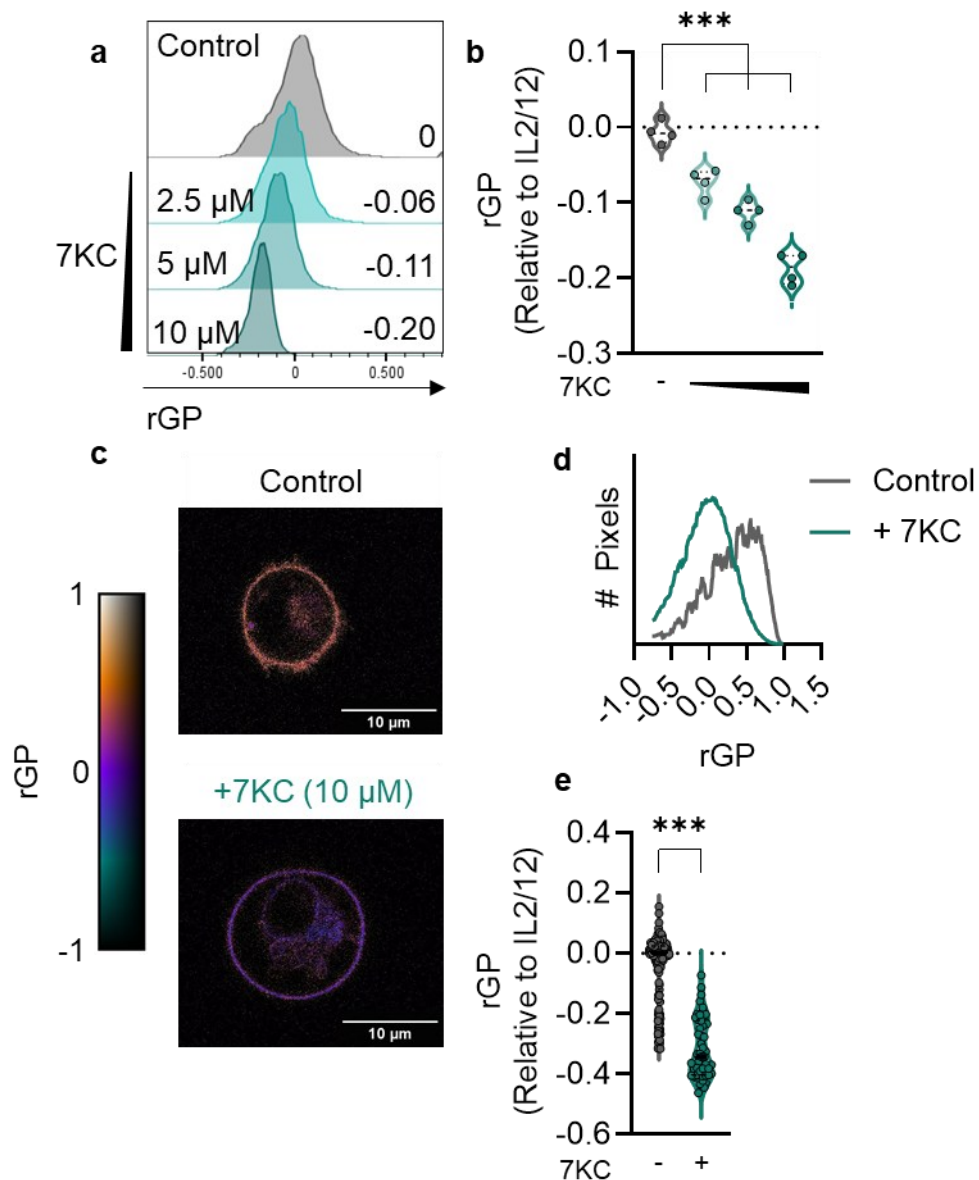


Figure 3.5 Exposure of previously stimulated NK cells to 7KC increases plasma membrane disorder.

IL15-expanded NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 24 h, before a further 18 h culture in the presence or absence of 7KC (10 μ M, unless otherwise indicated). Ratiometric generalised polarisation (rGP) of Di-4-ANEPPDHQ was used as a measure of plasma membrane order, by flow cytometry (a,b) or confocal microscopy (c-e). A pseudocolour mask was used to represent rGP values of each pixel in confocal images (c). Data is representative (a,c,d) or median of 4 biological replicates across 2 independent experiments (b) or of at least 50 cells from 3 biological replicates across 3 independent experiments (e). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (b) or a Mann-Whitney test (e) (**p < 0.01, ***p < 0.001).

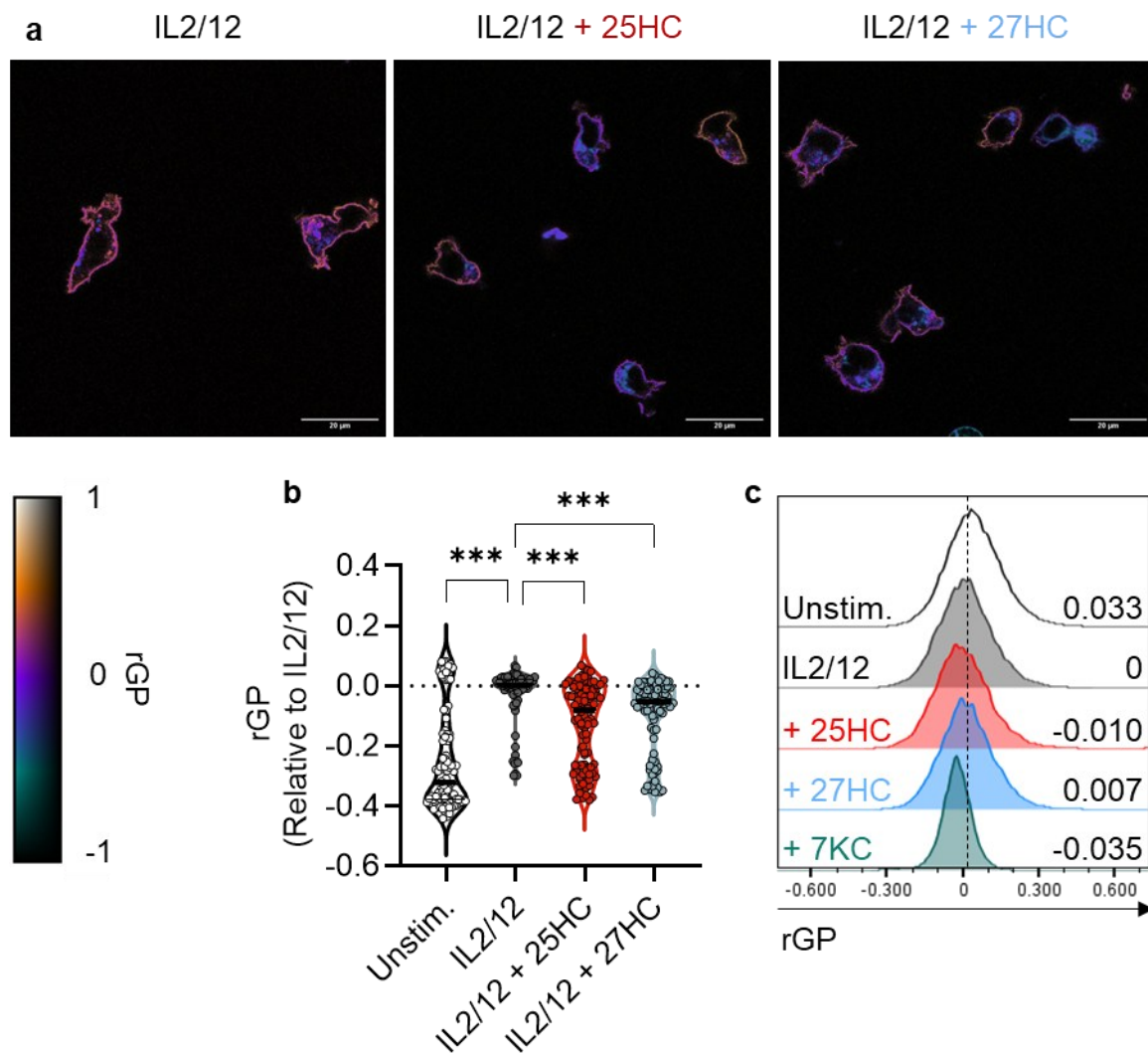


Figure 3.6 Exposure of previously activated NK cells to oxysterols for 4 h modestly increases plasma membrane disorder.

IL15-expanded NK cells were treated with low-dose human IL15 (5 ng/mL) or were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 24 h before a further 4 h culture in the presence or absence of 25HC (2.5 μ M), 27HC (5 μ M) or 7KC (5 μ M). Ratiometric generalised polarisation (rGP) of Di-4-ANEPPDHQ was used as an indicator of plasma membrane order, and was measured by confocal microscopy (a,b) or flow cytometry (c). A pseudocolour mask was used to represent rGP values of each pixel in confocal images (a). Data is representative (a,c) or median of at least 70 cells from 3 biological replicates across 3 independent experiments (e). Statistical analysis was performed using a Kruskal-Wallis test with Dunn's post-test (** $p < 0.001$).

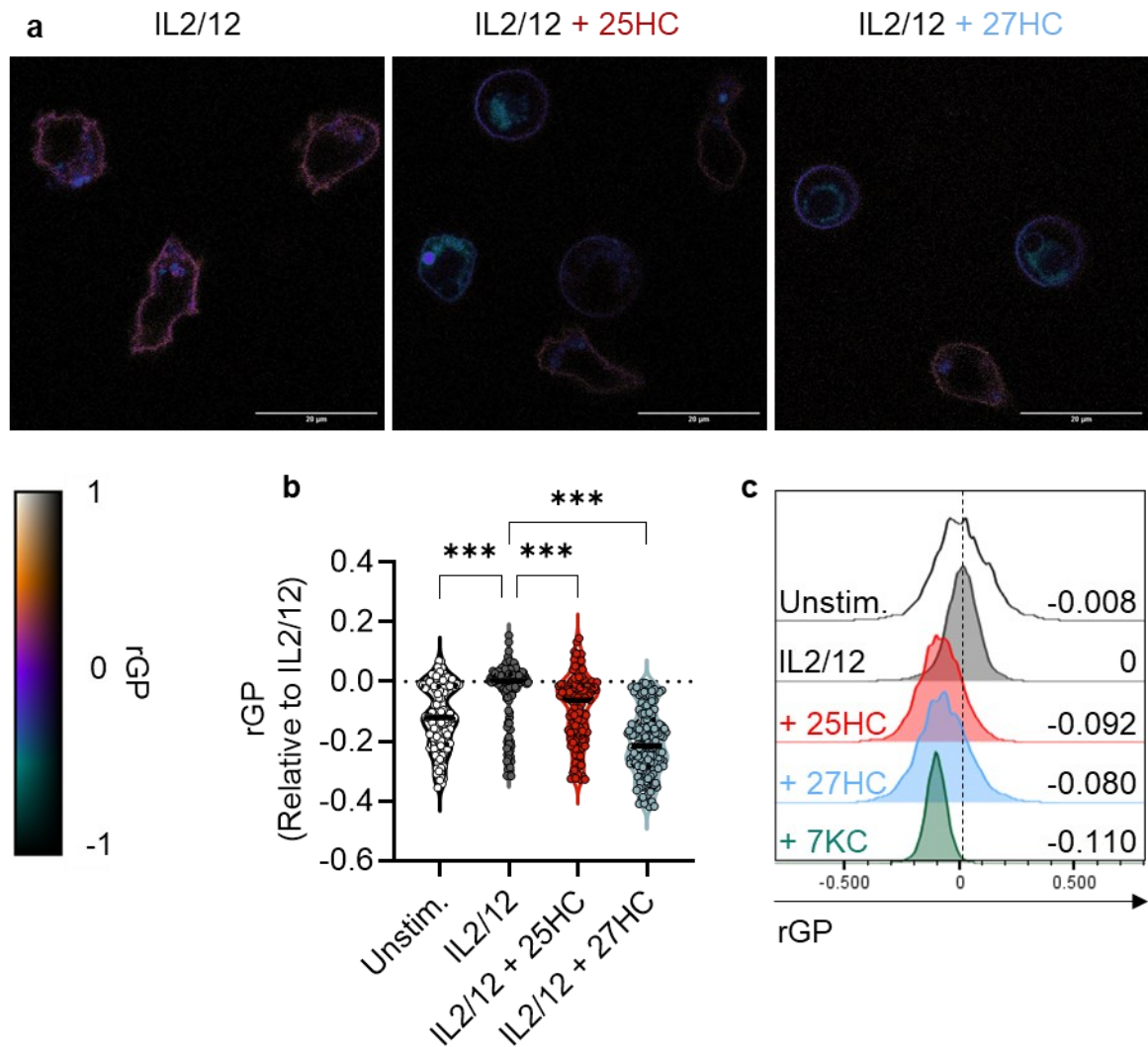


Figure 3.7 Exposure of previously activated NK cells to oxysterols for 18 h increases plasma membrane disorder.

IL15-expanded NK cells were treated with low-dose human IL15 (5 ng/mL) or were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 24 h before a further 18 h culture in the presence or absence of 25HC (2.5 μ M), 27HC (5 μ M) or 7KC (5 μ M). Ratiometric generalised polarisation (rGP) of Di-4-ANEPPDHQ was used as a measure of plasma membrane order, by confocal microscopy (a,b) or flow cytometry (c). A pseudocolour mask was used to represent rGP values of each pixel in confocal images (a). Data is representative (a,c) or median of at least 120 cells from 3 biological replicates across 3 independent experiments (e). Statistical analysis was performed using a Kruskal-Wallis test with Dunn's post-test (** $p < 0.001$).

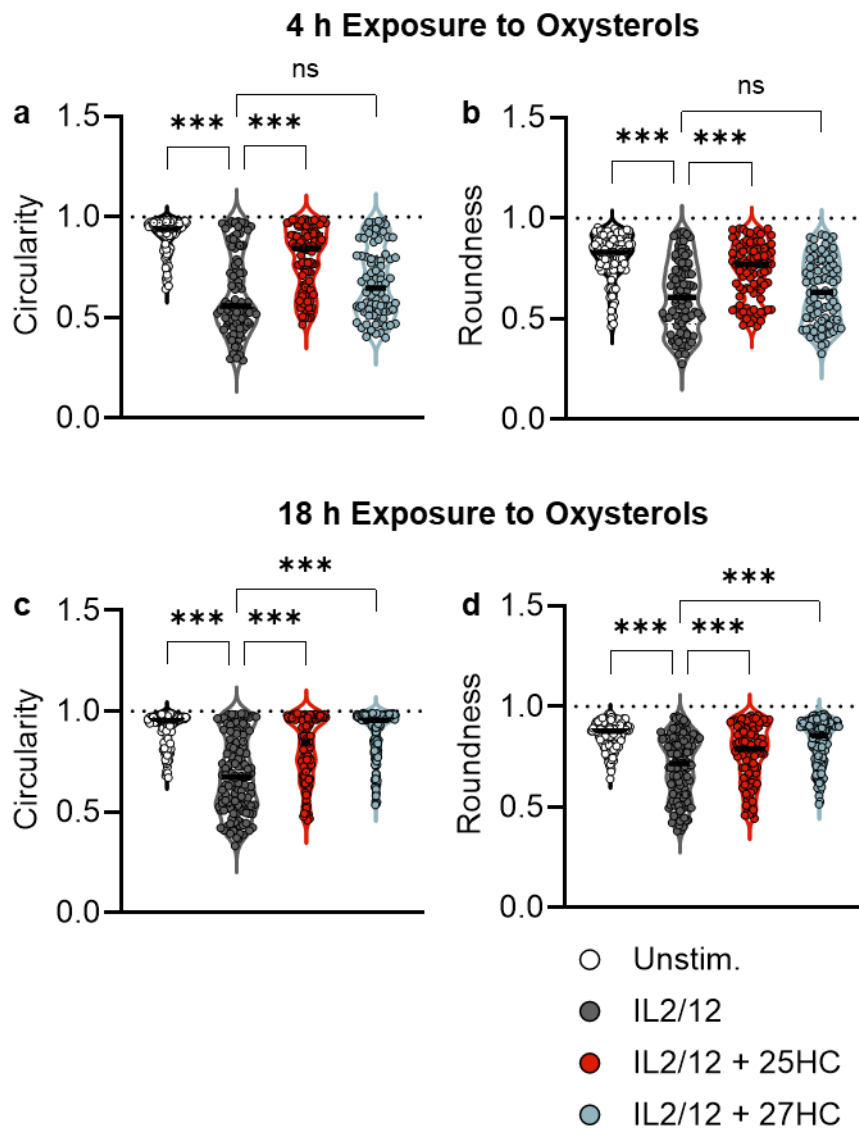


Figure 3.8 Exposure of previously activated NK cells to oxysterols impairs polarisation and plasma membrane ruffling.

IL15-expanded NK cells were treated with low-dose human IL15 (5 ng/mL) or were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 24 h before exposure to 25HC (2.5 μ M) or 27HC (5 μ M) for a further 4 h (a,b) or 18 h (c,d). Cells membranes were stained with Di-4-ANEPPDHQ and were imaged by confocal microscopy. Cell circularity was used as a measure of NK cell plasma membrane ruffling (a,c). Cell roundness was used as a measure of NK cell polarisation (b,d). Data is median of at least 70 cells from 3 biological replicates across 3 independent experiments. Statistical analysis was performed using a Kruskal-Wallis test with Dunn's post-test (** $p < 0.001$, ns=non-significant).

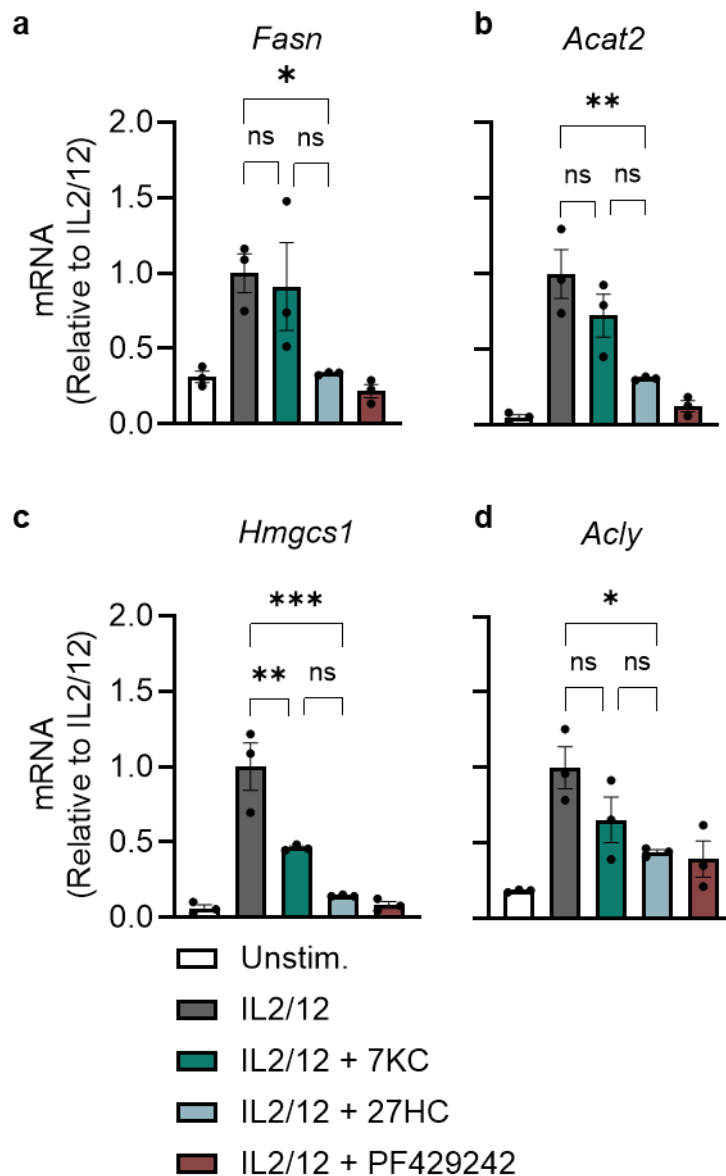


Figure 3.9 7KC is a weak inhibitor of SREBP activation by IL2/12-stimulated NK cells.

IL15-expanded NK cells were treated with low-dose human IL15 (5 ng/mL) or were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h in the presence or absence of 7KC (5 μ M), 27HC (5 μ M) or PF429242 (10 μ M). Cells were lysed and analysed by RT-qPCR for expression of *Fasn* (a), *Acat2* (b), *Hmgcs1* (c) and *Acly* (d). Data is normalised to levels of control genes *Gapdh*, *Hprt* and *Rplp0*. Data is mean $\pm$ SEM of 3 biological replicates across 2 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (* p <0.05, ** p <0.01, ns=non-significant).

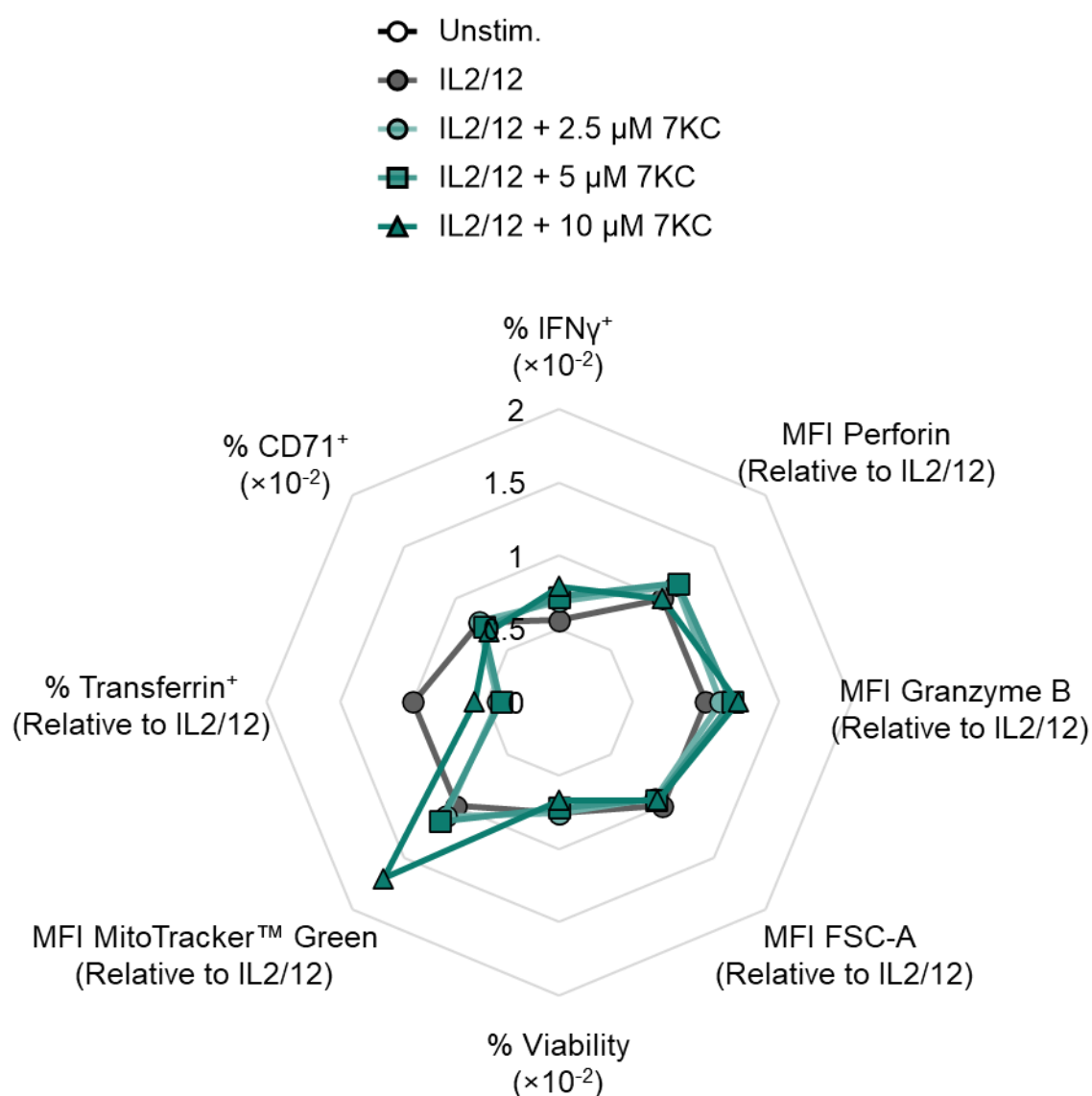


Figure 3.10 7KC does not significantly alter effector molecule expression or metabolic parameters of IL2/12-stimulated NK cells at lower doses.

IL15-expanded NK cells were treated with low-dose human IL15 (5 ng/mL) or were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h in the presence or absence of 7KC (2.5, 5, 10 μ M). IFN γ , perforin, granzyme B and CD71 expression were measured by flow cytometry. FSC-A was used as an indicator cell size and was measured by flow cytometry. Zombie NIR™ and MitoTracker™ Green staining were used as an indicator of viability and mitochondrial mass respectively and were measured by flow cytometry. Holotransferrin uptake was measured using fluorescently labelled holotransferrin by flow cytometry. as Data is mean of at least 4 biological replicates across at least 2 independent experiments.

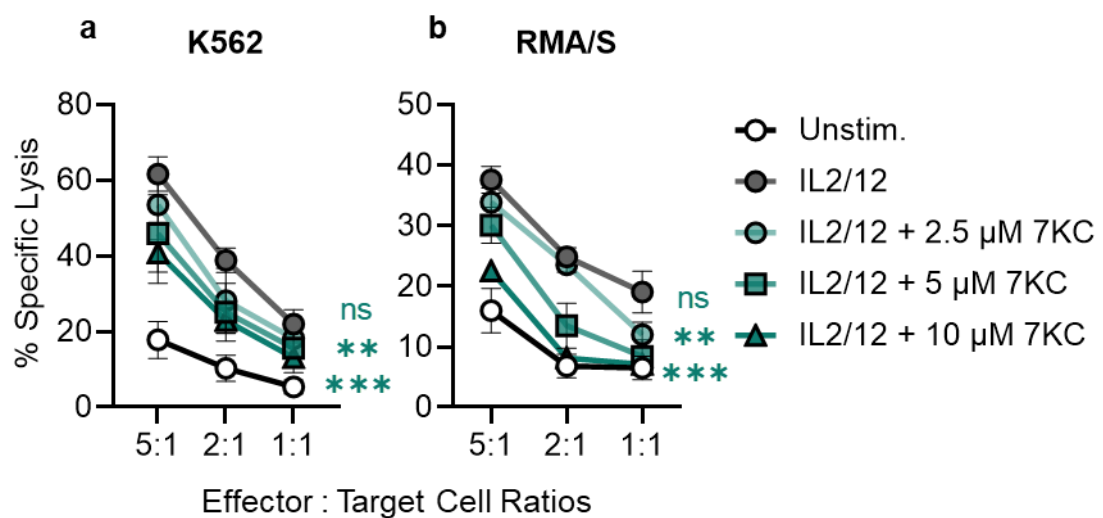


Figure 3.11 7KC impairs *in vitro* cytotoxicity of IL2/12-stimulated NK cells.

IL15-expanded NK cells were treated with low-dose human IL15 (5 ng/mL) or were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h in the presence or absence of 7KC (2.5 μ M, 5 μ M, 10 μ M). Cytotoxicity against K562 (a) and RMA/S (b) target cells was measured by fluorescence release cytotoxicity assay. Data is mean $\pm$ SEM of 4 biological replicates across 2 independent experiments.

3.3 Macrophages are a source of 25-hydroxycholesterol.

Pro-inflammatory macrophages and TAMs have been described as sources of 25HC, though whether this macrophage-derived 25HC has an effect on NK cell functions is not known. This study also investigated whether macrophage-derived 25HC could inhibit NK cell functions. Before this could be investigated *in vitro*, 25HC production by macrophages was assessed.

Measuring the expression of *Ch25h* mRNA can be useful to determine which cells might be producing 25HC in certain environments. Many studies have suggested that pro-inflammatory macrophages are potent producers of 25HC. Through interrogation of a publicly available dataset (GSE239696), a significant increase in expression of *Ch25h* mRNA was observed in peritoneal macrophages stimulated *in vitro* with LPS (Figure 3.12a,c). Alongside this, there was a significant decrease in expression of *Cyp27a1* mRNA indicating that 27HC does not have a role in supporting macrophage functions in response to LPS (Figure 3.12a,c). Furthermore, there was no significant change in expression of *Cyp7b1* mRNA (Figure 3.12a,c). The ratio of *Ch25h* versus *Cyp7b1* mRNA abundance significantly increases following LPS-stimulation suggesting that these macrophages are changing their gene expression to facilitate 25HC accumulation (Figure 3.12b). This data has shown that a physiological macrophage population exhibits an increase in *Ch25h* expression; however, in order to investigate the role of macrophage-derived 25HC *in vitro*, this study used BMDMs in co-culture with cytokine-expanded NK cells. Firstly, BMDMs were cultured to assess whether they expressed oxysterol-synthesising and oxysterol-eliminating enzymes by RT-qPCR. Enzymatic production of oxysterols species occurs through the activity of CYP7A1, CH25H and CYP27A1 on cholesterol, producing 7 α HC, 25HC and 27HC respectively. Other enzymes can act on cholesterol to produce other oxysterol species though they were not considered in these experiments as their expression has not been described by immune cells. In BMDMs there was much lower expression of *Cyp7a1* and *Cyp27a1* mRNA in comparison to *Ch25h* mRNA suggesting that among the oxysterol species produced by BMDMs 25HC is the most abundant (Figure 3.13). Furthermore, mRNA expression of oxysterol-eliminating enzymes did not suggest oxysterol degradation. Expression of *Hsd3b7* mRNA was very low, with

no expression of *Cyp7b1* mRNA, suggesting that BMDMs can accumulate 25HC (Figure 3.13). In order to assess whether this pattern of gene expression could support oxysterol accumulation, the expression of these genes was measured in the liver, a tissue specialised for cholesterol and oxysterol clearance. Indeed, the gene expression of the oxysterol synthesising enzymes in the liver was significantly lower than expression of *Cyp7b1* mRNA (Figure 3.14). Considering oxysterol production by macrophages has been linked with viral and bacterial infection, changes in expression of *Ch25h* mRNA were assessed when macrophages were stimulated with a variety of different TLR agonists. At an early time point, 6 h following stimulation there were no significant increases in the expression of *Ch25h* mRNA with any TLR agonist, although there is a trend towards increased expression in polyI:C (TLR3 agonist)-stimulated BMDMs (Figure 3.15). However, following stimulation of BMDMs with these TLR agonists for 24 h there were significant increases in expression of *Ch25h* mRNA in response to zymosan, polyI:C and LPS (Figure 3.15). Data not included in this chapter showed no expression of *Cyp7b1* mRNA when BMDMs were stimulated with each TLR agonist used.

In order to assess whether 25HC is actually being produced by BMDMs, expression of *Ch25h* mRNA was correlated with expression of *Fasn* and *Abca1* mRNA in BMDMs. In BMDMs *Fasn* expression has been shown to be under the control of SREBP signalling (Bidault *et al.*, 2021), meaning that increased levels of 25HC should inhibit expression of this gene. Similarly, *Abca1* expression has been shown to be under the control of LXR early following stimulation, meaning that increased levels of 25HC should enhance expression of this gene (Liebergall *et al.*, 2020). Following stimulation with polyI:C, there was an increase in *Abca1* mRNA after 6 h and a significant decrease in expression of *Fasn* mRNA after 24 h, arguing that 25HC was present and impacting on LXR and SREBP signalling (Figure 3.16). Taking all conditions into account there was significant but weak correlation between *Ch25h* expression and LXR activity or SREBP activity with R^2 values of 0.4997 and 0.5582, respectively (Figure 3.16). Although correlation of gene expression is useful to determine whether 25HC is present or not, quantification of the metabolite by LC/MS is essential to determine whether or not it is in fact being produced and released from the cell. Comparing the culture

medium of BMDMs to reference standards showed that there is detection of 25HC but not 27HC (Figure 3.17). Following stimulation of BMDMs with zymosan, LPS and CpG there were modest increases in the amount of 25HC in cell culture medium (Figure 3.18). However, there was a significant, ~12-fold increase in the amount of 25HC in the cell culture medium of BMDMs stimulated with polyI:C (Figure 3.18). This did not correlate with expression of *Ch25h* mRNA by TLR-stimulated BMDMs at the same 24 h time point (Figure 3.15).

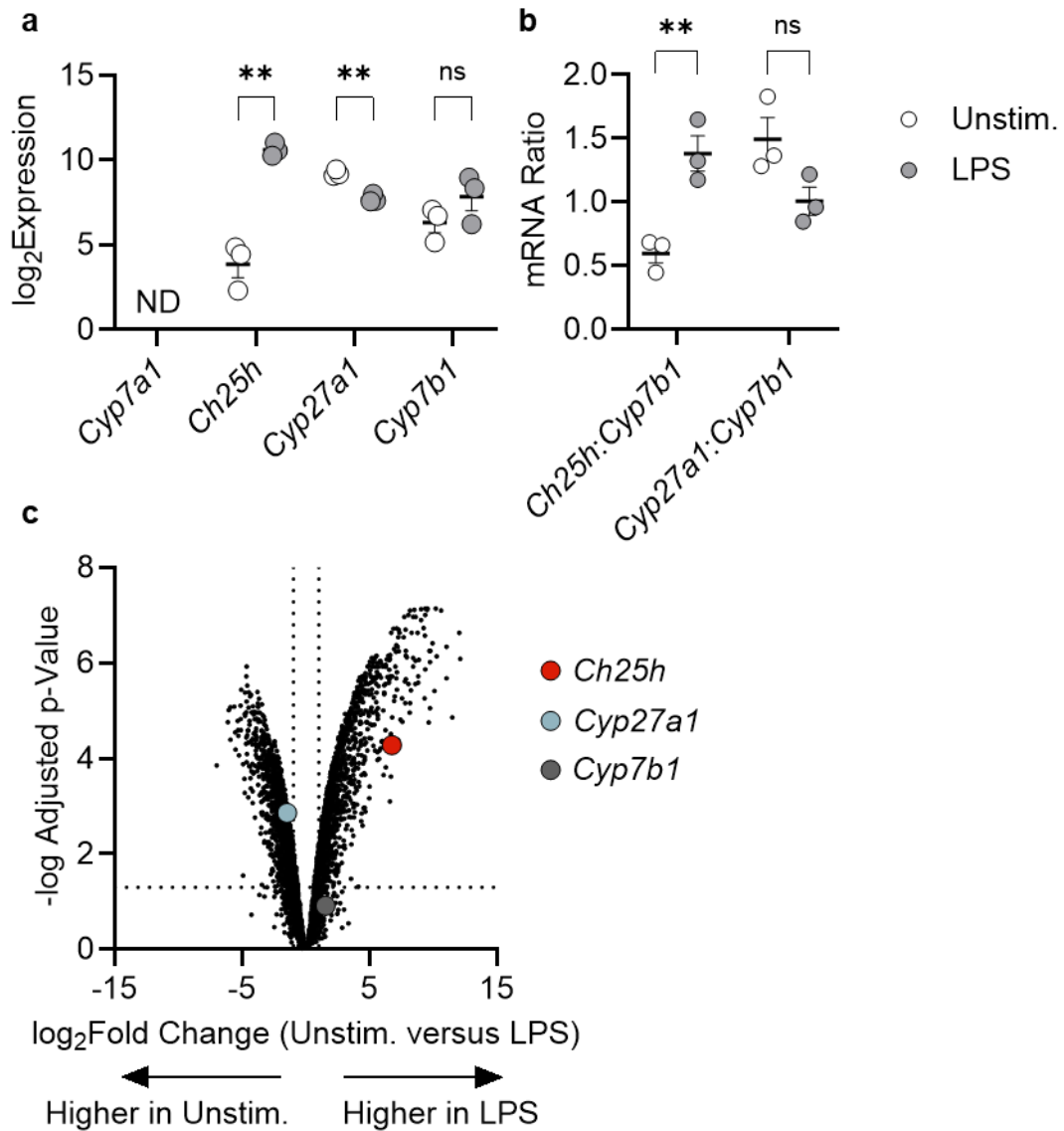


Figure 3.12 *Ch25h* expression by peritoneal macrophages increases following stimulation with LPS in vitro.

Peritoneal macrophages were left unstimulated or stimulated with LPS (10 ng/mL) for 24 h. mRNA abundance is shown as log₂expression (a), or log₂fold change with statistically significant genes having a –log adjusted p-value >1.3 (c). Ratios of *Ch25h* or *Cyp27a1* mRNA abundance versus *Cyp7b1* mRNA abundance are also shown (b). RNA-seq data was obtained from gene expression omnibus (GSE239696) and was reanalysed using three biological replicates. Data is mean +/- SEM of 3 biological replicates (a,b). Statistical analysis was performed using unpaired Student's t-test (**p<0.01, ns=non-significant).

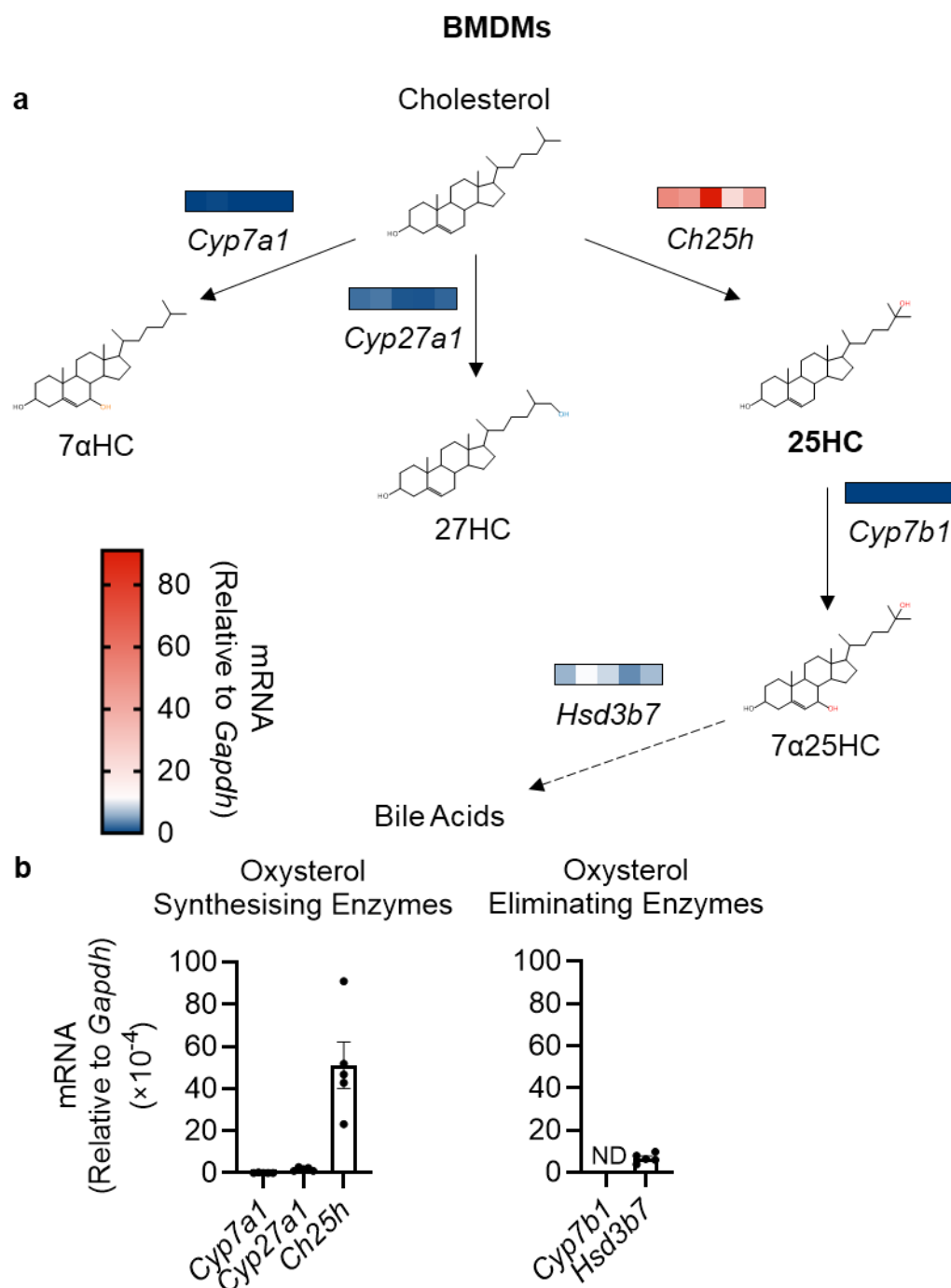


Figure 3.13 The expression pattern of oxysterol-synthesising and oxysterol-eliminating enzyme mRNA in **BMDMs** suggests that they can accumulate 25HC.

BMDMs were differentiated for 7 days before the cells were lysed and analysed by RT-qPCR for *Cyp7a1*, *Cyp27a1*, *Ch25h*, *Cyp7b1* and *Hsd3b7*. Data is normalised to levels of control gene *Gapdh*. Data is representative (a) or mean $\pm$ SEM of 5 biological replicates across 2 independent experiments (b). (ND=not detected)

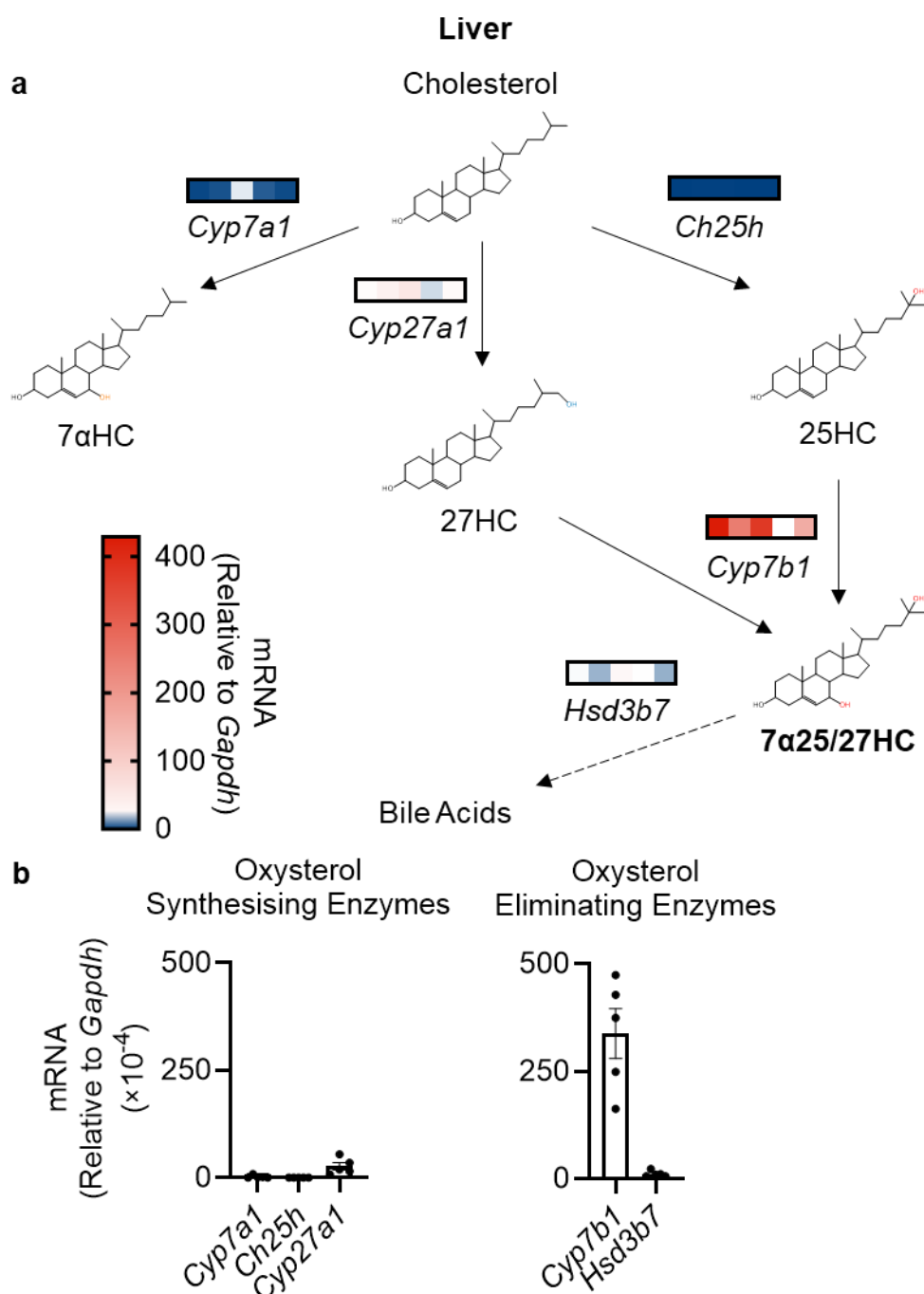


Figure 3.14 The expression pattern of oxysterol-synthesising and oxysterol-eliminating enzyme mRNA in liver suggests oxysterol clearance.

Liver mRNA was analysed by RT-qPCR for *Cyp7a1*, *Cyp27a1*, *Ch25h*, *Cyp7b1* and *Hsd3b7*. Data is normalised to levels of control gene *Gapdh*. Data is representative (a) or mean $\pm$ SEM of 5 biological replicates (b). Liver mRNA was provided by Dr Ciara Byrne, Fallon Laboratory, Trinity College Dublin.

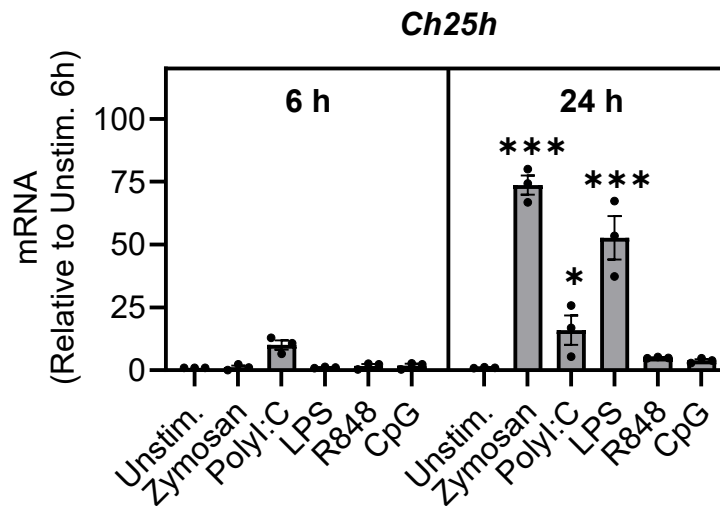


Figure 3.15 Stimulation of BMDMs with certain TLR agonists increases expression of *Ch25h* mRNA.

BMDMs were either left unstimulated or stimulated with zymosan (100 µg/mL), polyI:C (1 µg/mL), LPS (10 ng/mL) R848 (1 µg/mL) or CpG (4 µg/mL) for 6 h or 24 h before cells were lysed and analysed by RT-qPCR for *Ch25h*. Data is normalised to levels of control gene *Gapdh*. Data is mean $\pm$ SEM of 3 biological replicates across 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's post-test. Only statistically significant comparisons are included on the graph and are made against unstimulated BMDMs (* p <0.05, *** p <0.001).

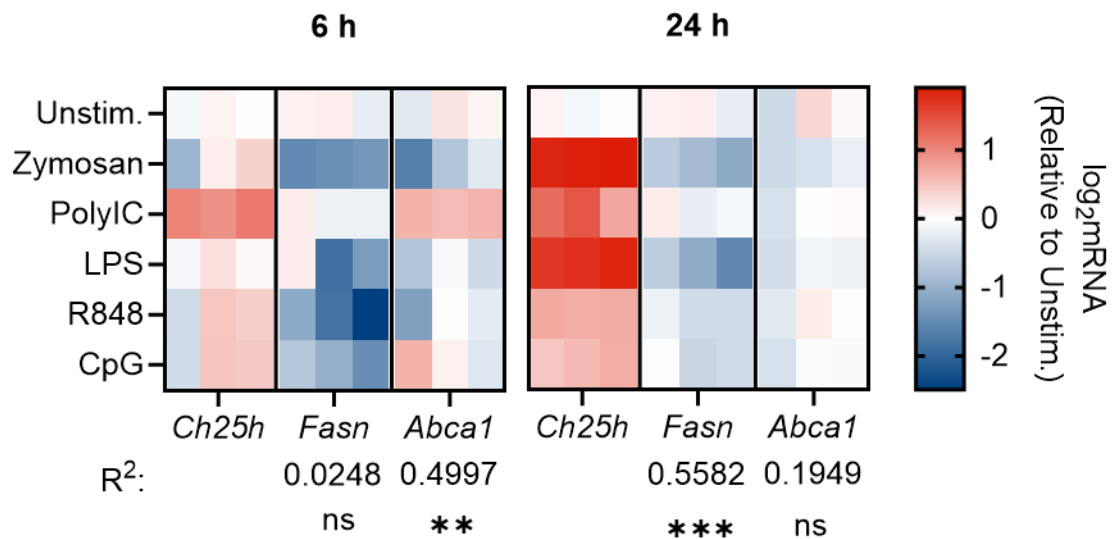


Figure 3.16 Expression of *Ch25h* mRNA weakly correlates with SREBP and LXR target gene expression in BMDMs stimulated with TLR agonists.

BMDMs were either left unstimulated or stimulated with zymosan (100 µg/mL), polyI:C (1 µg/mL), LPS (10 ng/mL) R848 (1 µg/mL) or CpG (4 µg/mL) for 6 h or 24 h before cells were lysed and analysed by RT-qPCR for *Ch25h*, *Fasn* and *Abca1*. Data is normalised to levels of control gene *Gapdh*. Data is representative of 3 biological replicates across 2 independent experiments. R^2 indicates the coefficient of determination from correlation analysis. Statistical analysis was performed on correlation coefficients using a t-test (**p<0.01, ***p<0.001, ns=non-significant).

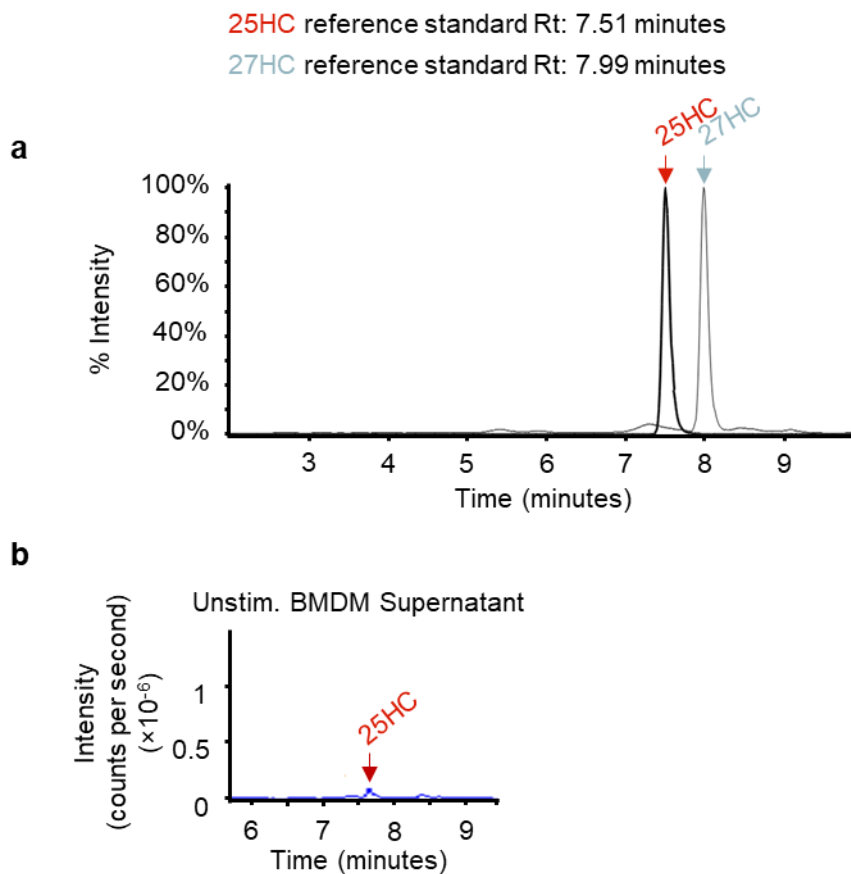


Figure 3.17 BMDMs produce 25HC but not 27HC.

BMDMs were cultured for 7 days before cell culture medium was harvested, and levels of secreted 25-hydroxycholesterol were quantified by LC/MS. Peak intensity was measured in counts per second (cps). Data shown is reference standards (a) or BMDM culture medium (b). Data is representative of three biological replicates. *This data was generated using samples provided by Cathal Keane to Dr Bernhard Drotleff, European Molecular Biology Laboratory, Heidelberg.*

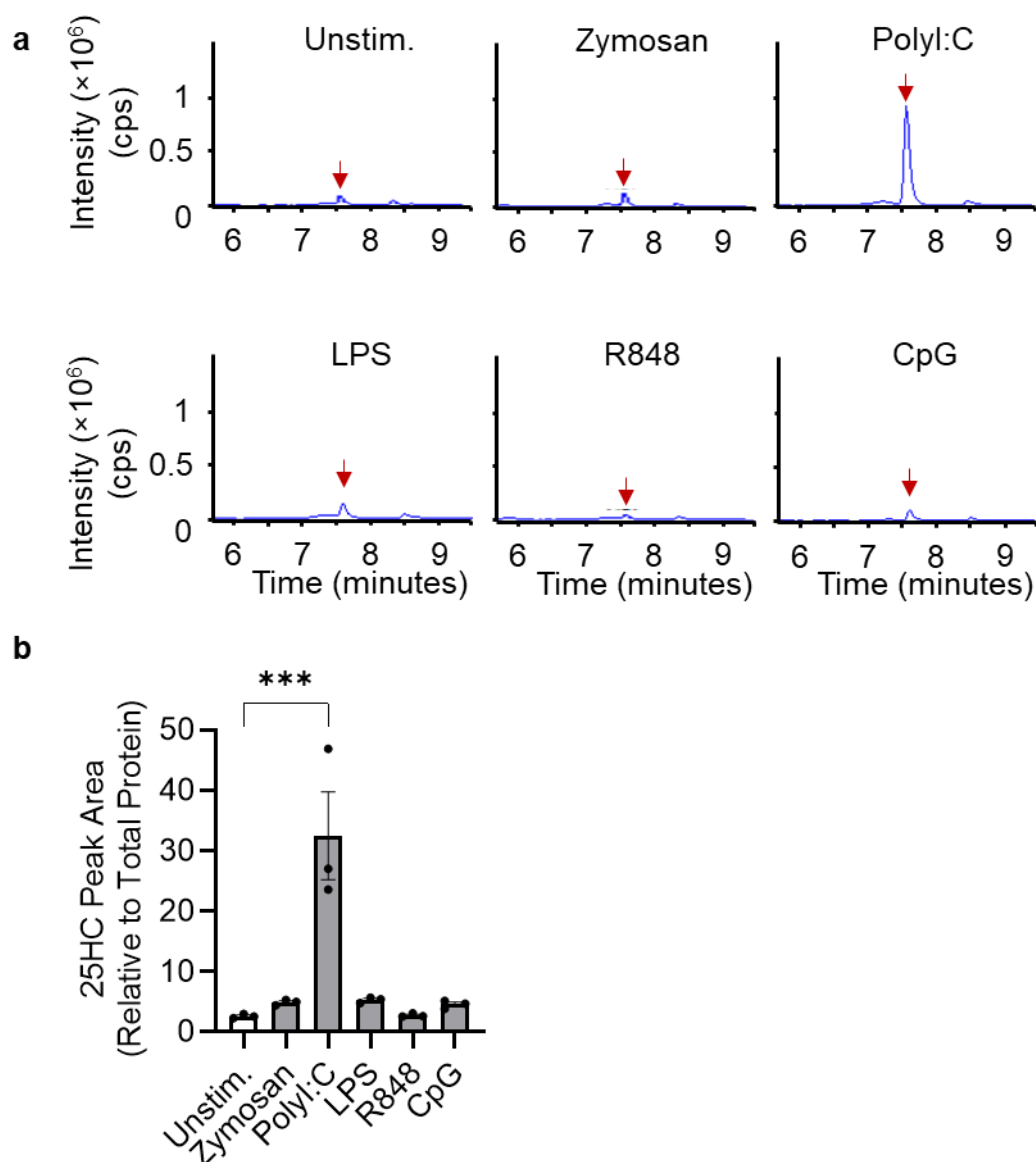


Figure 3.18 PolyI:C treatment increases 25HC production by BMDMs.

BMDMs were either left unstimulated or stimulated with zymosan (100 $\mu\text{g/mL}$), polyI:C (1 $\mu\text{g/mL}$), LPS (10 ng/mL) R848 (1 $\mu\text{g/mL}$) or CpG (4 $\mu\text{g/mL}$) for 24 h. Cell culture medium was harvested, and levels of secreted 25-hydroxycholesterol were quantified by LC/MS. Peak intensity was measured in counts per second (cps). Data is normalised to total protein measured using BCA assay. Data is representative (a) or mean $\pm$ SEM of three biological replicates (b). Statistical analysis was carried out using a one-way ANOVA with Tukey post-test (ns=non-significant, *** $p < 0.001$). Only statistically significant comparisons are included on the graph. *This data was generated using samples provided by Cathal Keane to Dr Bernhard Drotleff, European Molecular Biology Laboratory, Heidelberg.*

3.4 Macrophage-derived 25HC does not limit NK cell functional responses *in vitro*.

From the data shown so far, it could be inferred that the increased production of 25HC when macrophages are stimulated with polyI:C might create a microenvironment in which NK cells have impaired cytotoxicity and altered plasma membrane order. Because of this, co-cultures were performed with BMDMs and IL2-expanded NK (IL2-NK) cells. The IL2-expansion protocol involves culture of splenic NK cells in IL2 (100 ng/mL) for 6 days which increases the number of NK cells ~10-fold. These NK cells are also phenotypically different from NK cells *ex vivo* and IL15-expanded NK cells, as they receive activation signals during the culture. Stimulation of these NK cells with IL2 in combination with IL12 enhanced NK cell cytotoxicity, cytokine production and rates of metabolism. Importantly, these NK cells were also sensitive to the oxysterols 25HC and 27HC. Data not included in this chapter showed that 18 h exposure to these oxysterols reduced cytotoxicity against RMA/S target cells, impaired effector functions and decreased plasma membrane order. Together this shows that although IL2-expanded NK cells differ from the IL15-expansion model they are still a useful to investigate the effects of oxysterols on NK cell functions *in vitro*. Prior to co-culture, BMDMs were left unstimulated or stimulated with TLR agonists for 6 h to allow oxysterol accumulation before the addition of IL2-NK cells.

Although 25HC was shown to impair IFN γ production by previously stimulated NK cells (Figure 3.2), NK cells cultured with polyI:C-stimulated BMDMs did not show any significant decrease in IFN γ expression (Figure 3.19). The NK cells that showed significant decreases in IFN γ expression were those co-cultured with zymosan-, LPS- and R848-stimulated BMDMs. These particular TLR-stimulations were not associated with significant 25HC production (Figure 3.18). Furthermore, changes in expression of perforin and granzyme B did not correlate with the amount of 25HC in BMDM culture medium (Figure 3.20). Perforin expression did not change in any co-culture condition. Granzyme B expression was significantly decreased in the presence of zymosan-stimulated BMDMs ($p=0.02$) and R848-stimulated BMDMs ($p=0.005$). NK cell cytotoxicity against RMA/S target cells was also assessed in each of these conditions. NK cells were

taken from the BMDM co-cultures to prevent MHC class I recognition by the NK cells and co-cultured with tumour target cells. These killing assays revealed no correlation between 25HC production and levels of NK cell killing (Figure 3.21). In fact, when NK cells were co-cultured with polyI:C- and R848-stimulated BMDMs there was a significant increase in their cytotoxicity (Figure 3.21).

Taken together this data does not support the hypothesis that macrophage-derived 25HC can impair NK cell effector functions in the context of *in vitro* co-culture. Nevertheless, this chapter has revealed a novel 25HC-dependent effect on NK cell plasma membrane order. NK cells were taken from co-culture and plasma membrane order was assessed by flow cytometry. Following co-culture with zymosan-treated BMDMs NK cells showed a significant increase in plasma membrane disorder; however, the histograms revealed several NK cell populations emerging depending on the rGP of Di-4-ANEPPDHQ which is difficult to interpret (Figure 3.22). The only other condition that showed changes in NK cell plasma membrane order was following co-culture with polyI:C-stimulated BMDMs. In this condition there was a uniform, significant increase in plasma membrane disorder (Figure 3.22), mirroring the effect of direct 25HC-treatment.

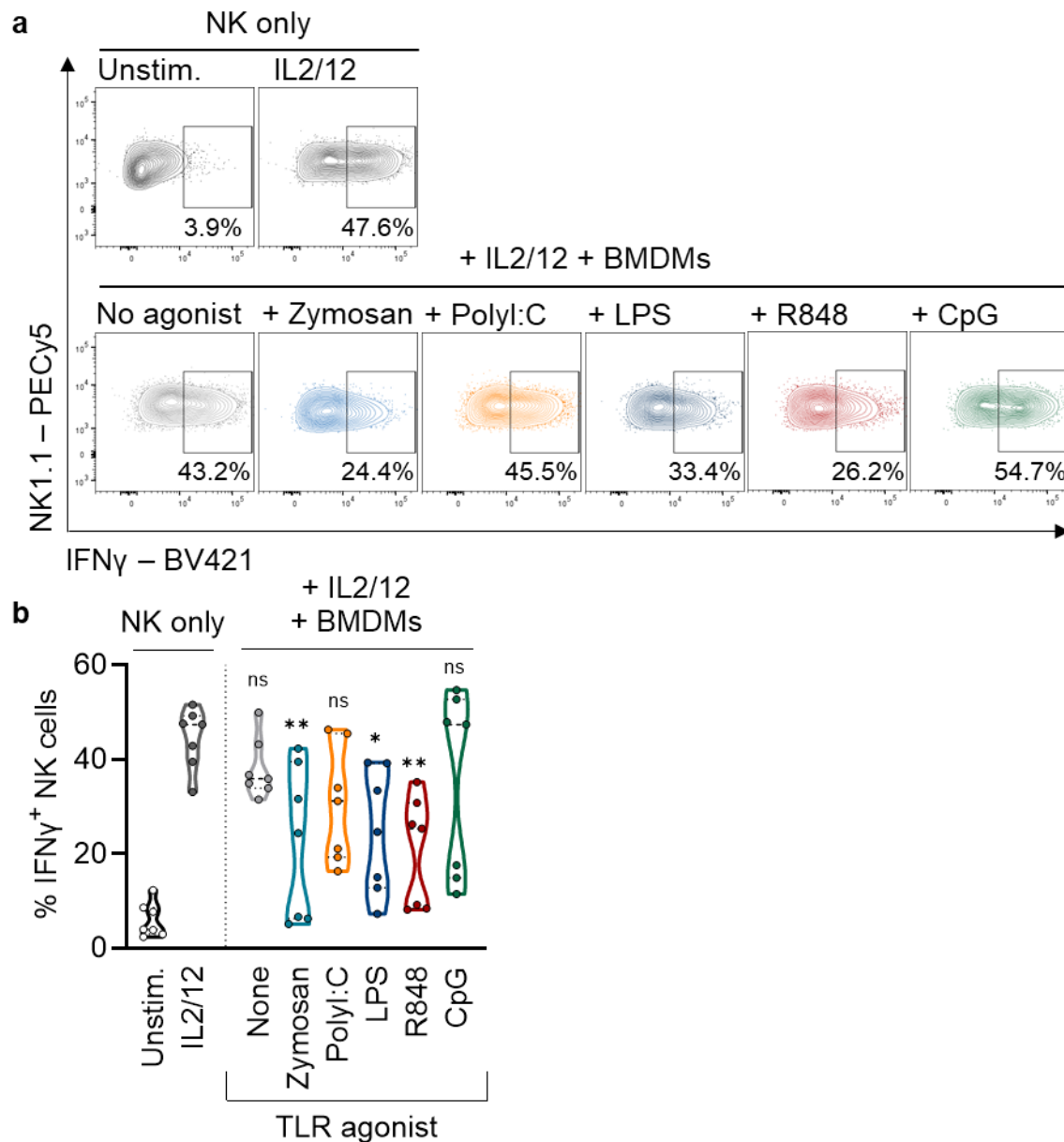


Figure 3.19 The presence of BMDMs stimulated with certain TLR agonists impairs NK cell IFN γ expression.

IL2-expanded NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and were co-cultured with BMDMs that were left unstimulated or pre-stimulated for 6 h with zymosan (100 μ g/mL), polyI:C (10 μ g/mL), LPS (10 ng/mL), R848 (1 μ g/mL) or CpG (4 μ g/mL). IFN γ expression was measured by flow cytometry. Data is representative (a) or pooled from 7 biological replicates across 3 independent experiments (b). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. All comparisons are made versus IL2/12-stimulated NK cells only (ns=non-significant, * p <0.05, ** p <0.01).

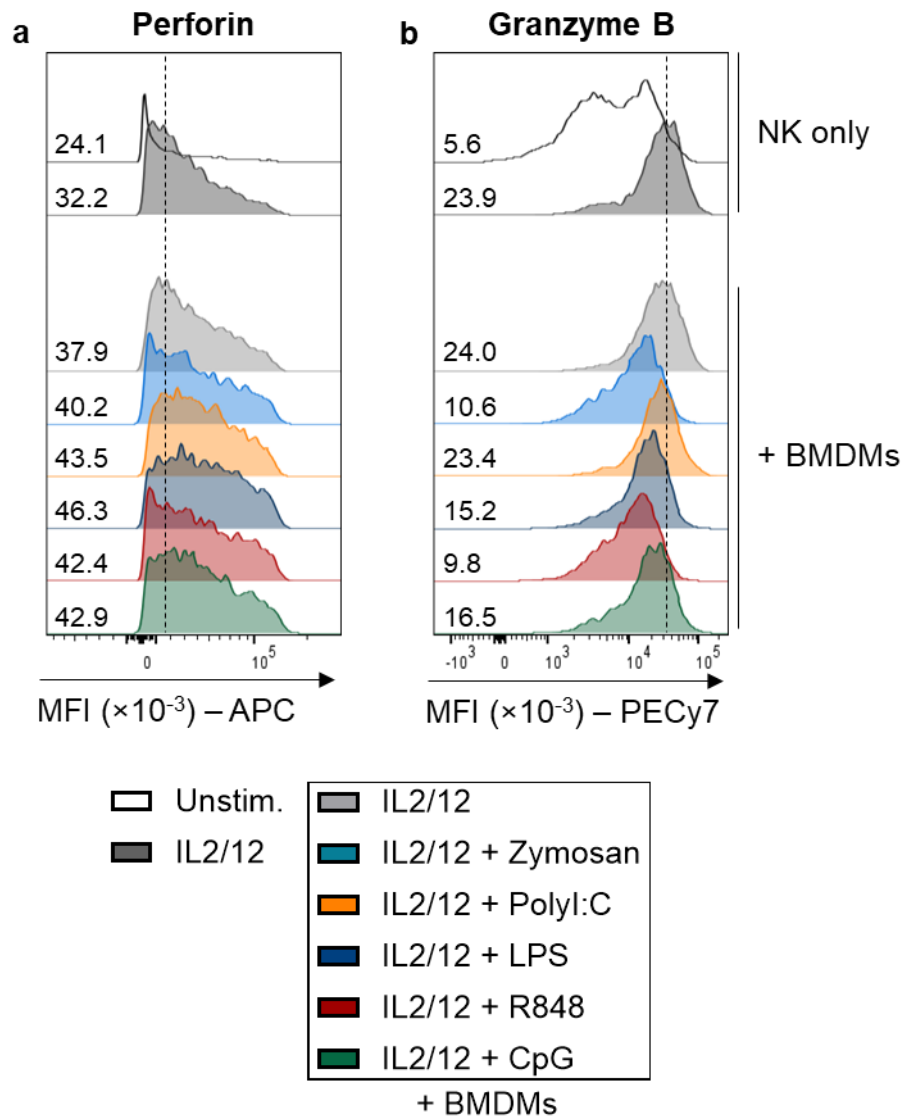


Figure 3.20 The presence of BMDMs does not cause loss of cytotoxic molecule expression by NK cells.

IL2-expanded NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and were co-cultured with BMDMs that were left unstimulated or pre-stimulated for 6 h with zymosan (100 μ g/mL), polyI:C (10 μ g/mL), LPS (10 ng/mL), R848 (1 μ g/mL) or CpG (4 μ g/mL). Perforin (a) and granzyme B (b) expression were measured by flow cytometry. Data is representative of 7 biological replicates across 3 independent experiments.

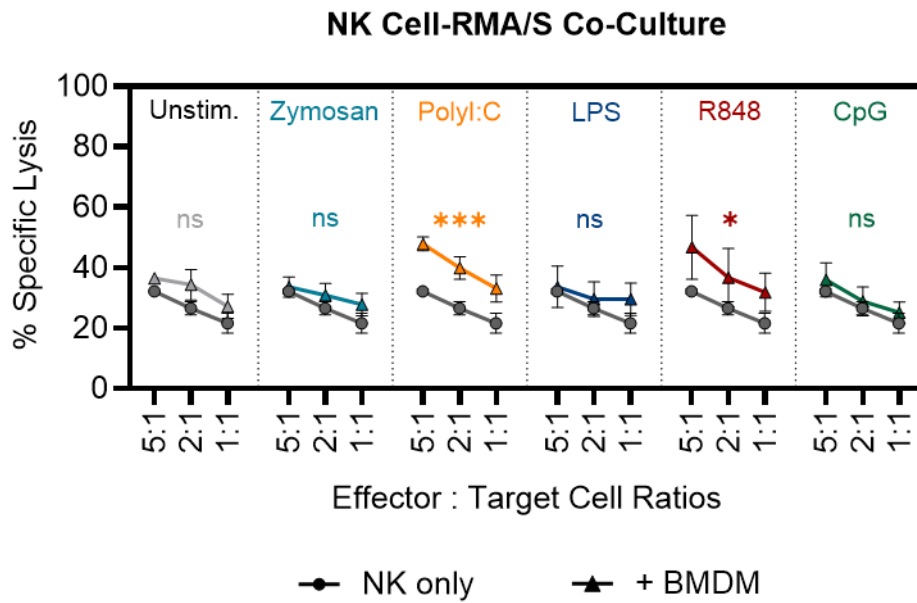


Figure 3.21 The presence of BMDMs stimulated with certain TLR agonists enhances NK cell killing of RMA/S target cells.

IL2-expanded NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and were co-cultured with BMDMs that were left unstimulated or pre-stimulated for 6 h with zymosan (100 µg/mL), polyI:C (10 µg/mL), LPS (10 ng/mL), R848 (1 µg/mL) or CpG (4 µg/mL). Cells were co-cultured for 18 h before NK cell cytotoxicity against RMA/S target cells was determined by fluorescence release assay. Data is mean $\pm$ SEM of 4 biological replicates across 2 independent experiments. Statistical analysis was performed using a two-way ANOVA (ns=non-significant, * p <0.05, *** p <0.001).

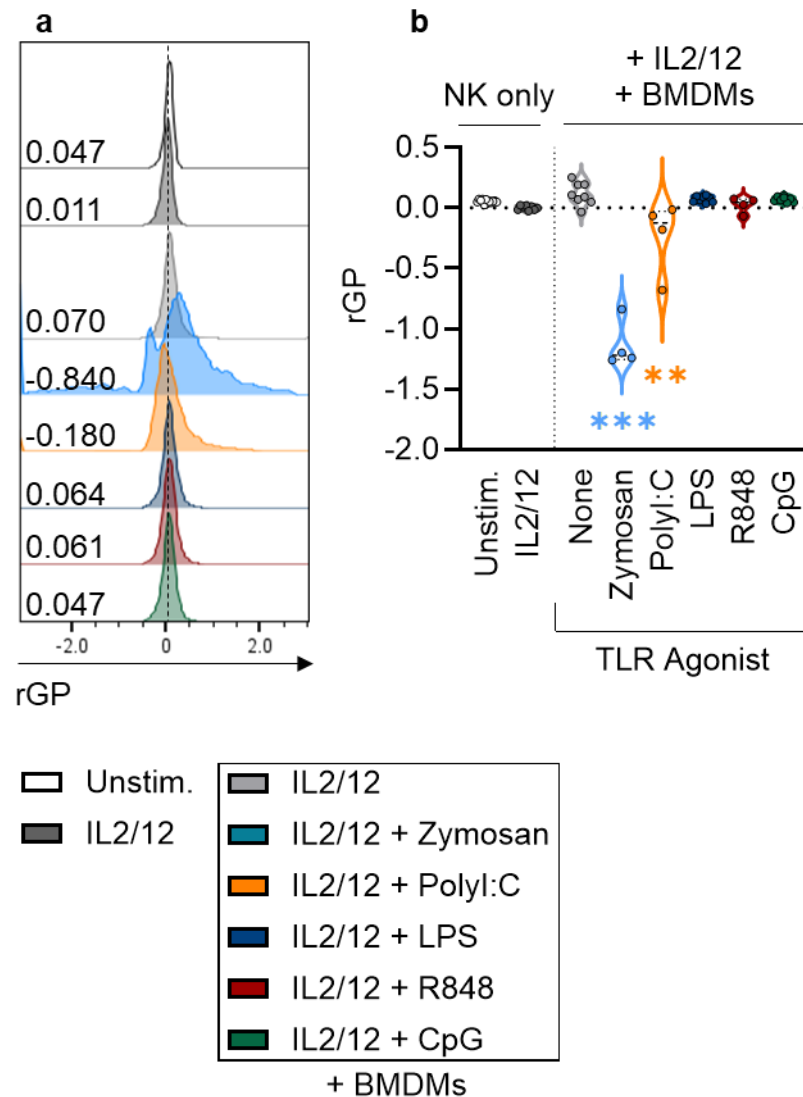


Figure 3.22 The presence of BMDMs stimulated with certain TLR agonists increases plasma membrane disorder in NK cells.

IL2-expanded NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and were co-cultured with BMDMs that were left unstimulated or pre-stimulated for 6 h with zymosan (100 µg/mL), polyI:C (10 µg/mL), LPS (10 ng/mL), R848 (1 µg/mL) or CpG (4 µg/mL). Ratiometric general polarisation of Di-4-ANEPPDHQ was used as an indicator of plasma membrane order and was measured by flow cytometry. Data is representative (a) or pooled from at least 4 biological replicates across 3 independent experiments (b). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. Only statistically significant comparisons are included on the graph. All comparisons are versus IL2/12-stimulated NK cells only (**p<0.01, ***p<0.01).

3.5 Discussion

Plasma membrane fluidity is an essential feature of cellular membranes, but fluidity must be regulated to maintain the barrier features of the membrane. Cholesterol is the most abundant single lipid species in plasma membranes accounting for 40% of lipids in the plasma membrane of erythrocytes (Lorent *et al.*, 2020). Its insertion into the plasma membrane maintains an ordered and fluid membrane through preventing conformational freedom of the adjacent phospholipids (McIntosh, 1978; Byfield *et al.*, 2004). This has been shown to thicken the membrane and increase its barrier function (Hung *et al.*, 2007; Frallicciardi *et al.*, 2022). However, not all sterol species have the same effects on the membrane. For example, cholesterol is more effective at thickening the plasma membrane than ergosterol (Urbina *et al.*, 1995; Hung *et al.*, 2016). In fact, liposomes treated with ergosterol showed increased permeability to small molecules in comparison to cholesterol-treated liposomes (Frallicciardi *et al.*, 2022). Cholesterol-sphingomyelin interactions are another common feature of plasma membranes, that are required for the formation of lipid rafts. These rafts are essential for the correct insertion of certain proteins into the membrane which can control receptor ligand interactions and downstream signalling (Codini *et al.*, 2021).

With respect to 25HC and 27HC the addition of the hydroxyl group on carbon-25 and -27 respectively, means that the normal insertion of the sterol backbone into the phospholipid bilayer would require large amounts of energy. Experimental studies and computer aided simulations have suggested that 25HC and 27HC can be solubilised in membranes, though the orientation that they insert into the membrane would have effects on membrane thickness, order and permeability (Holmes and Yoss, 1984; Kauffman *et al.*, 2000; Olsen *et al.*, 2009). In the case of 25HC it has been suggested that its insertion into the membrane would expand and thin the plasma membrane, likely causing functional consequences. In support of this, 25HC has been described to increase membrane permeability to small molecules in studies with liposomes (Holmes and Yoss, 1984; Theunissen *et al.*, 1986). This direct effect of oxysterols on plasma membrane structure agrees with data presented in this chapter. If 25HC and 27HC were directly inserting into the plasma membrane the expected result would be increases in

the plasma membrane disorder. Furthermore, the roundness of the cells with low membrane order could be attributed to increased permeability to small molecules, causing the cells to become turbid.

It should also be considered that oxysterols could alter plasma membrane structure through other indirect mechanisms. For example, changes in expression of genes involved in lipid synthesis or plasma membrane maintenance could have downstream effects on plasma membrane order. In macrophages loss of FASN was shown to decrease plasma membrane order through altering cellular phospholipid composition (Wei *et al.*, 2016). Therefore, oxysterol-mediated inhibition of SREBP activation could induce changes to plasma membrane order, through inhibiting expression of proteins such as FASN. Such changes could not happen within 4 h exposure to oxysterols, when changes to membrane order were first observed, but might contribute to the more substantial changes seen after 18 h exposure to oxysterols. Many lipid species act as co-factors for enzymes. In the case of 25HC, it is a known co-factor for ACAT2, a cholesterol esterifying enzyme which can remove cholesterol from the membrane for incorporation into lipid droplets or LDLs (Chang *et al.*, 2009; Yang *et al.*, 2012; Xu *et al.*, 2019; Gao *et al.*, 2019). In macrophages it has been shown that the increase in CH25H and subsequent production of 25HC following IFN γ stimulation resulted in esterification and removal of accessible cholesterol from the plasma membrane through the activity of ACAT2 (Abrams *et al.*, 2020). CH25H null macrophages also showed increased susceptibility to infection by the intracellular bacteria *L. monocytogenes* and *S. flexneri* through cell-cell transmission (Abrams *et al.*, 2020). Similar 25HC-dependent protection was seen for viral transmission. 25HC treatment was effective at protecting Huh7.5 cells from infection with Zika virus and human Coronavirus (Heisler *et al.*, 2023). Although the exact mechanism for this effect is not known, it is possible that 25HC-dependent removal of cholesterol from the plasma membrane could be reducing the fluidity of the plasma membrane and interrupting the formation of the protrusions required for pathogen entry. Lipids have also been described to alter plasma membrane structure through post-translational modification of membrane-associated proteins. Palmitoylation and *N*-myristylation of proteins can affect their interactions with the plasma membrane (Resh, 2013). Oxysterol

post-translational modifications of proteins have never been described, though it is possible that levels of these post-translational modifications could be altered through inhibition of SREBP activation at later time points.

Plasma membrane homeostasis is known to be essential for NK cell functions, particularly their migration and cytotoxicity. One striking effect that can be observed in images of NK cells treated with oxysterols for 18 h was the cells with decreased plasma membrane order being more round in shape. Although this could be due to increased permeability to small molecules inducing turbidity, it could also indicate disruptions to the cytoskeleton. Cytoskeletal organisation is highly dependent on correct interactions with the plasma membrane through proteins such as spectrin and ankyrin (Di Giaimo *et al.*, 2020). Disruptions to cytoskeletal organisation are inhibitory to lymphocyte functions. For example, spectrin has been shown to be essential for CD45 and CD3 expression by T cells (Pradhan and Morrow, 2002). It has also been shown to support interactions with APCs and the correct formation of immunological synapses (Meissner *et al.*, 2017). NK cells are constantly moving and interacting with their environment in order to remove transformed, virally infected or senescent cells. Consequently, NK cell polarisation of the actin cytoskeleton is essential to facilitate this movement (Carpén *et al.*, 1983; Vicente-Manzanares and Sánchez-Madrid, 2004). Taken together, this may explain why the NK cells with lower plasma membrane order are so round and circular. NK cell movement in the presence of oxysterols is significantly reduced following exposure to oxysterols for 4 h with a more pronounced effect following 18 h exposure (Choi, 2023). This effect could be due to impaired NK cell metabolism in the presence of oxysterols; however, data here suggests that changes in the plasma membrane could be interfering with the correct organisation of the cytoskeleton, which is essential for movement. This effect could potentially be impairing NK cell killing due to reduced frequency of target cell recognition.

Aside from NK cell movement, many other NK cell functions are dependent on an organised cytoskeleton. Many NK cell receptors require the recruitment of adaptor proteins via the cytoskeleton and in turn require cytoskeletal rearrangement in order to activate downstream mitogen-activated protein kinases (MAPKs) (Watzl and Long, 2010). Furthermore, polarisation of the MTOC

is essential to facilitate NK cell degranulation and target cell lysis (Prager and Watzl, 2019; Gwalani and Orange, 2018). This suggests that oxysterol-mediated cytoskeletal disruption could be preventing receptor mediated recognition of target cells and subsequent activation of NK cells, as well as preventing degranulation into the immunological synapse.

Aside from the effects that can be caused by global changes in plasma membrane order, discrete changes to the plasma membrane structure also occur during degranulation. Following the release of perforin and granzymes into the immunological synapse, NK cells need to protect themselves from perforin-mediated autolysis. Once released from the environment of cytotoxic granules, perforin will create pores in any membrane that it encounters (Fraser *et al.*, 2000). Consequently, NK cells increase plasma membrane order at the immunological synapse to make the membrane thicker and more rigid (Li and Orange, 2021; Andronico *et al.*, 2024). Disruptions to plasma membrane order induced by 7KC led to increased NK cell death following exposure to recombinant perforin *in vitro* (Li and Orange, 2021), demonstrating the importance of plasma membrane homeostasis to protect NK cells from perforin-mediated autolysis. This suggests that the defect in killing observed in oxysterol-treated NK cells could be attributed to increased death following initial degranulation events, preventing serial killing by NK cells in cytotoxicity assays.

4 h exposure of NK cells to oxysterols was sufficient time to impair NK cell killing of target cells, despite no other impairments detected in NK cell functional parameters. In order to understand whether oxysterol-mediated disruptions to plasma membrane order alone are sufficient to impair NK cell killing 7KC was also used as a control in this chapter. Because of this oxysterol species' weak binding to both SCAP and INSIG (Radhakrishnan *et al.*, 2007), it is a weak inhibitor of SREBP activation and is unlikely to cause an energy crisis in NK cells. The fact that 7KC induced a dose-dependent decrease in NK cell cytotoxicity supports this hypothesis. However, this oxysterol's potent ability to alter membrane structure likely has many other effects in the cell. There is evidence that 7KC can insert into lysosomal membranes (Li *et al.*, 2011). This has the potential to cause a number of downstream effects such as impaired mitophagy, ER stress and ROS accumulation, all of which may potentially impair NK cell

functions. However, at lower doses there were no significant changes to NK cell viability or metabolism, indicating that the NK cells are not under any 7KC-induced stress at these concentrations. Nonetheless the most striking effect induced by this oxysterol was inducing plasma membrane disorder, visualised by confocal microscopy. These images also show very striking changes to cellular morphology, supporting the hypothesis that alterations to plasma membrane order may be sufficient to impair NK cell cytotoxicity.

Having investigated the direct effects of oxysterols on NK cell functions, this chapter also investigated whether oxysterol-producing cells could use these metabolites as paracrine signalling molecules to dampen NK cell responses. Many metabolites have been described to act in a paracrine manner to alter the function of cells in the local environment. For example, extracellular ATP has been described to act as a danger signal for phagocytes to move to damaged tissue (Iyer *et al.*, 2009). Moreover, myeloid-derived itaconate has been described to act on respiratory epithelial cells to promote mucociliary clearance in viral infections (Zeng *et al.*, 2023). Whether oxysterols act as immunosuppressive metabolites in discrete environments *in vivo* is not yet known. 27HC in particular has been associated with many cancers. It is known to be a SERM in ER⁺ breast cancer, promoting tumour growth through autocrine signalling (Nelson *et al.*, 2013; Wu *et al.*, 2013). NK cells are also known to be dysfunctional in breast cancer patients (Slattery *et al.*, 2021), and it is possible that 27HC is a contributor to this dysfunction. Macrophages are producers of 25HC, and have been shown to secrete 25HC into the extracellular environment (Bauman *et al.*, 2009; Diczfalusy *et al.*, 2009; Blanc *et al.*, 2013; Ngo *et al.*, 2022). Communication between immune cells via soluble factors is canonically described in terms of cytokines and chemokines, but metabolites may be playing an important role in this process. Although there are a number of studies that have described the role of endogenous 25HC production by macrophages (Gold *et al.*, 2014; Abrams *et al.*, 2020; Canfrán-Duque *et al.*, 2023), the role of secreted 25HC is not as well understood. Because many oxysterols are membrane permeable, the release of 25HC from macrophages may be happening in a passive uncontrolled manner leading to potential consequences for neighbouring cells.

One potential role of secreted 25HC could involve co-operative cholesterol metabolism by multiple cell types. Although the pattern of expression of oxysterol-metabolising enzymes in BMDMs and peritoneal macrophages in this chapter did not suggest cholesterol clearance, it is possible that other cells could be expressing CYP7B1. The product of this reaction is 7 α -25HC, a chemokine which binds to G protein-coupled receptor (GPR) 183, causing cells that express this receptor to migrate into 7 α -25HC-rich environments. An example of this occurs in the spleen, where CYP27A1-expressing type I conventional dendritic cells (cDC1) in the marginal zone produce 27HC which is metabolised by T cells, inducing migration of GPR183-expressing cDC1 into the T cell zone where antigen cross-presentation can occur. This co-operative metabolism of oxysterols by cDC1 and CD8⁺ T cells within splenic architecture effectively promotes T cell activation. Disruption of this communication axis resulted in increased viral loads in the liver and spleen of mice infected with MCMV (Ghilas *et al.*, 2021). Human blood NK cells have also been shown to express GPR183 (Rosenkilde *et al.*, 2006), suggesting that they can respond to this oxysterol gradient in certain environments.

Another potential role of secreted oxysterols can be seen in the tumour microenvironment. TAMs are known to express CH25H and produce 25HC to much higher levels than any other immune cell in MC38 tumours (Xiao *et al.*, 2024). Levels of CH25H expressing TAMs were negatively correlated with patient survival in a number of different tumours including pancreatic ductal carcinoma, colon carcinoma and uterine corpus endometrial carcinoma (Xiao *et al.*, 2024). This study also revealed that in MC38 tumours given to CH25H-null mice, there was increased infiltration of CD8⁺ T cells, and these T cells had increased IFN γ and TNF α production in comparison to tumours given to wild-type mice. The same effects were shown in B16F10 tumour models, widely considered a cold tumour model, indicating that oxysterols levels in tumours can have significant impacts on TILs (Xiao *et al.*, 2024). Notably, the immunosuppressive effects of 25HC in this study were not linked with direct effects on T cells, rather the endogenous 25HC production by TAMs was associated with enhanced arginase (ARG) 1 expression. Arginine depletion by tumour cells has been shown to limit T cell proliferation and anti-tumour responses (Fletcher *et al.*, 2015; Geiger *et al.*,

2016; Vonwirth *et al.*, 2021). It has also been shown that tumour-derived 27HC drove metastasis in a murine breast cancer model through enhancing the frequency of neutrophils, rather than direct effects on CD8⁺ T cells. The exact mechanism for the reduction in CD8⁺ T cell frequency is not fully understood (Baek *et al.*, 2017). One potential cause for the reduction in CD8⁺ T cell numbers is oxysterol-mediated cholesterol depletion. 27HC production by tumour cells was shown to activate LXR signalling in intratumoral CD8⁺ T cells resulting in depletion of cholesterol from the plasma membrane. This lack of cholesterol in CD8⁺ T cells was shown to reduce proliferation and survival, and could be rescued through deletion of LXR β (Yan *et al.*, 2023). Both 25HC and 27HC have been shown to have strong affinity for LXR, suggesting that 25HC in the TME may be able to elicit similar effects (Radhakrishnan *et al.*, 2007). Most studies investigating the role of oxysterols in tumours have focused on understanding their endogenous role in the oxysterol-producing cells. However, the results in this chapter highlight that in discrete microenvironments, such as in the TME, secreted oxysterols may be eliciting immunosuppressive effects on NK cells, leading to poor outcomes in certain cancers.

Before investigating whether secreted 25HC, produced by macrophages could be altering NK cells response *in vitro*, the production of 25HC by BMDMs stimulated with various TLR agonists was investigated. Pro-inflammatory macrophages were chosen for this initial investigation as they have been described to express high levels of *Ch25h* mRNA (Diczfalusy *et al.*, 2009; Gold *et al.*, 2012; Dang *et al.*, 2017; Abrams *et al.*, 2020). Demonstration of this effect *in vitro* through the generation of TAMs from BMDM cultures, was also considered. This was not feasible due to the complex growth medium required to effectively turn a BMDM into a TAM-like cell *in vitro*. The initial assessment of expression of oxysterol-synthesising and oxysterol-eliminating gene mRNA supported that BMDMs *in vitro* were accumulating oxysterols. However, the data showing that certain TLR agonists could induce expression of *Ch25h* mRNA whereas others did not was surprising. CH25H has been described to be associated with type I interferon signalling following TLR stimulation. Supporting evidence for this comes from IFNAR-null mice being unable to increase expression of *Ch25h* mRNA (Liu *et al.*, 2013), and the requirement for STAT1-

binding to *Ch25h* promoter being required for gene expression (Blanc *et al.*, 2013). Among the TLR agonists used in this study, all have been described to induce a type I interferon response in BMDMs. TLR4 signalling through TRIF is a potent inducer of type I interferons. Recognition of RNA by the endosomal TLRs: TLR3, TLR7, TLR8 and TLR9 is also associated with a strong type I interferon response (McNab *et al.*, 2015). Although type I interferon production by macrophages was not measured, expression of the cytokines IL6 and TNF α were comparable across all stimulations given. Furthermore, expression of co-stimulation molecules: CD40, CD80 and CD86 were also comparable, suggesting that the concentrations of each TLR agonist given were not limiting the expression of CH25H. The expression of the different TLRs at protein level by BMDMs also did not correlate with levels of *Ch25h* mRNA (Brenes *et al.*, 2023), suggesting that the expression is linked with the strength of the type I interferon response.

Initial supporting evidence for the production of 25HC by macrophages in this study came from correlation of *Ch25h* mRNA expression with expression of SREBP- and LXR-target gene mRNA. Expression of these genes was initially used as a surrogate measure of 25HC levels because much of the 25HC produced by BMDMs is maintained within the cells (Diczfalusy *et al.*, 2009). These weak correlations did suggest that increases in *Ch25h* were associated with enhanced *Abca1* mRNA expression and decreased *Fasn* expression, though this did not correlate with direct measurements of 25HC by LC/MS. This highlights that the use of mRNA to assess cellular metabolism should be considered with caution. The levels of 25HC in the cell culture medium measured in this chapter were compared to what was known in literature to validate that the measurements were accurate. It is known that treatment of BMDMs with the same dose of LPS used in this study was sufficient to double the levels of 25HC in the cell culture medium (Diczfalusy *et al.*, 2009), which agrees with the results presented here. However, the strongest induction of 25HC production was seen following stimulation of BMDMs with polyI:C. In this study, polyI:C was used to target TLR3, but it is also known that this synthetic dsRNA molecule can also bind to the RIG-I-like receptors (RLRs): RIG-I and MDA5 which induce a strong type I interferon response in BMDMs (Dutta *et al.*, 2017). It is possible that the

parallel TLR and RLR signalling, induced by polyI:C, may have synergistic effects promoting type I interferon production leading to enhanced 25HC synthesis by macrophages. The ability of polyI:C to bind RLRs is not shared with CpG or R848 (Hemmi *et al.*, 2000; Hemmi *et al.*, 2002), which may explain why these TLR agonists were not sufficient to promote equivalent levels of 25HC synthesis.

25HC and 27HC were the two oxysterols measured in this analysis because of the complexity in resolving many oxysterol species simultaneously before detection by mass spectrometry. Furthermore, evidence from previous studies and measurement of oxysterol-synthesising and oxysterol-eliminating gene expression suggested that these were the only candidate oxysterol species present in BMDM culture medium. CYP46A1 is another oxysterol-synthesising protein that produces 24HC, an oxysterol mainly associated with cholesterol clearance from neurons in the brain (Lund *et al.*, 1999). The only immune cells described to express this enzyme are microglia, so it was not investigated in this study (The Immunological Genome Project Consortium *et al.*, 2008). Furthermore, there was no detected expression of *Cyp7b1* in BMDMs, suggesting that 7 α -25HC or 7 α -27HC would not be present in the BMDM culture medium. The low expression of *Cyp7a1* mRNA also suggests that 7 α HC would be present in very low amounts. Furthermore, this oxysterol is not described to have strong SCAP- or INSIG-binding, indicating it would not have implications for NK cell activation (Radhakrishnan *et al.*, 2007). 27HC was not detected at all in the cell culture medium, which leaves 25HC as the only side chain oxysterol species produced by BMDMs, in agreement with previous studies (Blanc *et al.*, 2013). These experiments informed subsequent co-cultures which investigated whether macrophage-derived 25HC could have implications for NK cell activation *in vitro*.

Experiments from this chapter show that in the presence of oxysterols, NK cells have reduced cytotoxicity, reduced expression of IFN γ and perforin, and have alterations to the plasma membrane. The stimulation of BMDMs with all TLR agonists and subsequent co-culture with NK cells was carried out to assess whether there was a correlation between levels of 25HC in the culture medium and NK cell dysfunction. Surprisingly, despite polyI:C-stimulated BMDMs having the highest levels of oxysterol production, NK cells had no defect in effector

molecule expression and exhibited enhanced killing of target cells. Evaluation of BMDM activation with all of the TLR agonists used in this study showed comparable levels of activation when assessing expression of TNF α and IL6, however, it is possible that polyI:C-stimulated BMDMs have an entirely different cytokine expression profile because of their ability to activate both TLR and RLR signalling. The increase in type I interferons associated with polyI:C activation can also activate NK cells and work synergistically with other cytokines to enhance cytotoxicity. The addition of the exogenous cytokines IL2 and IL12 may have additive effects with macrophage-derived IFN α and IFN β , to increase NK cell killing specifically in these co-cultures. In fact, human NK cells have been shown to have enhanced IFN γ production when exposed to both IL12 and IFN β (Duluc *et al.*, 2009). IFNAR-null NK cells also showed impaired killing of RMA/S target cells *in vitro*, and mice with specific deletion of IFNAR in NK cells showed enhanced tumour burden in the lung following injection of B16F10 melanoma cells (Mizutani *et al.*, 2012).

The plasma membrane order of NK cells in co-culture with macrophages was also assessed to determine whether 25HC, derived from polyI:C-stimulated BMDMs could be impacting NK cell polarisation or plasma membrane ruffling. The only BMDM stimulations that caused changes to plasma membrane order were zymosan and polyI:C, with zymosan inducing opposing changes to the plasma membrane with some NK cells increasing order and others increasing disorder. The reasons for this are not understood, though it has been suggested that zymosan can directly target plasma membranes and prevent transfer of many small molecules across the membrane (Derbocio *et al.*, 2005). However, polyI:C-stimulated BMDMs did cause the NK cell population to skew towards increased plasma membrane disorder which could be attributed to 25HC accumulation. The fact that the plasma membrane disorder was observed in combination with an increase in cytotoxicity in these co-cultures highlights that strong disruptions to the plasma membrane structure may be required to impair NK cell anti-tumour responses, such as the dramatic changes seen when NK cells are treated with 7KC. Furthermore, it also highlights that oxysterol-mediated inhibition of NK cells responses are likely due to multiple effects occurring simultaneously to dampen cytotoxicity. It is also possible that in this simplified co-

culture, the activation of BMDMs will lead to enhanced production of both positive and negative signals for NK cells activation. In the case of polyI:C-stimulated BMDMs, the balance of these signals may favour NK cell activation. However, *in vivo* there are much more complex signalling pathways that exist to shape the immune response with the spectrum of macrophage activation being much more complex than observed *in vitro* (Strizova *et al.*, 2023). Therefore, using pro-inflammatory BMDMs stimulated with TLR agonists *in vitro* to assess the effect of macrophage-derived 25HC on NK cells, may not capture the effects this metabolite may be having in *in vivo* environments such as the TME. However, these experiments did reveal that BMDMs can impair IFN γ production by NK cells, following stimulation with certain TLR agonists, which will be further investigated in the following chapters in this thesis.

Chapter 4

Direct interactions with macrophages limit NK cell effector functions.

4.1 Introduction

Macrophages and NK cells co-exist in a number of different tissues and disease states due to macrophages being present in virtually every tissue, from as early as embryonic development (Hoeffel and Ginhoux, 2015). Work evaluating NK cell-macrophage interactions in this thesis so far has focused on a wide variety of TLR agonists used to model viral, bacterial and fungal infections. Although NK cells have been known to have roles in all of these infections, work in this chapter will specifically use LPS and CpG to stimulate macrophages. These particular TLR agonists were chosen because of the different effects that LPS and CpG-stimulated BMDMs had on IL2-NK cells. In the presence of LPS-stimulated BMDMs NK cells had dampened production of IFN γ , whereas IFN γ production was largely unaffected when NK cells were co-cultured with CpG-stimulated BMDMs (Figure 3.19). Although there was no correlation between 25HC synthesis and impaired NK cell cytotoxicity, it is still possible that other communication axes exist to regulate NK cell effector functions.

TLR4 and TLR9 activation are both associated with bacterial infection (Hoshino *et al.*, 1999; Dalpke *et al.*, 2006). The roles of NK cells in bacterial infection can vary based on the extent of the infection. For example, in the early stages of infection NK cells can have a supporting role, but as infections progress to sepsis NK cells are described to have more of an aggravating role (Badgwell *et al.*, 2002; Goldmann *et al.*, 2005; Enoh *et al.*, 2006). In particular, NK cell-derived IFN γ has been shown to be involved in NK cell-mediated death in murine models of hyperinflammation (Heremans *et al.*, 1994; Guo *et al.*, 2015). IFN γ is known to activate macrophages in bacterial infections and is additive to LPS to further enhance polarisation of pro-inflammatory macrophages. Therefore, the loop of NK cell-IFN γ production and macrophage-derived IL12, IL15 and IL18 has been linked with cytokine storm and tissue damage (Souza-Fonseca-Guimaraes *et al.*, 2012). This chapter further investigates whether macrophages might be altering

NK cell metabolism to dampen NK cell IFN γ production. Due to the link between NK cell production of IFN γ and cellular metabolism, the effect of macrophages on NK cell metabolic parameters will also be assessed.

4.2 Direct interactions with LPS-stimulated macrophages limit NK cell effector function *in vitro*.

In order to determine whether the effects that macrophages were eliciting on NK cell functions were cell-mediated or *via* a soluble factor, IL2-NK cells were co-cultured with BMDMs either through direct co-cultures or co-cultures across a 0.4 μ m trans-well. Alternatively, IL2-NK cells were stimulated overnight in 70% BMDM-conditioned medium (Figure 4.1). Firstly, NK cell cytotoxicity was assessed to determine whether macrophages were influencing this process. In direct co-cultures BMDMs had no impact on IL2-NK cell cytotoxicity (Figure 4.2a), whereas trans-well co-cultures and IL2-NK cells cultured in BMDM-conditioned medium enhanced killing of target cells (Figure 4.2b,c). These results may indicate a limitation of NK cell killing in direct co-culture with BMDMs. In these killing assays IL2-NK cells were removed from co-cultures with BMDMs before being cultured with target cells to specifically measure NK cell-dependent killing. Importantly, the presence of BMDMs did not kill IL2-NK cells, as assessed by Zombie™ NIR viability staining (Figure 4.3). To determine whether changes in NK cell cytotoxicity could be attributed to changes in expression of cytotoxic molecules, granzyme B and perforin were measured, showing no change in perforin expression by IL2-NK cells in the presence of BMDMs, either through direct interactions or soluble factors (Figure 4.4a). Granzyme B expression by IL2-NK cells was impaired in the presence of LPS-stimulated BMDMs in direct co-cultures but was significantly impaired in the presence of BMDMs regardless of activation status in trans-well co-cultures (Figure 4.4b). Culture of IL2-NK cells in BMDM-conditioned medium had no impact on cytolytic molecule expression. When IL2-NK cells were directly co-cultured with LPS-stimulated BMDMs there was as a significant decrease in IFN γ expression, but not in the case of CpG-stimulated BMDMs (Figure 4.5). There were conflicting results when investigating the role of soluble factors on NK cell IFN γ expression also. In trans-well co-cultures, there was a significant decrease in IFN γ expression by IL2-NK cells in

the presence of BMDMs regardless of their activation status, but no change when NK cells were cultured in BMDM-conditioned medium (Figure 4.5).

To confirm whether any parallels could be made between these co-cultures and co-cultures using more physiological sources of cells, splenic NK cells were co-cultured with peritoneal macrophages for 24 h in the presence of the cytokines IL12, IL15 and IL18, to assess the impact macrophages were having on activation of these cells. These NK cells differ from the cultured NK cells as they only receive activation for 24 h, whereas IL2-NK cells receive some stimulation from IL2 for 6 days. The cytokines IL12, IL15 and IL18 were also chosen for these activations as they are more associated with an early, innate immune response, when NK cells and macrophages are interacting at the site of inflammation. These experiments focused on splenic NK cells in direct co-culture with peritoneal macrophages, due to the effect direct co-culture with LPS-stimulated BMDMs had on NK cell effector function. Assessing cytotoxicity of splenic NK cells in direct culture with peritoneal macrophages revealed no changes in killing of RMA/S target cells (Figure 4.6). Perforin expression by splenic NK cells was significantly decreased in the presence of macrophages, (Figure 4.7a,b), though the cells were still producing considerably more perforin than unstimulated controls. There was no detectable change in granzyme B expression in the presence of peritoneal macrophages (Figure 4.7c,d). IFN γ production by splenic NK cells was significantly decreased in the presence of peritoneal macrophages, with LPS- and CpG-stimulated macrophages causing the greatest reduction (Figure 4.8).

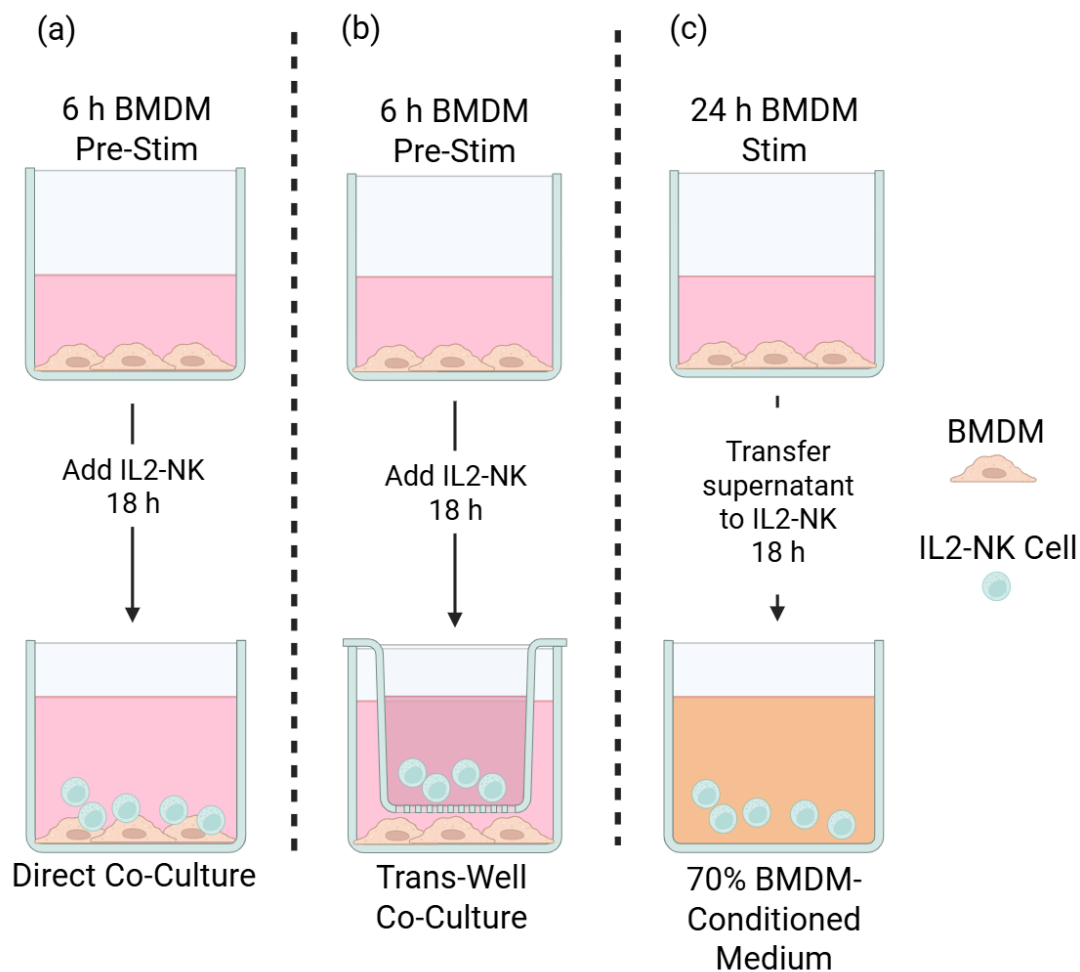


Figure 4.1 Experimental design for IL2-NK cell-BMDM co-cultures.

BMDMs were stimulated with LPS (10 ng/mL) or CpG (4 µg/mL) or left unstimulated for 6 h. Then IL2-NK cells were either directly co-cultured with BMDMs (a) or co-cultured in a trans-well (b) for 18 h in the presence of NK cell-activating cytokines IL2 (20 ng/mL) and IL12 (10 ng/mL). Alternatively, NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h in 70% conditioned medium collected from BMDMs previously stimulated with LPS (10 ng/mL), CpG (4 µg/mL) or unstimulated for 24 h (c). *This figure was made using Biorender.com.*

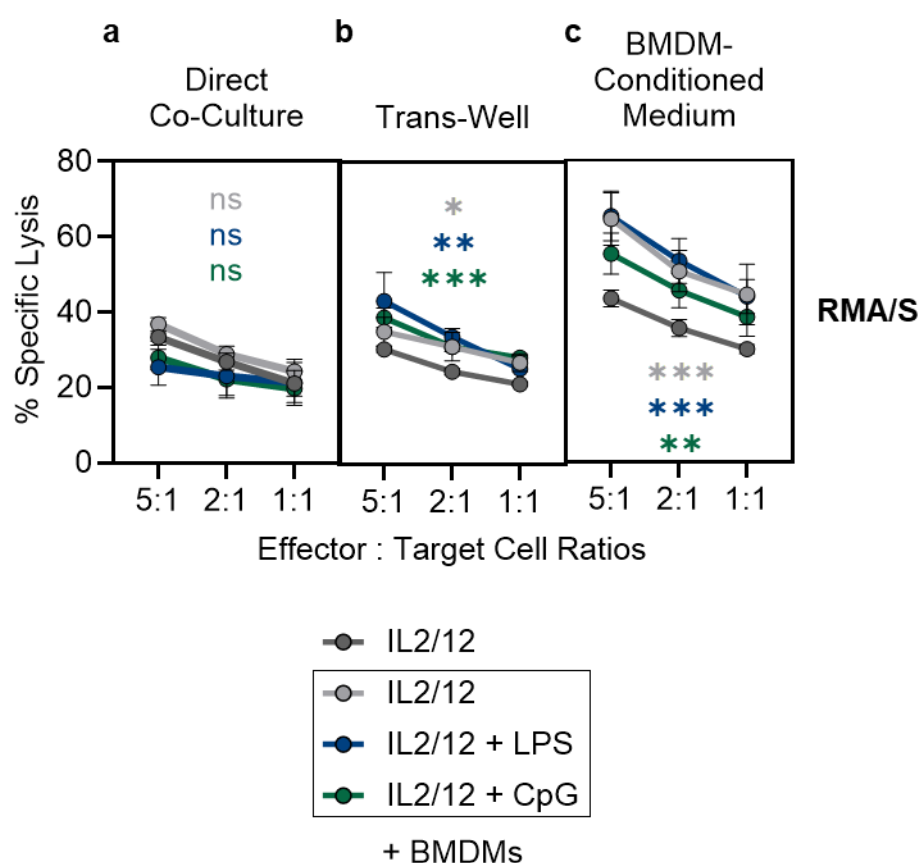


Figure 4.2 Direct co-culture of IL2-NK cells with BMDMs limits NK cell cytotoxicity.

BMDMs were stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL) or left unstimulated for 6 h. Then IL2-NK cells were either directly co-cultured with BMDMs (a) or co-cultured in a trans-well (b) for 18 h in the presence of NK cell-activating cytokines IL2 (20 ng/mL) and IL12 (10 ng/mL). Alternatively, NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h in 70% conditioned medium collected from BMDMs previously stimulated with LPS (10 ng/mL), CpG (4 μ g/mL) or unstimulated for 24 h (c). NK cells were then collected from culture and cytotoxicity against RMA/S target cells was determined by fluorescence release assay. Data is mean $\pm$ SEM of 4 biological replicates across two independent experiments (a,c) or one independent experiment (b). Statistical analysis was performed using a two-way ANOVA. All comparisons made on this graph are versus IL2/12-stimulated NK cells only (* p <0.05, ** p <0.01, *** p <0.001, ns=non-significant).

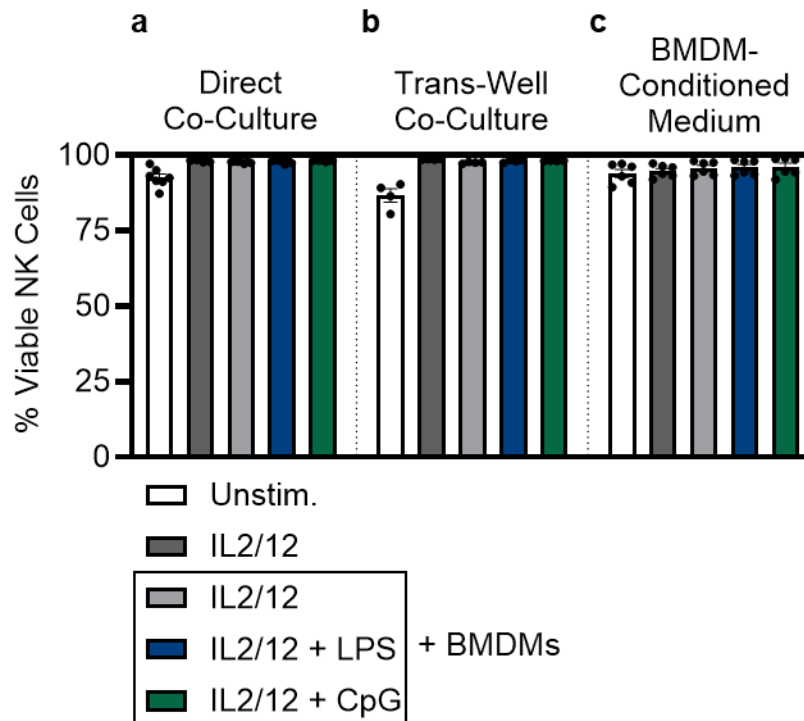


Figure 4.3 IL2-NK cell viability does not change in the presence of BMDMs.

BMDMs were stimulated with LPS (10 ng/mL) or CpG (4 µg/mL) or left unstimulated for 6 h. Then IL2-NK cells were either directly co-cultured with BMDMs or co-cultured in a trans-well for 18 h in the presence of NK cell-activating cytokines IL2 (20 ng/mL) and IL12 (10 ng/mL). Alternatively, NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h in 70% conditioned medium collected from BMDMs previously stimulated with LPS (10 ng/mL), CpG (4 µg/mL) or unstimulated for 24 h. Zombie NIR™ staining was used as an indicator of NK cell death and was measured by flow cytometry. Data is mean +/- SEM of at least 4 biological replicates across 2 independent experiments (a,c) or 1 independent experiment (b). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test.

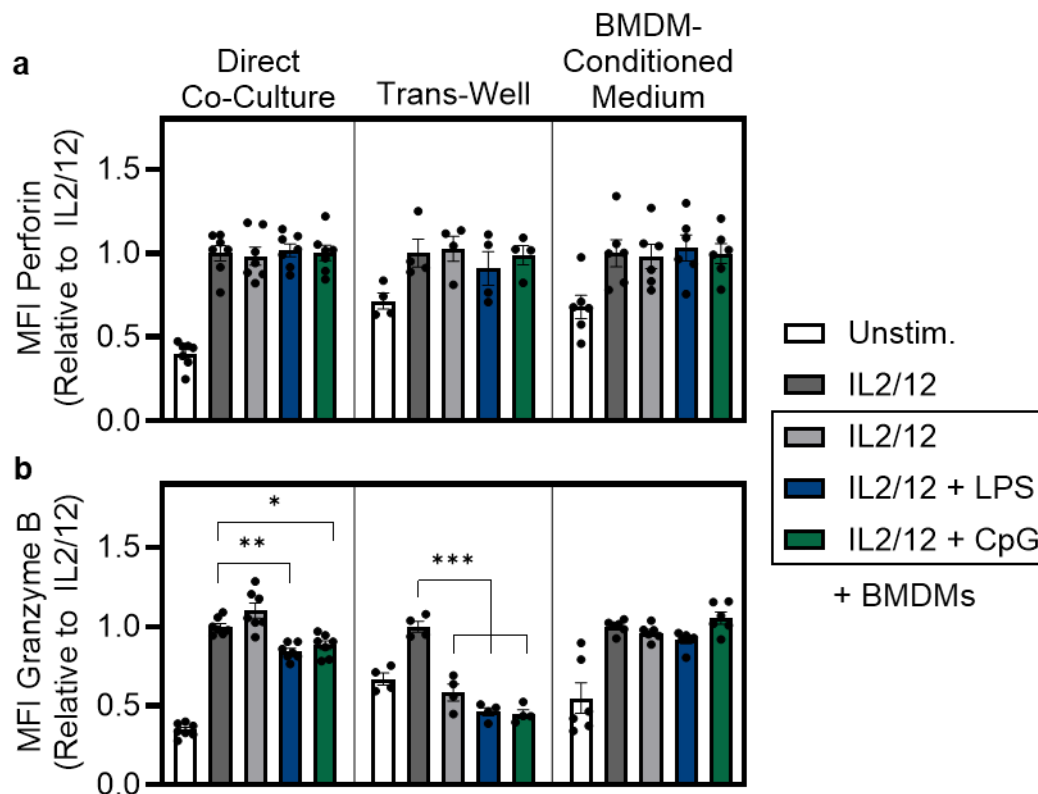


Figure 4.4 Granzyme B expression by IL2-NK cells is impaired in direct co-cultures with LPS- and CpG-stimulated BMDMs.

BMDMs were stimulated with LPS (10 ng/mL) or CpG (4 µg/mL) or left unstimulated for 6 h. Then IL2-NK cells were either directly co-cultured with BMDMs or co-cultured in a trans-well for 18 h in the presence of NK cell-activating cytokines IL2 (20 ng/mL) and IL12 (10 ng/mL). Alternatively, NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h in 70% conditioned medium collected from BMDMs previously stimulated with LPS (10 ng/mL), CpG (4 µg/mL) or unstimulated for 24 h. Perforin (a) and granzyme B (b) expression were measured by flow cytometry. Data is mean $\pm$ SEM of at least 4 biological replicates across 3 independent experiments for direct co-cultures and BMDM-conditioned medium, or 1 independent experiment for trans-well co-cultures. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. Only statistically significant comparisons are included on the graph (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

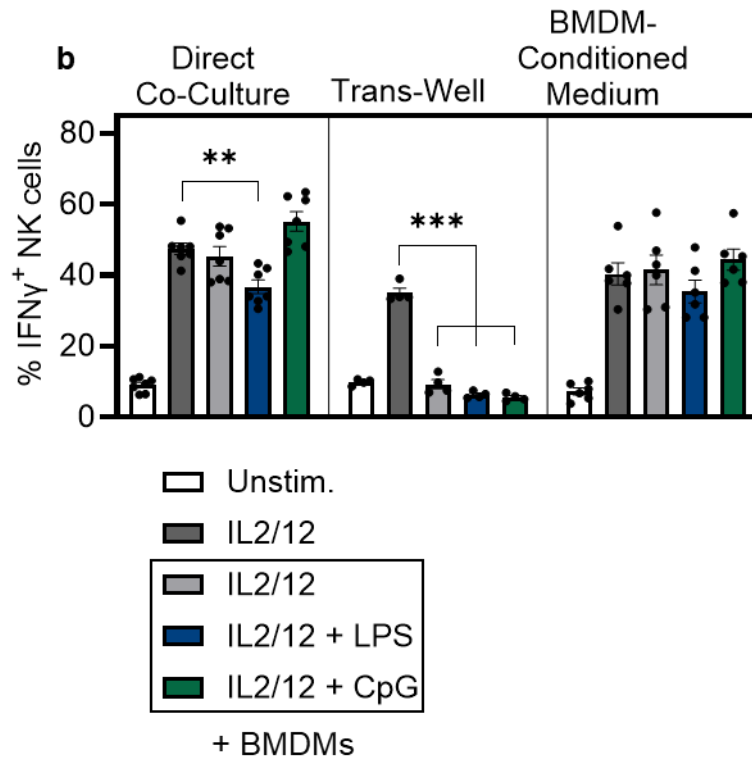
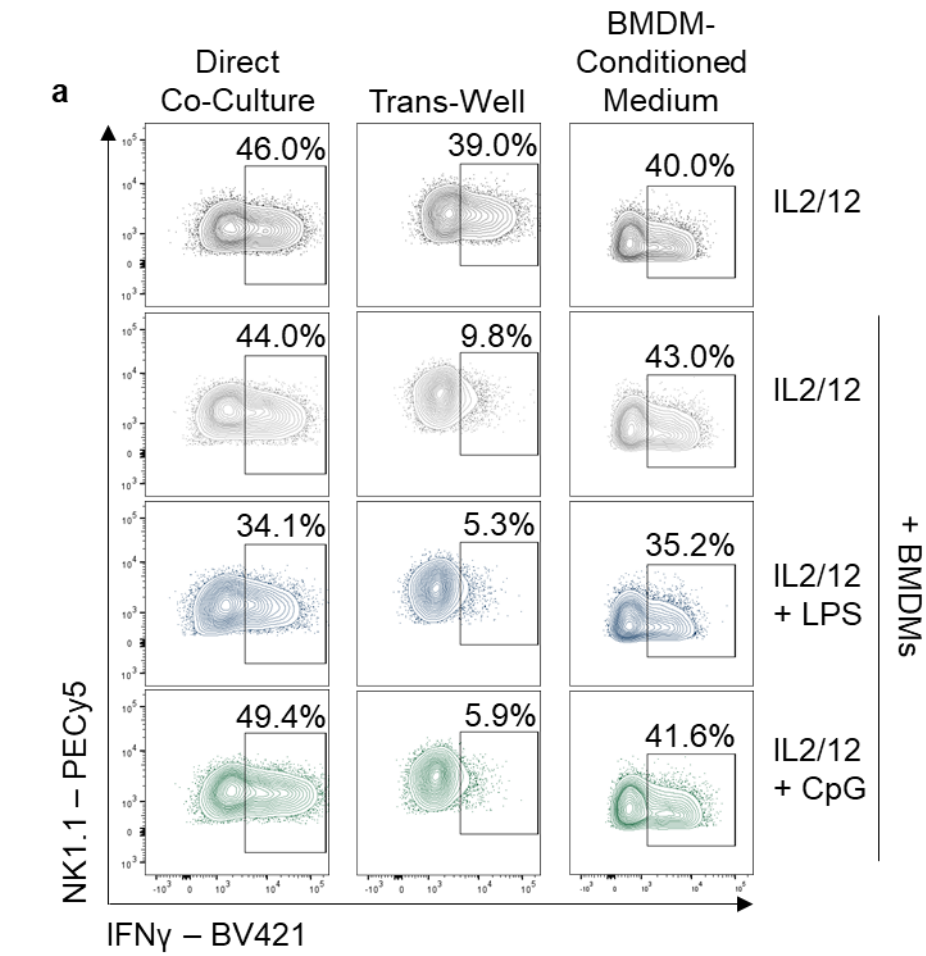


Figure 4.5 IFN γ production by IL2-NK cells is impaired in direct co-culture with LPS-stimulated BMDMs.

BMDMs were stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL) or left unstimulated for 6 h. Then IL2-NK cells were either directly co-cultured with BMDMs or co-cultured in a trans-well for 18 h in the presence of NK cell-activating cytokines IL2 (20 ng/mL) and IL12 (10 ng/mL). Alternatively, NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h in 70% conditioned medium collected from BMDMs previously stimulated with LPS (10 ng/mL), CpG (4 μ g/mL) or unstimulated for 24 h. IFN γ was measured by flow cytometry. Data is mean $\pm$ SEM of at least 4 biological replicates across 3 independent experiments for direct co-cultures and BMDM-conditioned medium, or 1 independent experiment for trans-well co-cultures. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (** $p < 0.01$, *** $p < 0.001$, ns=non-significant).

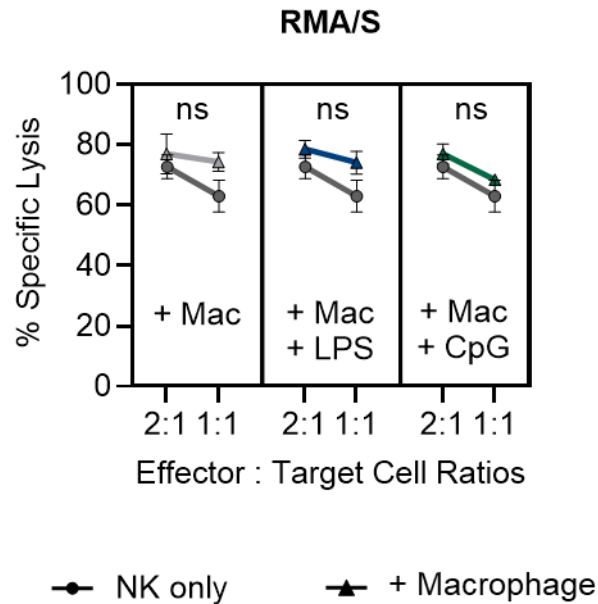


Figure 4.6 Splenic NK cells co-cultured with peritoneal macrophages do not have altered cytotoxicity.

NK cells were purified from the spleen and were stimulated with IL12 (10 ng/mL), IL15 (10 ng/mL) and IL18 (50 ng/mL) for 18 hours in the presence or absence of macrophages which were purified from the peritoneal cavity. Macrophages were left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 µg/mL). NK cell cytotoxicity against RMA/S target cells was determined by fluorescence release assay. Data is mean +/- SEM of 4 biological replicates across 2 independent experiments. Statistical analysis was performed using a two-way ANOVA (ns=non-significant).

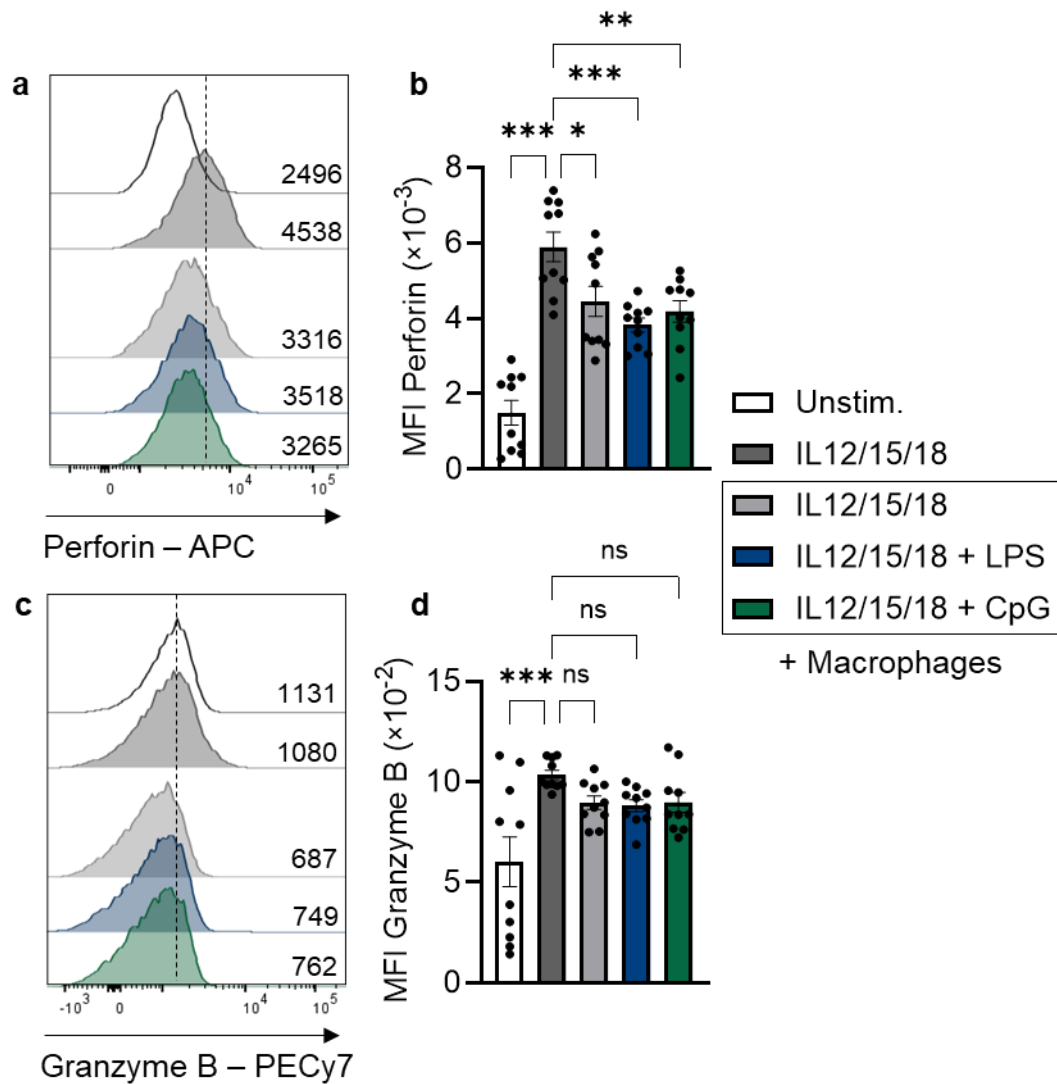


Figure 4.7 Splenic NK cells co-cultured with peritoneal macrophages have impaired perforin expression.

NK cells were purified from the spleen and were stimulated with IL12 (10 ng/mL), IL15 (10 ng/mL) and IL18 (50 ng/mL) for 18 hours in the presence or absence of macrophages which were purified from the peritoneal cavity. Macrophages were left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL). Perforin (a,b) and granzyme B (c,d) expression were measured by flow cytometry. Data is representative (a,c) or mean $\pm$ SEM of 10 biological replicates across 4 independent experiments (b,d). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). *Some replicates were performed with Shauna Mayock, Finlay Laboratory, Trinity College Dublin.*

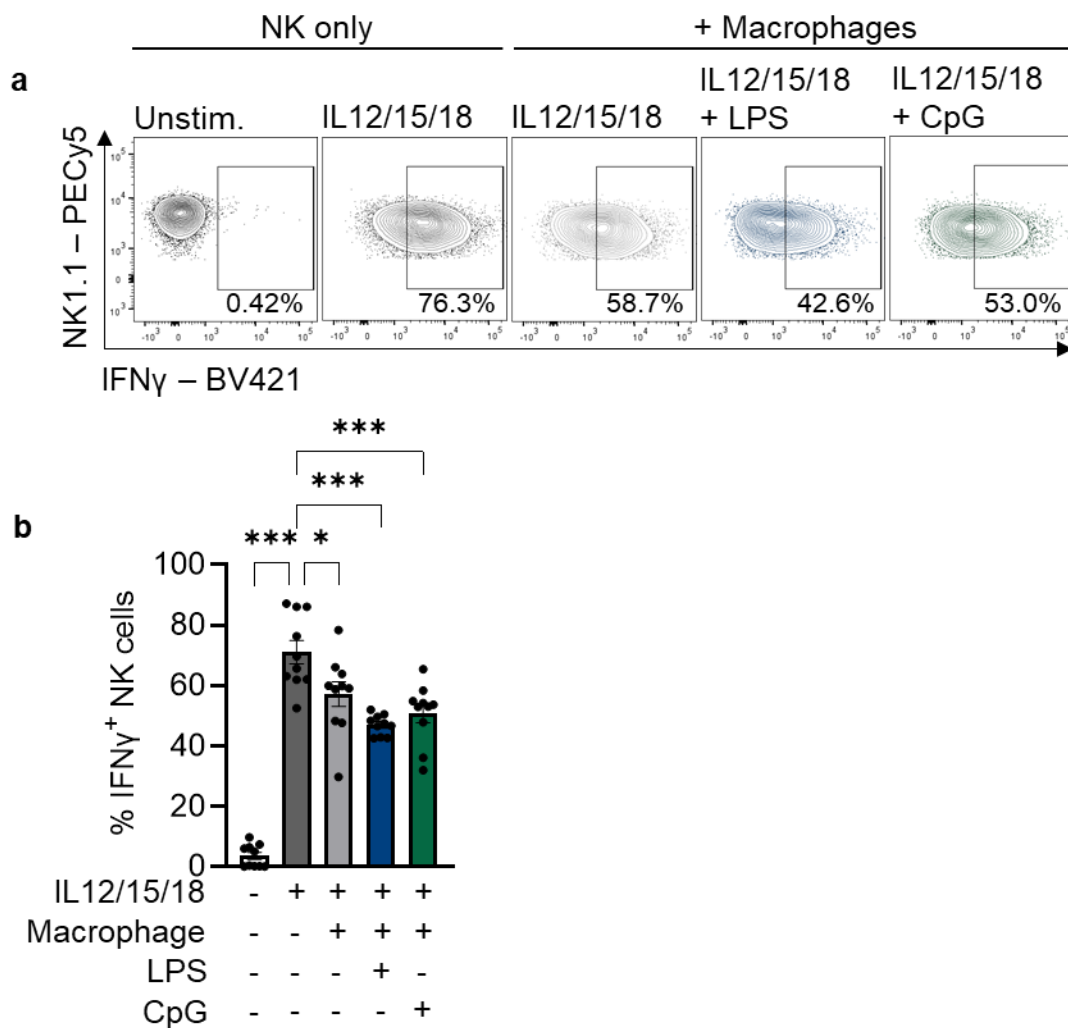


Figure 4.8 Splenic NK cells co-cultured with peritoneal macrophages have impaired IFN γ production.

NK cells were purified from the spleen and were stimulated with IL12 (10 ng/mL), IL15 (10 ng/mL) and IL18 (50 ng/mL) for 18 hours in the presence or absence of macrophages which were purified from the peritoneal cavity. Macrophages were left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL). IFN γ expression was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 10 biological replicates across 4 independent experiments (b). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (* p <0.05, *** p <0.001). *Some replicates were performed with Shauna Mayock, Finlay Laboratory, Trinity College Dublin.*

4.3 LPS-immunisation activates peritoneal NK cells *in vivo*.

Work shown so far in this thesis has investigated NK cell-macrophage interactions *in vitro*. However, in order to determine whether any effects being observed could be seen in an *in vivo* setting a peritonitis model was induced using LPS from *E. coli* (Figure 4.9). A range of doses of LPS were used to investigate whether the activation status of the macrophages in the peritoneum could impact NK cells in that environment. These ranged from 0.6 mg/kg to 2.4 mg/kg LPS, with higher doses inducing sepsis in the mice. TLR expression was assessed in a proteomics dataset of splenic NK cells in the Finlay lab, showing that splenic NK cells do not express TLR4, and therefore, should not respond directly to LPS. Nevertheless, NK cells were purified from the spleen and cultured in the presence of LPS (10, 100 ng/mL) for 24 h, confirming that LPS alone does not activate murine NK cells (Figure 4.10).

Cytokine production was measured in the peritoneal lavage fluid to confirm that there was evidence of inflammation. IL1 β , IL6 and TNF α were measured as they are canonically considered markers of inflammation. Although the signal is quite low due to the dilution of the peritoneal fluid during the lavage, there is evidence of elevated IL6 24 h following LPS immunisation, indicating an inflammatory environment (Figure 4.11b). To determine whether there was an increase in the abundance of cytokines that influence NK cell activation in the peritoneum following LPS-immunisation, IL12p70, IL15, IL18, IL2, TGF β 1 and IL10 were measured in the peritoneal lavage fluid. The only cytokine that could be detected was IL2 which was significantly increased following LPS-immunisation (Figure 4.12d). Again, it should be considered that the other cytokines measured in the peritoneal lavage fluid may still be present and are not detected because of the considerable dilution of peritoneal fluid required to collect the lavage. The abundance of chemokines which are known to act on NK cells was also measured in the lavage fluid to determine whether NK cells were possibly trafficking to the peritoneum. This showed that following LPS-immunisation there was a significant increase in the levels of CCL2, CCL5 in the peritoneal cavity, suggesting increased trafficking of NK cells to the peritoneal cavity, or increased contacts between NK cells and other cells within the peritoneum (Figure 4.13).

Cytokine production by peritoneal macrophages was assessed to determine whether macrophages were still producing NK cell-activating cytokines 24 h after injection of LPS. Among the three cytokines measured, IL12p40/p70 expression was the highest at this time point, indicating that NK cells in the environment were likely still receiving activating signals from macrophages (Figure 4.14a,c). The IL12p40/p70 antibody used will detect the active heterodimer form of the cytokine IL12p70 but it is not known whether it interacts with the IL12p40 monomer. IL12p40 can form part of IL23 so cross-reactivity with IL23 cannot be disregarded. Expression of *Il23a*, the gene for the other component of IL23 was expressed to a much lower extent than *Il12a* and *Il12b*, the genes for IL12p35 and IL12p40 in peritoneal macrophages stimulated with LPS *in vitro* (GSE239696). Taken together this suggests the IL12p40 staining will primarily detect IL12p70. The frequency of IL12p40/p70-producing macrophages was comparable across all doses of LPS used. There were very modest increases in IL6 and TNF α expression by these macrophages although there was likely much higher expression of these cytokines earlier following LPS-immunisation (Figure 4.14b,d).

Canonically, CD69 is commonly used as an activation marker for lymphocytes. However, while this interpretation may be true for lymphocytes in circulation, in tissue niches CD69 can also be interpreted as a marker of residency. Indeed, in this study there was very little increase in the frequency of CD69-expressing NK cells in both the spleen and the peritoneum (Figure 4.15a,b), despite the fact that other NK cell functional parameters such as IFN γ and perforin expression were elevated (Figure 4.16, Figure 4.17). Therefore, another surface marker: CD71, the receptor for iron-bound transferrin (holotransferrin) was used as a marker of NK cell activation in the peritoneum (Figure 4.15a,c).

Although expression of activation markers is useful to assess whether the NK cells in the peritoneum are increasing their effector functions, measurement of cytokines and cytolytic molecules are important to determine whether they are carrying out their particular functions. In the peritoneum there was significant increases in expression of IFN γ 24 h after LPS-immunisation, whereas this increase was not seen in splenic NK cells (Figure 4.16). Furthermore, perforin expression was significantly increased in peritoneal NK cells following LPS-

immunisation (Figure 4.17a,b), whereas granzyme B expression did not significantly increase across the range of doses of LPS used (Figure 4.17c,d).

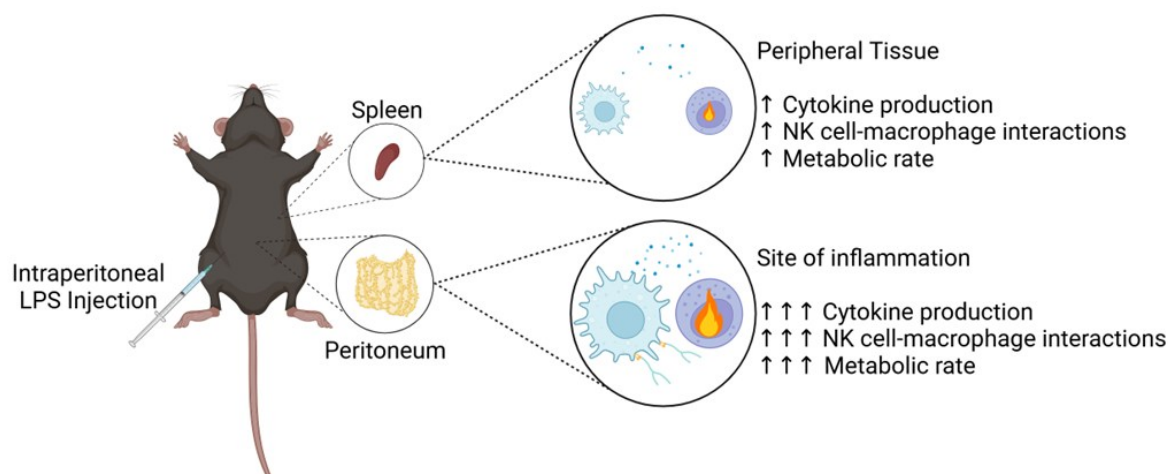


Figure 4.9 LPS-induced peritonitis model.

Mice were administered LPS by intraperitoneal injection across a range of doses before the spleen and peritoneum were harvested for further analysis. These two tissues were chosen as the peritoneum is the site of inflammation with increased numbers of cytokine-producing macrophages (blue) following exposure to LPS. There is also migration of NK cells (purple) to the peritoneum following LPS injection allowing for increased numbers of NK cell-macrophage interactions. The spleen is a peripheral tissue from the site of inflammation. Following LPS-immunisation macrophages migrate from the spleen meaning there are less instances of direct NK cell-macrophage interactions. These two tissues were compared to better understand the effect of direct interactions with pro-inflammatory macrophages on NK cell activation and metabolism following LPS-immunisation. *This figure was created using Biorender.com.*

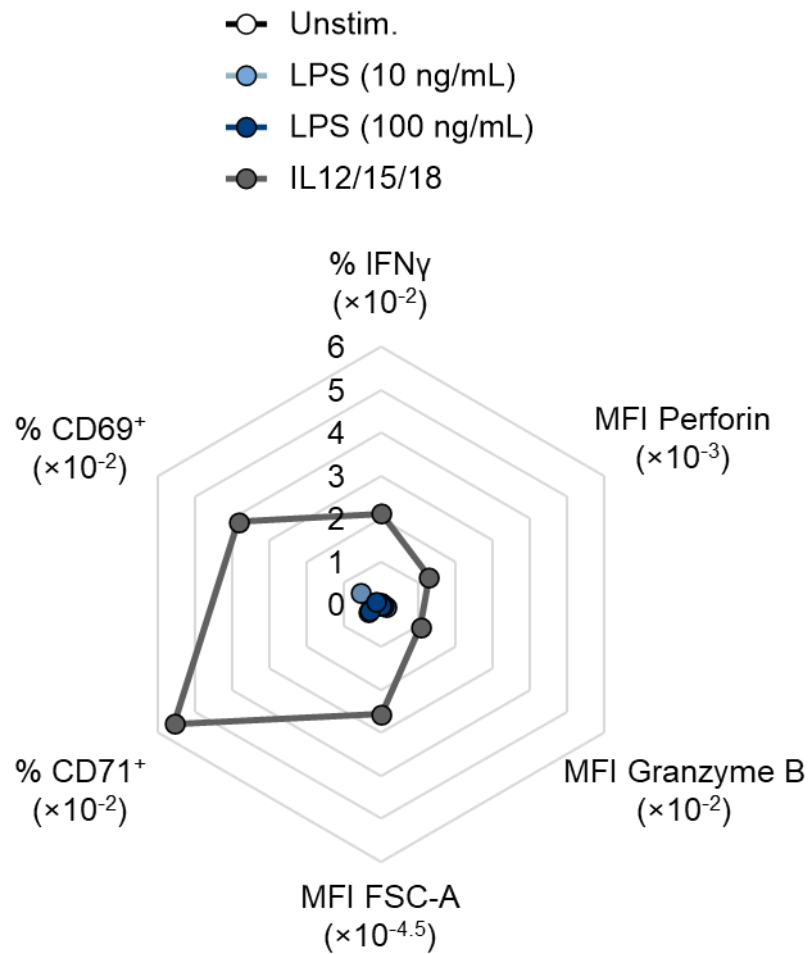


Figure 4.10 LPS does not directly activate splenic NK cells *ex vivo*.

NK cells were purified from the spleen and were left unstimulated or stimulated with LPS (10, 100 ng/mL) or IL12 (10 ng/mL), IL15 (10 ng/mL) and IL18 (50 ng/mL) for 24 h. IFN γ , perforin, granzyme B, CD71 and CD69 expression were measured by flow cytometry. FSC-A was used as an indicator of cell size and was measured by flow cytometry. Data is mean of 4 biological replicates. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. Unstimulated NK cells were compared to LPS-stimulated NK cells. There were no statistically significant comparisons.

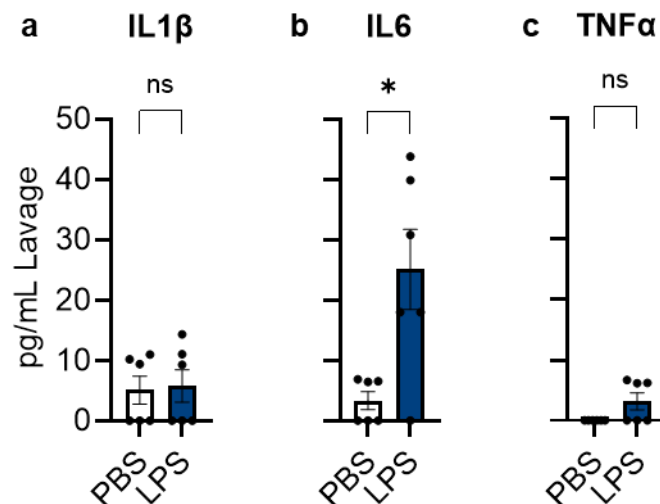


Figure 4.11 There is increased abundance of IL6 in the peritoneum 24 h following LPS-immunisation.

Mice were administered PBS or LPS (2.4 mg/kg) by intraperitoneal injection. Peritoneal lavages were performed 24 h post-administration before IL1 β , IL6 and TNF α were measured in the lavage using a cytokine bead array by flow cytometry. Data is mean $\pm$ SEM of 6 mice per group across 2 independent experiments. Statistical analysis was performed using a two-tailed Welch's t-test (* p <0.05, ns=non-significant). *Cytokine bead array was performed with Carrie Corkish, Finlay Lab, Trinity College Dublin.*

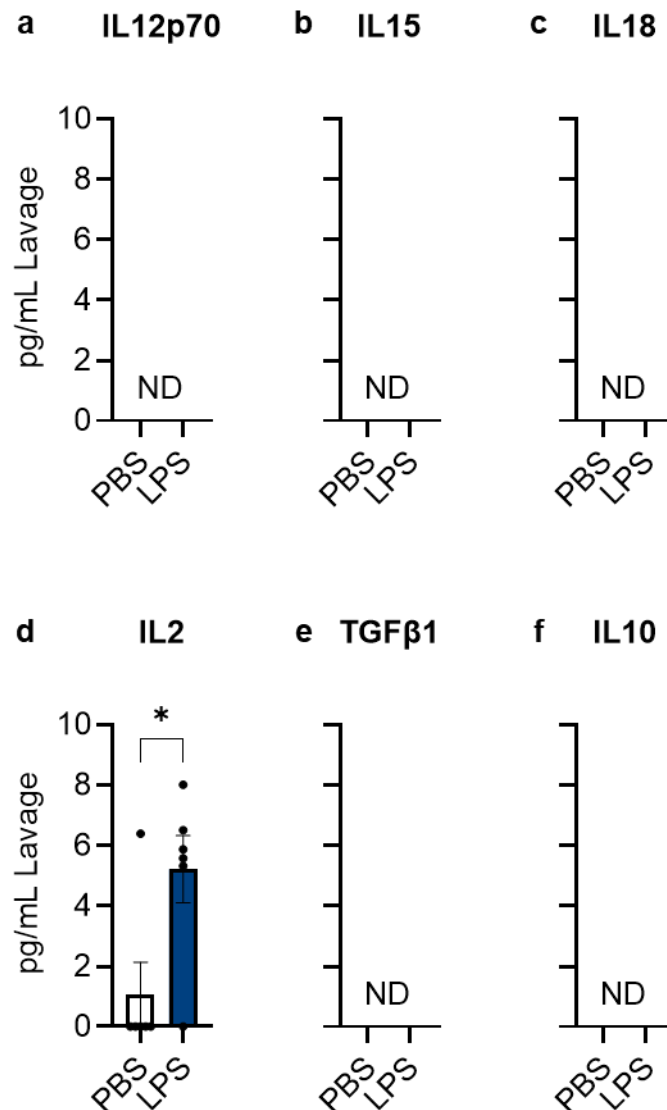


Figure 4.12 There is increased abundance of IL2 in the peritoneum 24 h following LPS-immunisation.

Mice were administered PBS or LPS (2.4 mg/kg) by intraperitoneal injection. Peritoneal lavages were performed 24 h post-administration before IL12p70, IL15, IL18, IL2, TGFβ1 and IL10 were measured in the lavage using a cytokine bead array by flow cytometry. Data is mean +/- SEM of 6 mice per group across 2 independent experiments. Statistical analysis was performed using a two-tailed Welch's t-test (*p<0.05, ns=non-significant). (ND=not detected) *Cytokine bead array was performed with Carrie Corkish, Finlay Lab, Trinity College Dublin.*

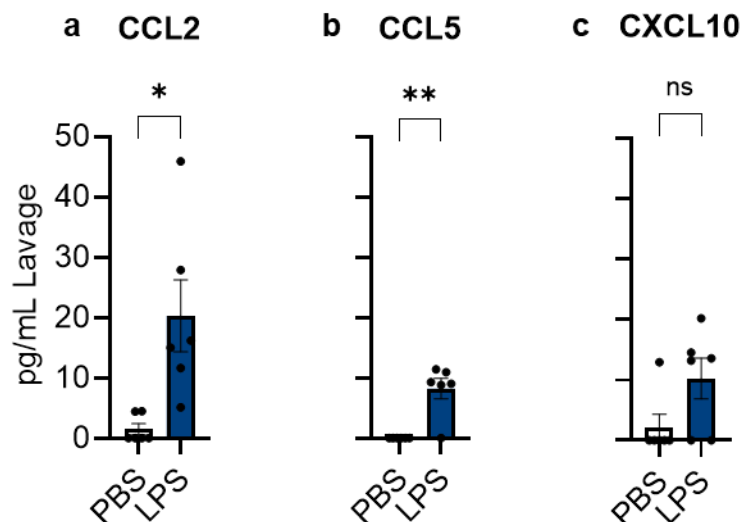


Figure 4.13 There is increased abundance of NK cell-recruiting chemokines in the peritoneum of LPS-immunised mice.

Mice were administered PBS or LPS (2.4 mg/kg) by intraperitoneal injection. Peritoneal lavages were performed 24 h post-administration before CCL2, CCL5 and CXCL10 were measured in the lavage using a cytokine bead array by flow cytometry. Data is mean $\pm$ SEM of 6 mice per group across 2 independent experiments. Statistical analysis was performed using a two-tailed Welch's t-test (* p <0.05, ** p <0.01, ns=non-significant). *Cytokine bead array was performed with Carrie Corkish, Finlay Lab, Trinity College Dublin.*

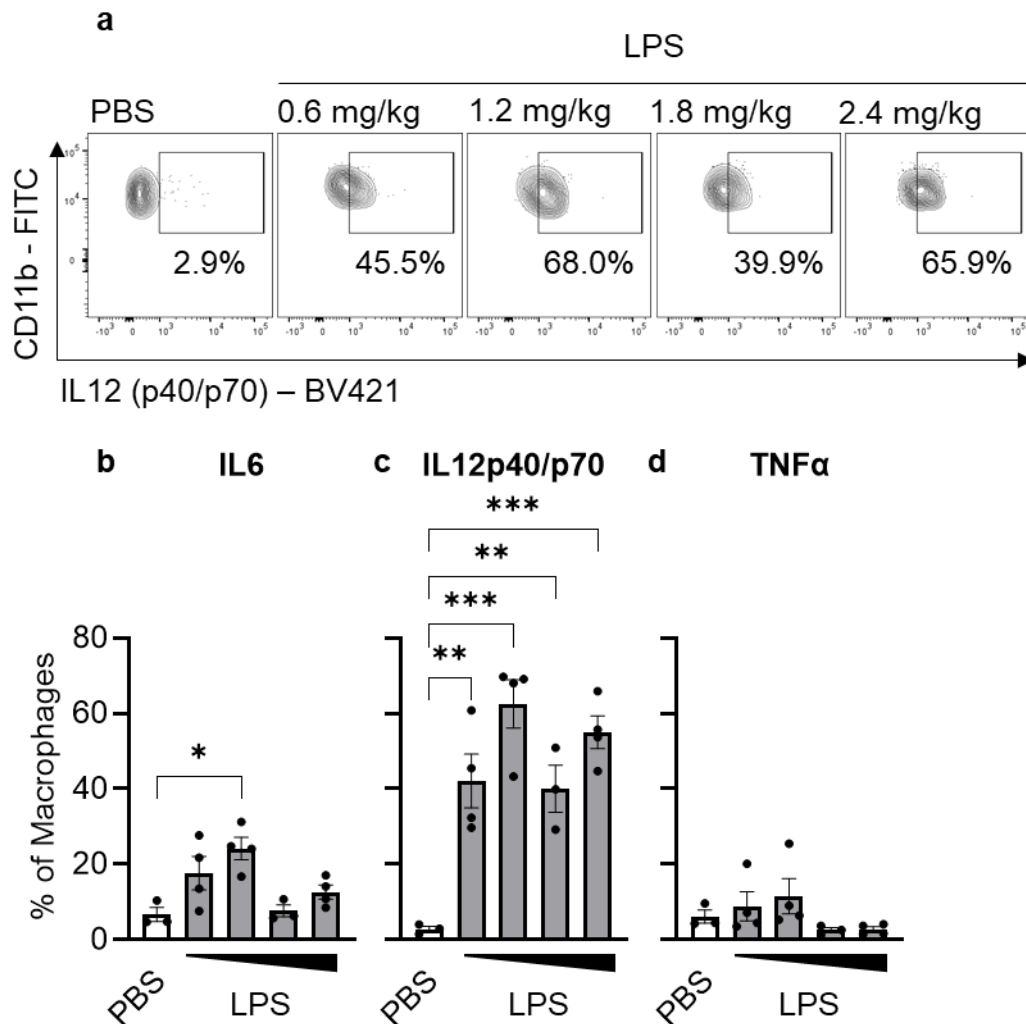


Figure 4.14 Peritoneal macrophages are a source of IL12p40/p70 following LPS-immunisation.

Mice were administered PBS or LPS (0,6 mg/kg, 1.2 mg/kg, 1.8 mg/kg, or 2.4 mg/kg) by peritoneal injection. Peritoneal cells were harvested 24 hours post-administration. IL6 (b), IL12 (a,c) and TNF α (d) production by small peritoneal macrophages (CD3⁻, CD19⁻, CD11b^{high}, F4/80⁺) was measured by flow cytometry. A minimum of 3 mice were used per group. Data is representative (a) or mean $\pm$ SEM (b,d). Statistical analysis was carried out using a one-way ANOVA with Tukey's post-test. Only statistically significant comparisons were included on the graph (* p <0.05, ** p <0.01, *** p <0.001).

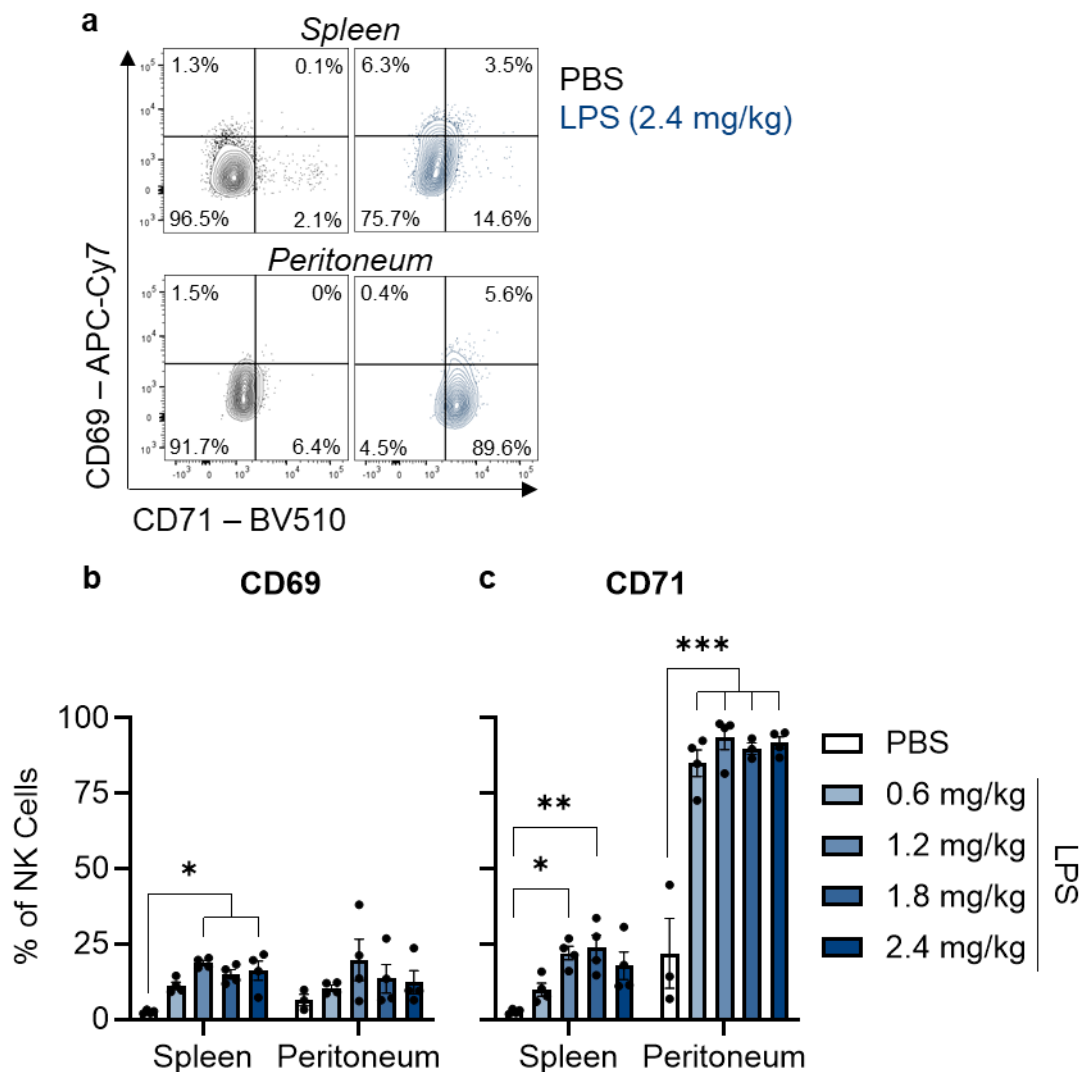


Figure 4.15 CD71 expression can be used as an activation marker for peritoneal NK cells following LPS-immunisation.

Mice were administered PBS or LPS (0,6 mg/kg, 1.2 mg/kg, 1.8 mg/kg, or 2.4 mg/kg) by peritoneal injection. Spleens and peritoneal cells were harvested 24 hours post-administration. CD69 (a,b) and CD71 (a,c) expression by NK cells (CD3-, CD19-, NK1.1+, CD49b+) was measured by flow cytometry. A minimum of 3 mice were used per group. Data is mean +/- SEM. Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test. Only statistically significant comparisons are included on the graph (*p<0.05, **p<0.01, ***p<0.001, ns=non-significant).

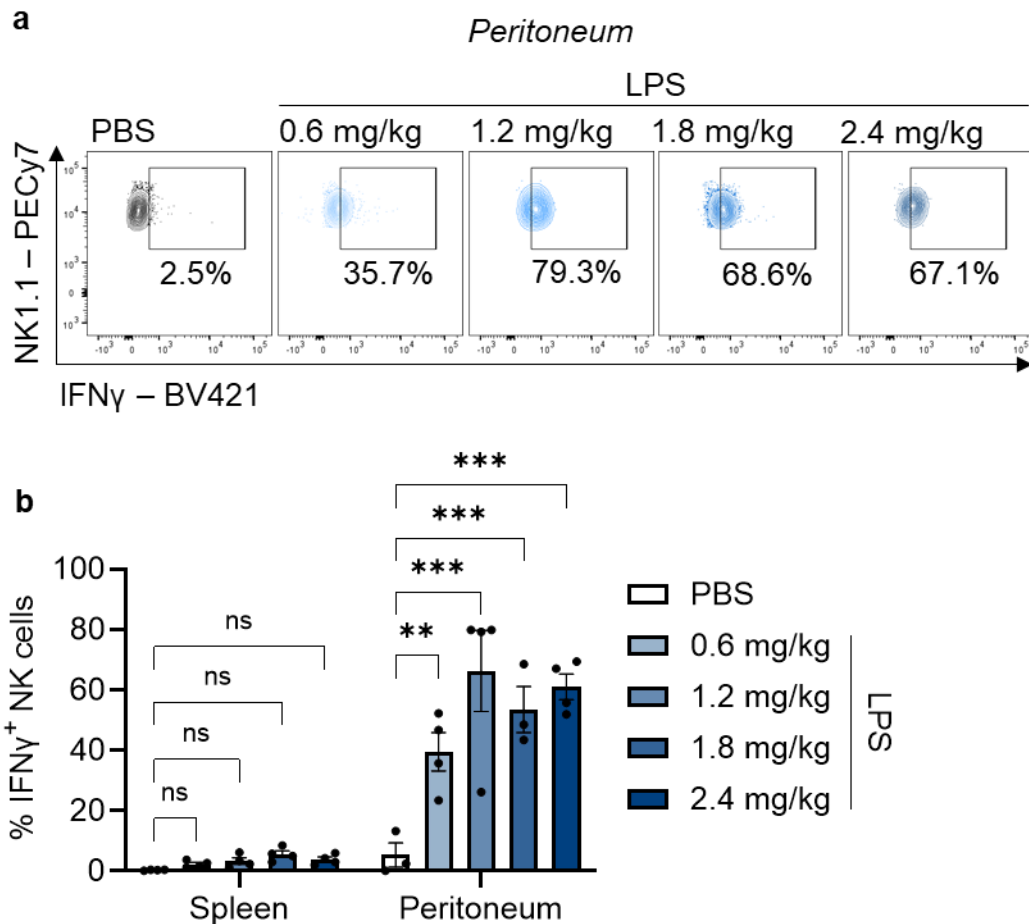


Figure 4.16 Peritoneal NK cells increase expression of IFN γ following LPS-immunisation.

Mice were administered PBS or LPS (0,6 mg/kg, 1.2 mg/kg, 1.8 mg/kg, or 2.4 mg/kg) by peritoneal injection. Spleens or peritoneal cells were harvested 24 hours post-administration. IFN γ expression by NK cells (CD3⁻, CD19⁻, NK1.1⁺, CD49b⁺) was measured by flow cytometry. A minimum of 3 mice were used per group. Data is mean $\pm$ SEM. Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test (**p<0.01, ***p<0.001, ns=non-significant).

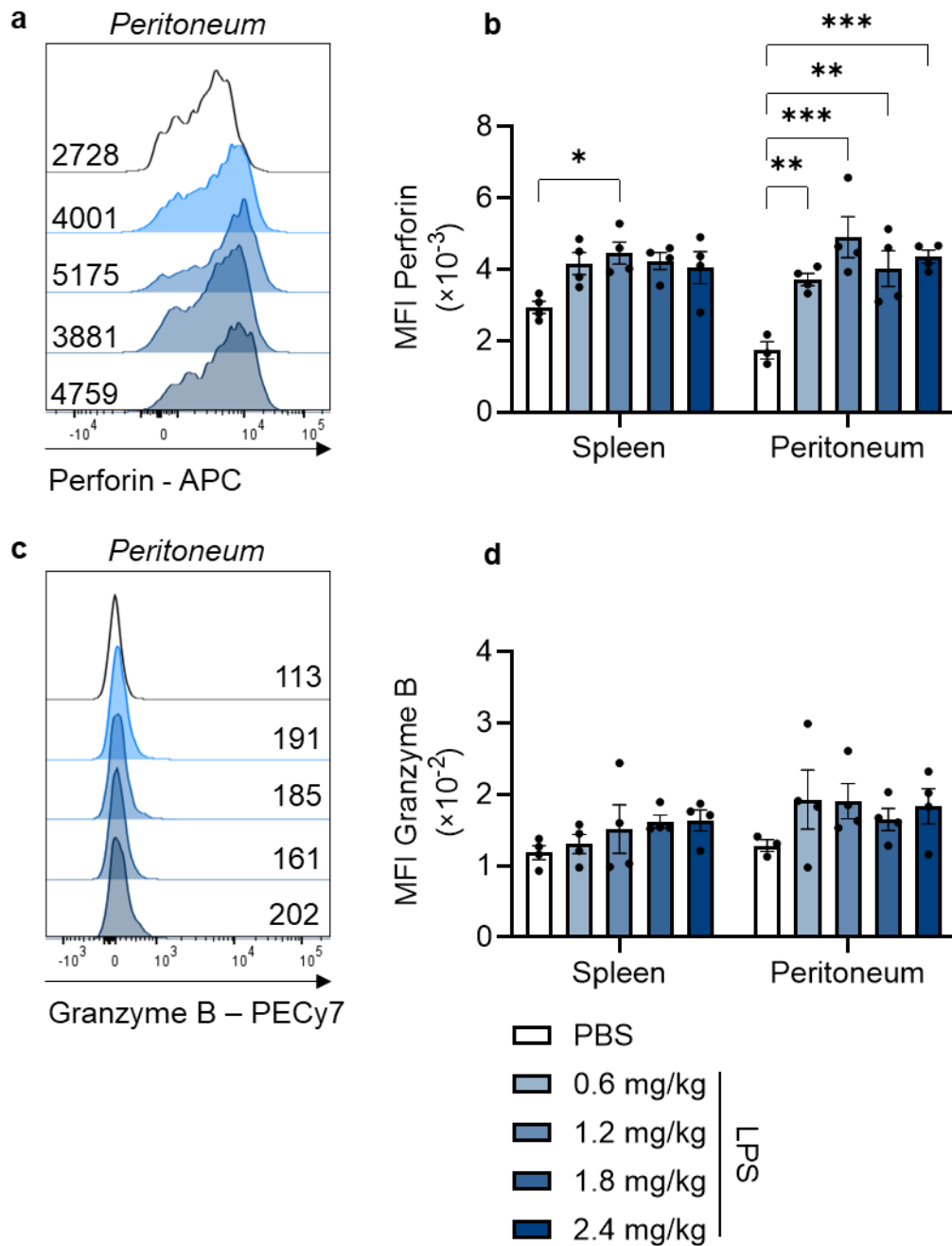


Figure 4.17 Peritoneal NK cells increase expression of perforin following LPS-immunisation.

Mice were administered PBS or LPS (0.6 mg/kg, 1.2 mg/kg, 1.8 mg/kg, or 2.4 mg/kg) by peritoneal injection. Spleens or peritoneal cells were harvested 24 hours post-administration. Perforin and granzyme B expression by NK cells ($CD3^+$, $CD19^-$, $NK1.1^+$, $CD49b^+$) was measured by flow cytometry. A minimum of 3 mice were used per group. Data is mean $\pm$ SEM. Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test. Only statistically significant comparisons are included on the graphs (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.4 NK cell metabolism is altered in the presence of macrophages *in vitro*.

Protein translation is the most ATP-demanding process that occurs in the cell, meaning that measuring rates of protein translation can act as a surrogate indicator of ATP production (Buttgereit and Brand, 1995; Argüello *et al.*, 2020). Protein translation was assessed through measuring incorporation of O-propargyl puromycin (OPP) into translated proteins of IL2-NK cells co-cultured with BMDMs. There was a trend towards impaired rates of protein translation when IL2-NK cells were co-cultured with BMDMs, though a significant decrease was observed in the presence of LPS- and CpG-stimulated BMDMs (Figure 4.18). In order to understand what metabolic processes may be impaired in the co-cultures, IL2-NK cells were stimulated with IL2/12 and exposed to 2DG and oligomycin for 40 mins to inhibit glycolysis and OxPhos respectively, before protein translation was measured. This revealed that although some cells had decreased protein translation rates when OxPhos was inhibited, this process is highly dependent on glycolysis (Figure 4.19).

The signals provided to the NK cells in these co-cultures to increase metabolic rate are due to IL2 signalling. This is facilitated by IL12 which induces expression of the high-affinity IL2 receptor (Nguyen *et al.*, 2000). CD25 (IL2R α) is a component of this receptor and can be measured by flow cytometry (Liu *et al.*, 1996). Co-culture of NK cells with BMDMs did not reveal any change in CD25 expression (Figure 4.20). Looking at signalling downstream of the IL2 receptor, transcription factor activation is a useful measure of signalling. Activated NK cells have been described to use SREBP-controlled glucose metabolism to support their effector functions, and this has been shown to be downstream of IL2-signalling (Assmann *et al.*, 2017). Expression of SREBP target genes were assessed throughout the duration of co-cultures with IL2-NK cells and BMDMs, showing no changes in expression of *Acat2*, *Hmgcs1* or *Acly* mRNA (Figure 4.21). *Acly* expression was of particular interest because it encodes ACLY, a component of the CMS (Figure 4.21c).

Assessment of rates of protein translation showed that glycolysis is essential to support this process (Figure 4.19), suggesting that glucose is an essential fuel

for IL2-NK cells. Therefore, in order to assess whether impairments in NK cell effector functions might be due to changes in expression of essential proteins in the glycolytic pathway, RT-qPCR was performed to measure expression of *Slc2a1*, *Hk2*, *Gapdh* and *Ldha* mRNA (Figure 4.22). This analysis revealed no changes in expression of these genes which supports the hypothesis that changes in effector function are occurring independently of any impairment in expression of glycolytic machinery.

Although OxPhos was not shown to be essential to support rates of protein translation, decreases in IFN γ expression could be explained by a change in the mitochondria of NK cells co-cultured with BMDMs. MitoTracker™ Green was used to measure NK cell mitochondrial mass in the co-cultures, showing that NK cells had less mitochondria in the presence of BMDMs although this was not statistically significant (Figure 4.23a,b). When assessing mitochondrial inner membrane potential using tetramethyl rhodamine methyl ester (TMRM) it could be seen that although the NK cells had lower mitochondrial mass, their mitochondrial inner membranes were more polarised, which could compensate for this (Figure 4.23c,d). The decrease in mitochondrial mass could be attributed to changes in nutrient uptake. For example, iron has been shown to be essential to maintain mitochondrial mass in cultured NK cells (Littwitz-Salomon *et al.*, 2021). Direct co-culture with BMDMs reduced CD71 expression by NK cells (Figure 4.24). CD71 is responsible for the endocytosis of holotransferrin, making iron available to the cell to support DNA synthesis and mitogenesis. In the presence of LPS-stimulated BMDMs there was a significant decrease in the ability of the NK cells to endocytose holotransferrin (Figure 4.25). Expression of *Tfrc* and *Slc40a1* mRNA were measured by RT-qPCR (Figure 4.26). These are the genes for CD71 and ferroportin (an iron exporter) respectively and were measured to determine whether any change in intracellular iron availability was due to changes in uptake or export. This analysis revealed that alongside a decrease in expression of CD71 at protein level there was also a significant decrease in gene expression. There was no detection of *Slc40a1* mRNA in these NK cells suggesting that any changes in total intracellular iron are due to an impairment in iron uptake as opposed to a decrease in iron export (Figure 4.26). To directly measure intracellular iron content samples were provided to the

Cloonan laboratory in Trinity College Dublin for measurement of total intracellular iron by GFAAS. These revealed no significant changes to NK cell iron content following co-culture with BMDMs, regardless of their activation status (Figure 4.27). To determine whether this was also occurring in a more physiological co-culture, splenic NK cells were co-cultured with peritoneal macrophages in the presence of IL12, IL15 and IL18 before CD71 expression was measured. This revealed a significant decrease in expression of CD71 in the presence of macrophages (Figure 4.28). NK cells in the presence of LPS-stimulated peritoneal macrophages had a significant decrease in holotransferrin uptake (Figure 4.29), though it was not as striking as observed in IL2-NK cells in co-culture with BMDMs.

To determine whether any of these metabolic defects were due to a soluble factor being produced by BMDMs, IL2-NK cells were cultured in 70% BMDM-conditioned medium, revealing that the decrease in mitochondrial mass, CD71 expression and holotransferrin uptake were all due to a soluble factor produced by BMDMs (Figure 4.30). Given NK cell effector functions were not impaired in BMDM-conditioned medium, it was determined that the defects in these parameters were not the cause for dampened NK cell effector functions in direct co-cultures. Changes in cMyc expression, due to limited amino acid availability, have been associated with changes in iron uptake and oxidative metabolism (Loftus *et al.*, 2018). Therefore, it was investigated whether amino acid availability in the co-cultures could be limiting, leading to impaired cMyc expression in NK cells in BMDM-conditioned medium. Although there were changes in the amino acid availability in the co-culture medium, they are still present in excess (Figure 4.31).

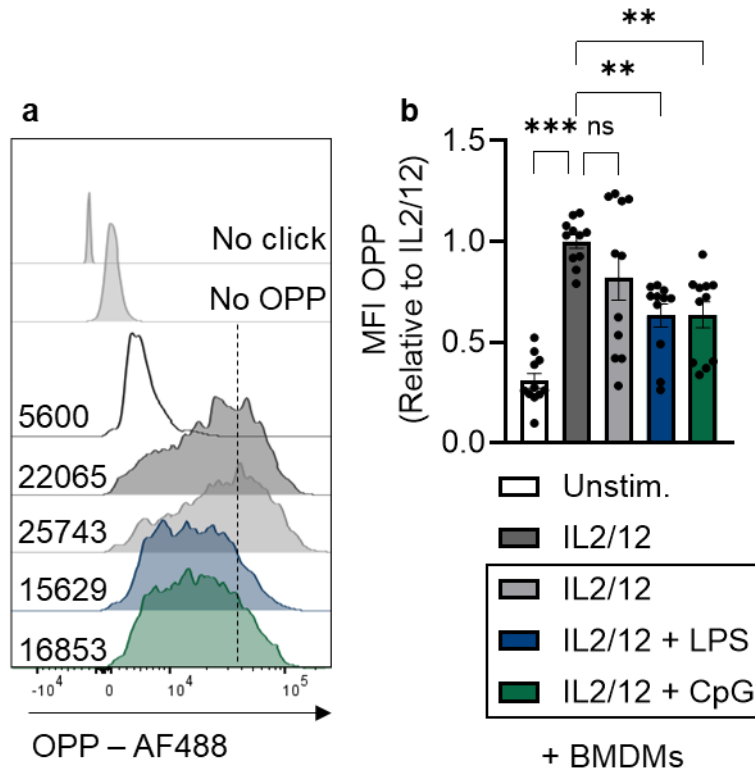


Figure 4.18 IL2-NK cells have impaired rates of protein translation in the presence of BMDMs.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 μ g/mL) for 6 h before co-culture with IL2-NK cells stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for a further 18 h. O-propargyl puromycin (OPP) was administered for 30 mins before cells were fixed. Incorporation of OPP into translated proteins of NK cells was measured using click chemistry and flow cytometry. Data is representative (a) or mean $\pm$ SEM of 11 biological replicates across 4 independent experiments (b). Statistical analysis was done using a one-way ANOVA with Tukey's post-test (** $p < 0.01$, *** $p < 0.001$).

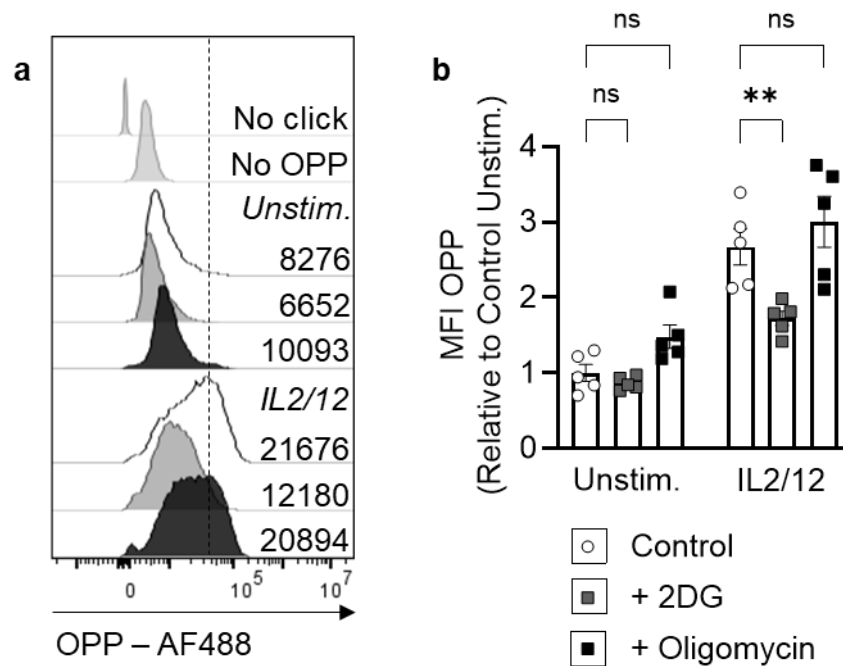


Figure 4.19 IL2-NK cells are reliant on glucose metabolism to support protein translation.

IL2-NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h. Cells were left untreated (white) or treated with 2DG (100 mM) (grey) or oligomycin (1 μ M) (black) for 40 mins. O-propargyl puromycin (OPP) was administered for the final 30 mins of the incubation. OPP incorporation into translated proteins was measured using click chemistry and flow cytometry. Data is representative (a) or mean $\pm$ SEM of 5 biological replicates across 2 independent experiments (b). Statistical analysis was performed using a two-way ANOVA with Tukey's post-test (**p<0.01, ns=non-significant). *Some replicates were performed by Ella Rogerson, Finlay Lab, Trinity College Dublin.*

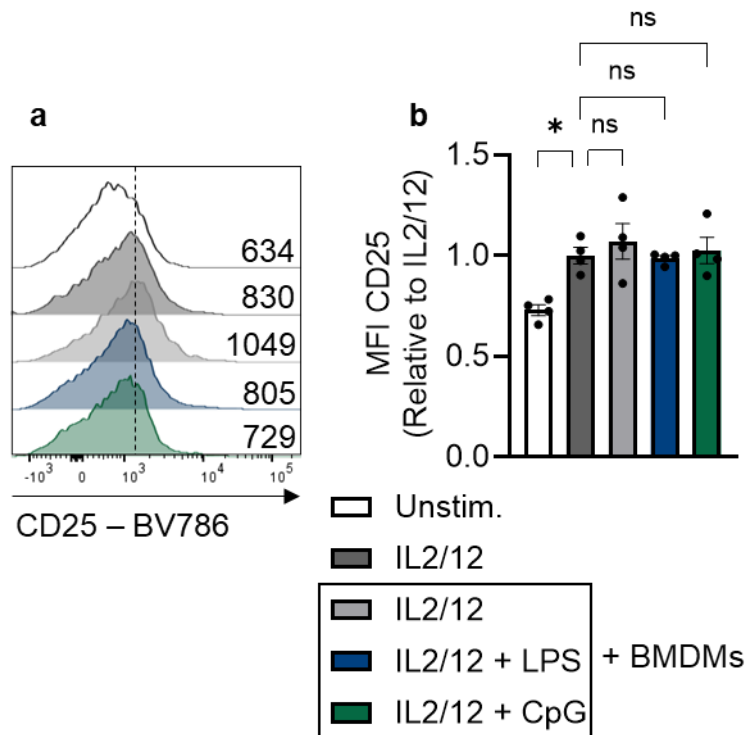


Figure 4.20 IL2-NK cells do not change expression of CD25 in the presence of BMDMs.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 μ g/mL) for 6 h before co-culture with IL2-NK cells that were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h. CD25 expression was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 4 biological replicates across 2 independent experiments (b). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (* p <0.05, ns=non-significant).

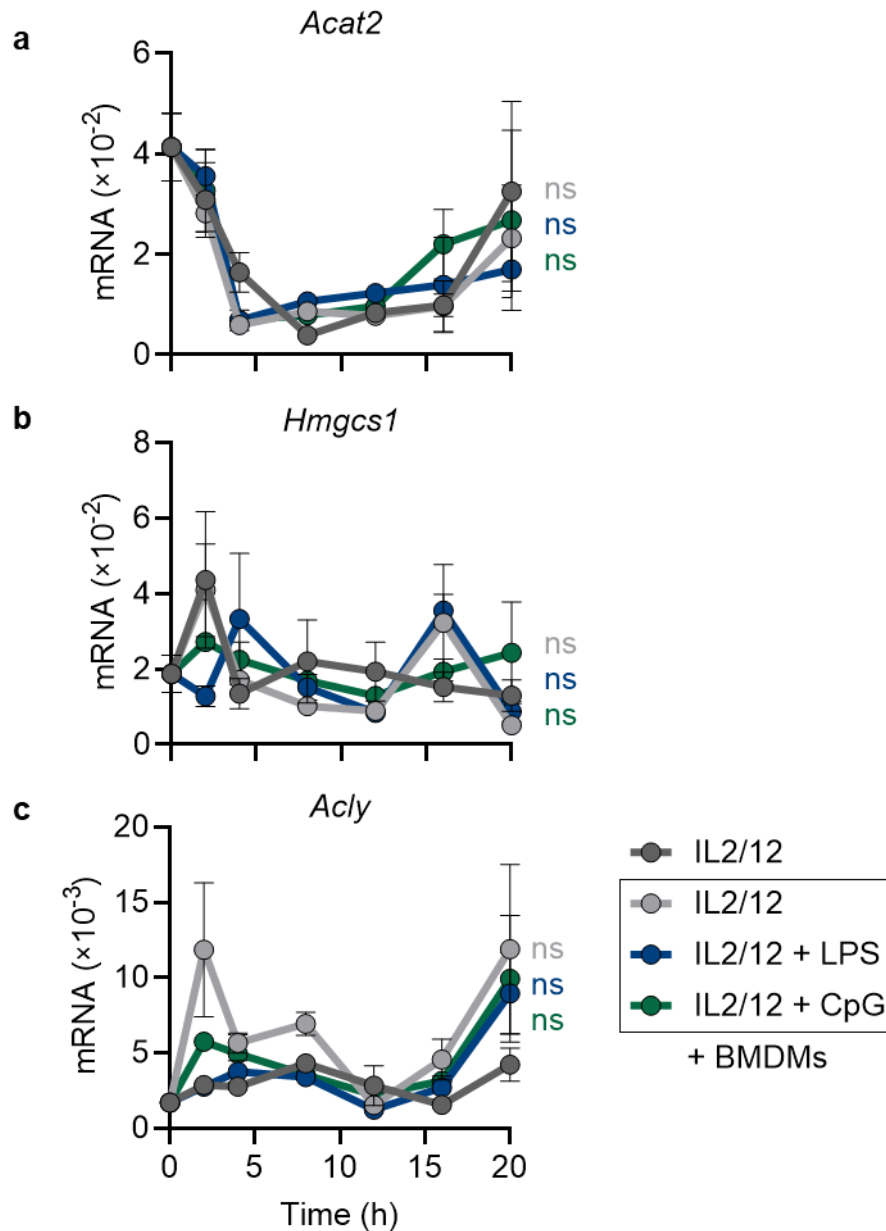


Figure 4.21 IL2-NK cells do not have altered expression of SREBP target genes when co-cultured with BMDMs.

IL2-NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and were co-cultured with BMDMs that were either unstimulated or pre-stimulated for 6 h with LPS (10 ng/mL) or CpG (4 µg/mL). Cells were co-cultured for 18 h before being lysed and analysed by RT-qPCR for expression of *Acat2* (a), *Hmgcs1* (b), and *Acly* (c) mRNA. Data is normalised to levels of control genes *Gapdh* and *Rplp0*. Data is mean $\pm$ SEM of 4 biological replicates. Statistical analysis was performed using a two-way ANOVA. All comparisons shown on the graph are made versus IL2/12-stimulated NK cells only (ns=non-significant).

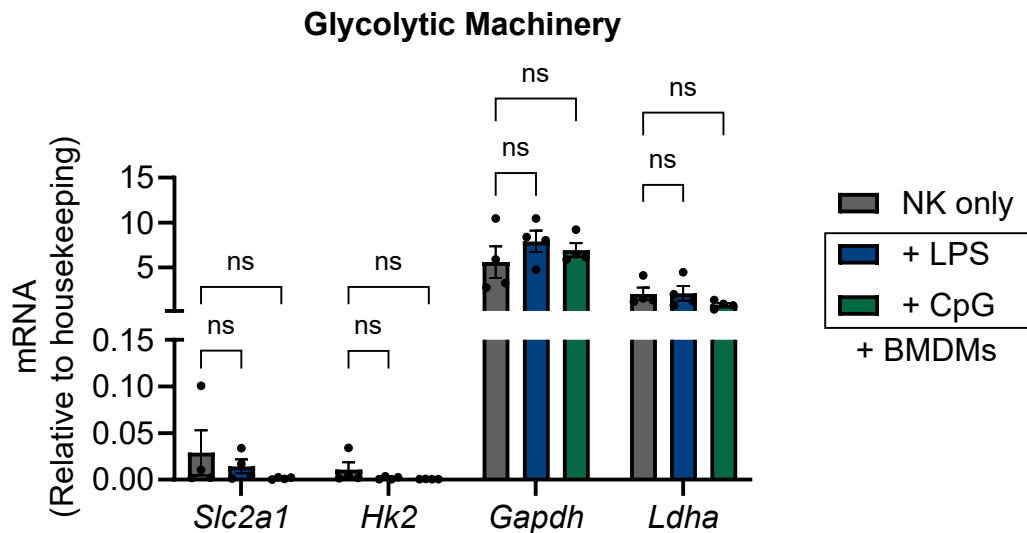


Figure 4.22 IL2-NK cells do not have altered expression of glycolytic machinery mRNA following co-culture with BMDMs.

IL2-NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and were co-cultured with BMDMs that were pre-stimulated for 6 h with LPS (10 ng/mL) or CpG (4 µg/mL). Cells were co-cultured for 18 h before being lysed and analysed by RT-qPCR for expression of *Slc2a1*, *Hk2*, *Gapdh* and *Ldha* mRNA. Data is normalised to levels of control genes *Hprt* and *Rplp0*. Data is mean $\pm$ SEM of 4 biological replicates across 2 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (ns=non-significant).

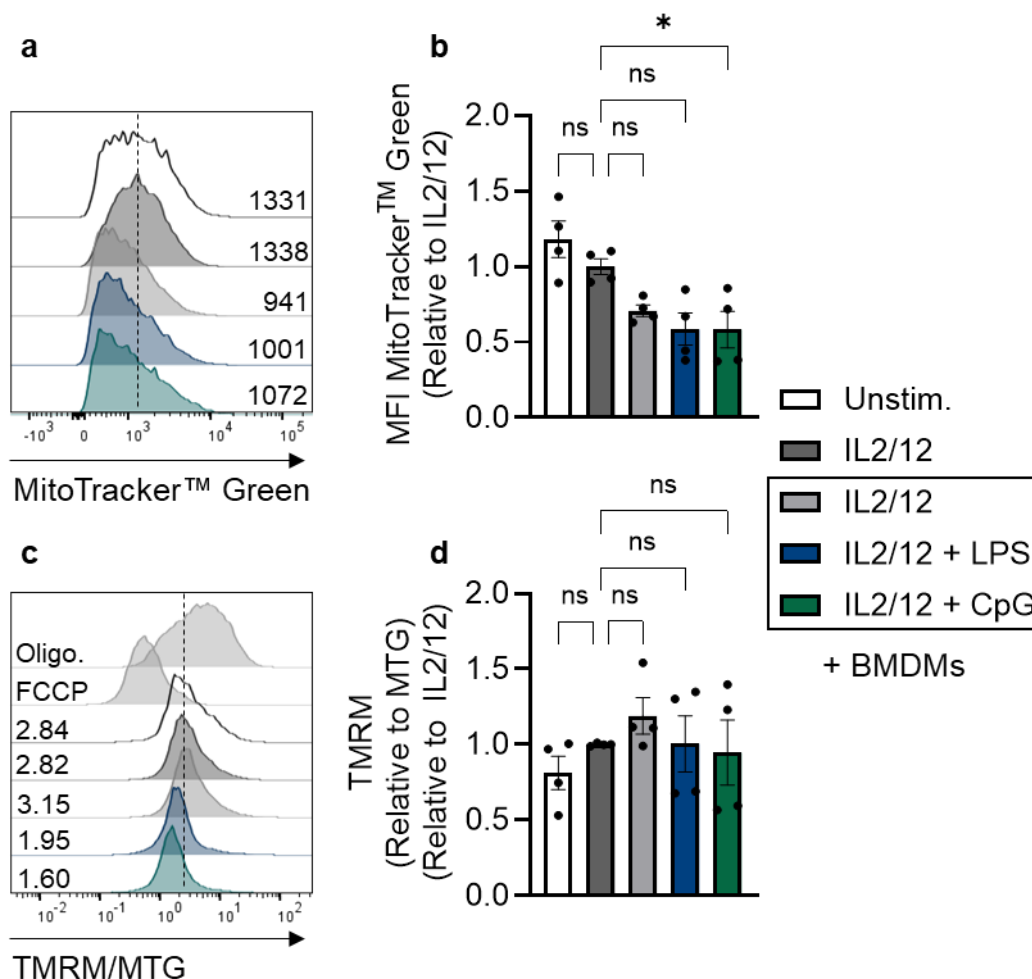


Figure 4.23 IL2-NK cells have reduced mitochondrial mass in the presence of BMDMs.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 µg/mL) for 6 h before co-culture with IL2-NK cells that were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h. MitoTracker™ Green was used as a measure of mitochondrial mass and tetramethyl rhodamine methyl ester (TMRM) was used as a measure of mitochondrial inner membrane potential. Both were measured using flow cytometry. FCCP (2.5 µM) and oligomycin (2 µM) were administered to control IL2/12-stimulated cells to induce hypopolarisation and hyperpolarisation respectively. Data is representative (a,c) or mean $\pm$ SEM of 4 biological replicates across 2 independent experiments (b,d). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (* p <0.05, ns=non-significant).

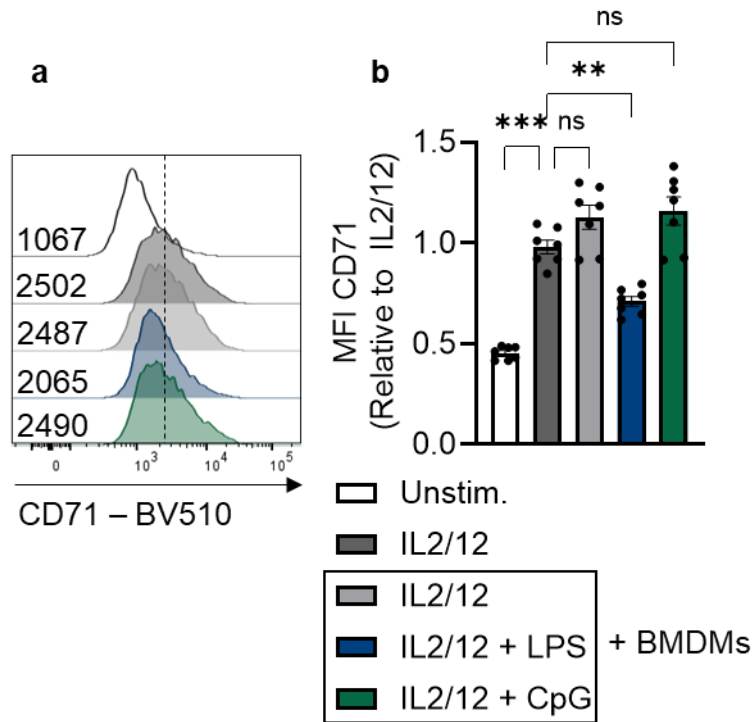


Figure 4.24 IL2-NK cells have impaired CD71 expression in the presence of LPS-stimulated BMDMs.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 μ g/mL) for 6 h before co-culture with IL2-NK cells that were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h. CD71 expression was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 7 biological replicates across 3 independent experiments (b). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (** $p < 0.01$, *** $p < 0.001$, ns=non-significant).

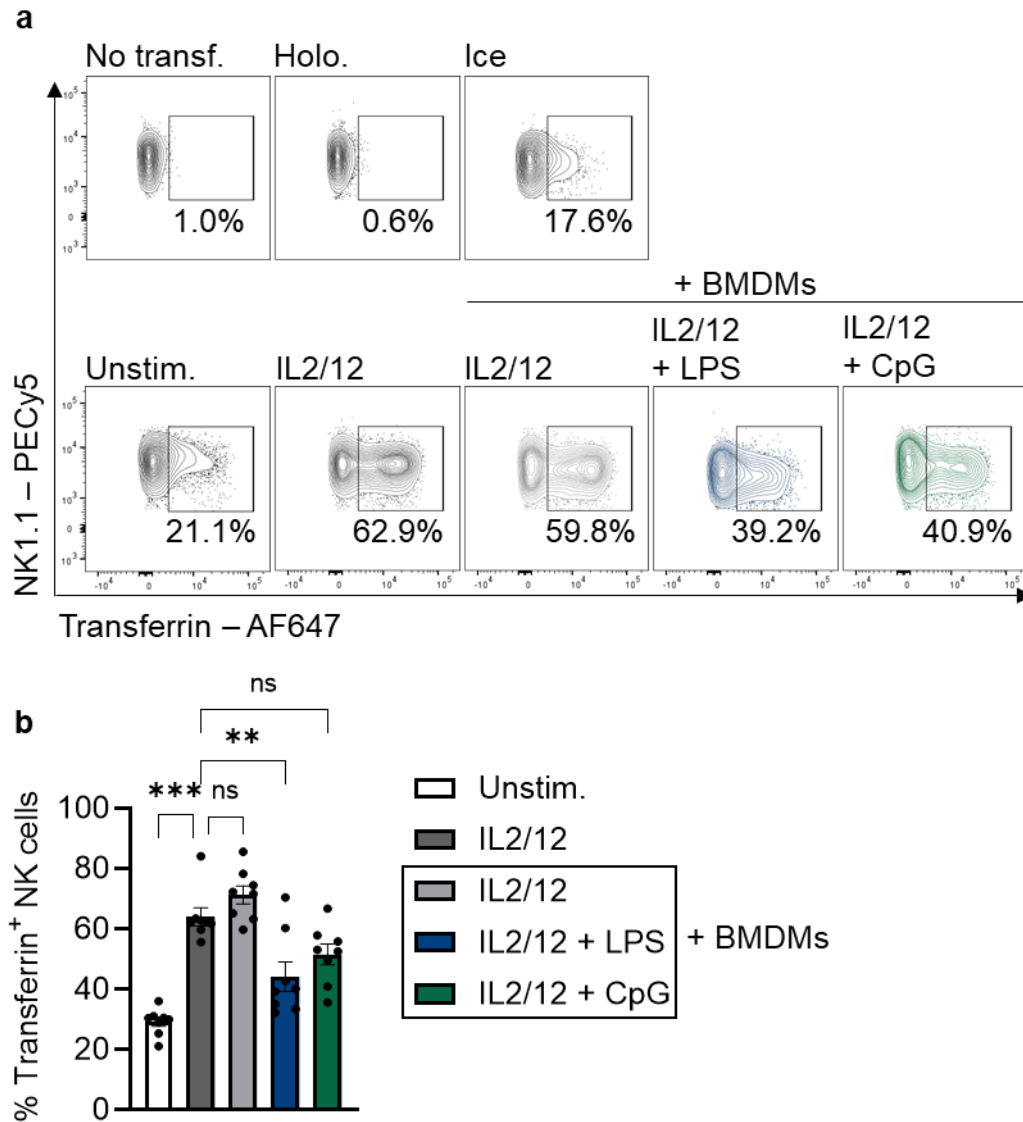


Figure 4.25 IL2-NK cells have impaired holotransferrin uptake in the presence of LPS-stimulated BMDMs.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 µg/mL) for 6 h before co-culture with IL2-NK cells that were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h. Holotransferrin uptake by NK cells was determined by flow cytometry using fluorescently labelled transferrin. Unlabelled holotransferrin was used as a competition control using IL2/12-stimulated NK cells. Data is representative (a) or mean $\pm$ SEM of 8 biological replicates across 3 independent experiments (b). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (** $p < 0.001$, *** $p < 0.001$, ns=non-significant).

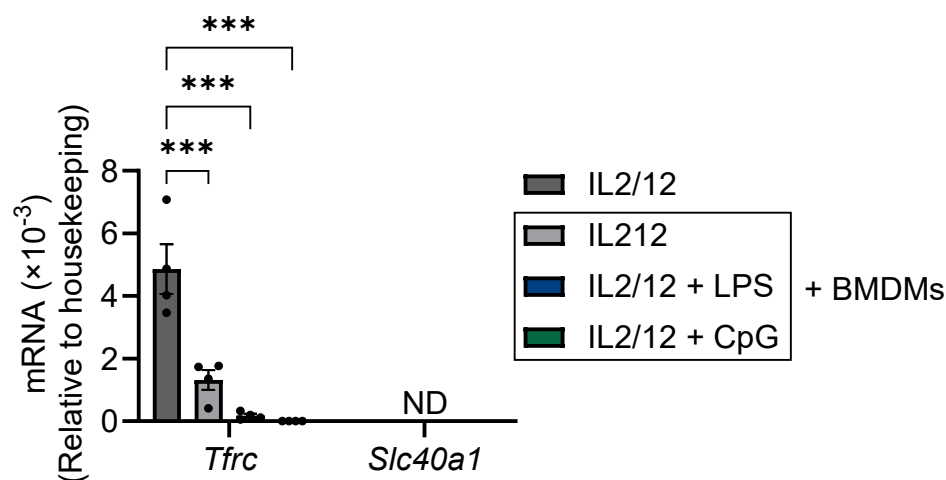


Figure 4.26 IL2-NK cells co-cultured with BMDMs have decreased expression of *Tfr* mRNA.

IL2-NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and were co-cultured with BMDMs that were left unstimulated or pre-stimulated for 6 h with LPS (10 ng/mL) or CpG (4 μ g/mL). Cells were lysed and analysed by RT-qPCR for expression of *Tfr* and *Slc40a1* mRNA. Data is normalised to levels of control genes *Gapdh* and *Rplp0*. Data is mean $\pm$ SEM of 4 biological replicates across 2 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (** $p < 0.001$).

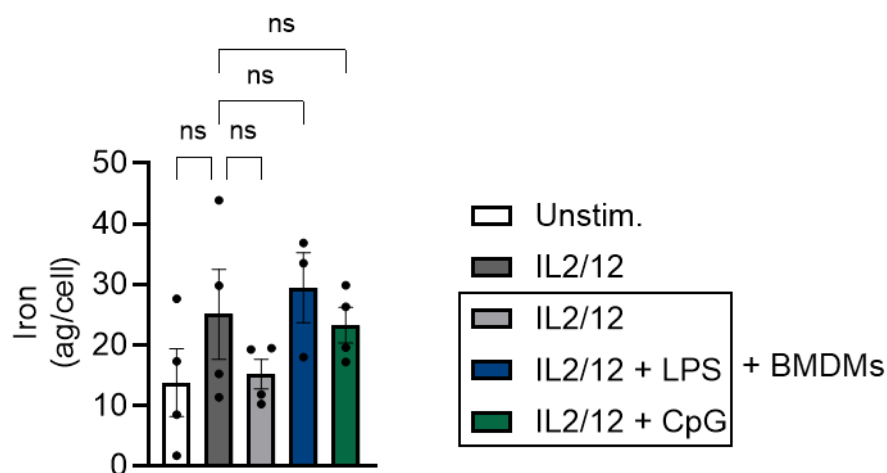


Figure 4.27 IL2-NK cells co-cultured with BMDMs do not alter intracellular iron content.

IL2-NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and were co-cultured with BMDMs that were left unstimulated or pre-stimulated for 6 h with LPS (10 ng/mL) or CpG (4 μ g/mL). Cells were then co-cultured for 18 h before being lysed and analysed by GFAAS for total iron. Data is mean $\pm$ SEM of at least 3 biological replicates across 3 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (ns=non-significant). *This data was generated using samples provided by Cathal Keane to Dr Sarah Kenny, Cloonan Laboratory, Trinity College Dublin.*

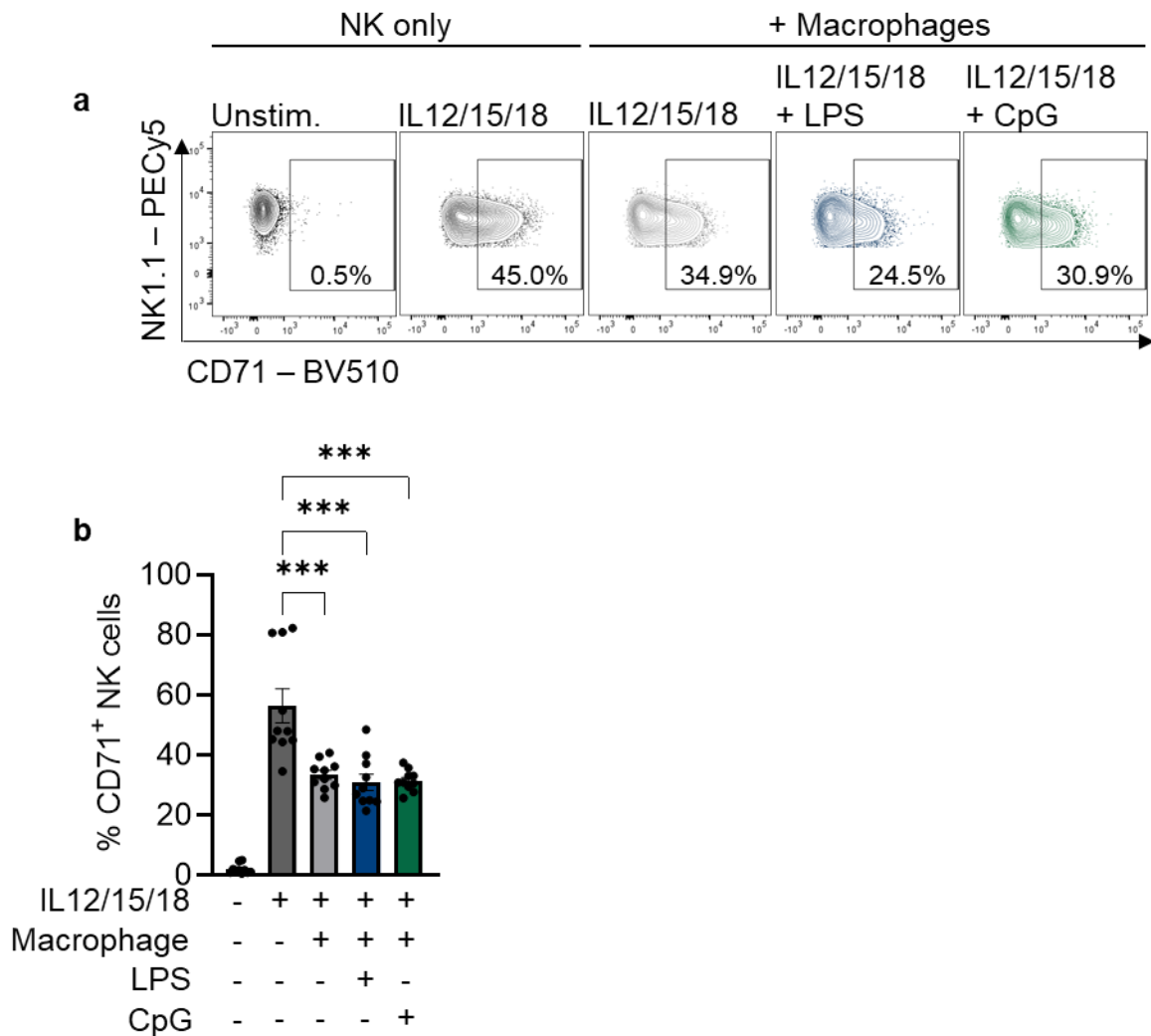


Figure 4.28 Splenic NK cells have reduced CD71 expression in the presence of peritoneal macrophages,

NK cells were purified from the spleen and were stimulated with IL12 (10 ng/mL), IL15 (10 ng/mL) and IL18 (50 ng/mL) for 18 hours in the presence or absence of macrophages which were purified from the peritoneal cavity. Macrophages were left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL). CD71 expression was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 10 biological replicates across 4 independent experiments (b). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (** p <0.001, * p <0.05 ns=non-significant). *Some replicates were performed with Shauna Mayock, Finlay Laboratory, Trinity College Dublin.*

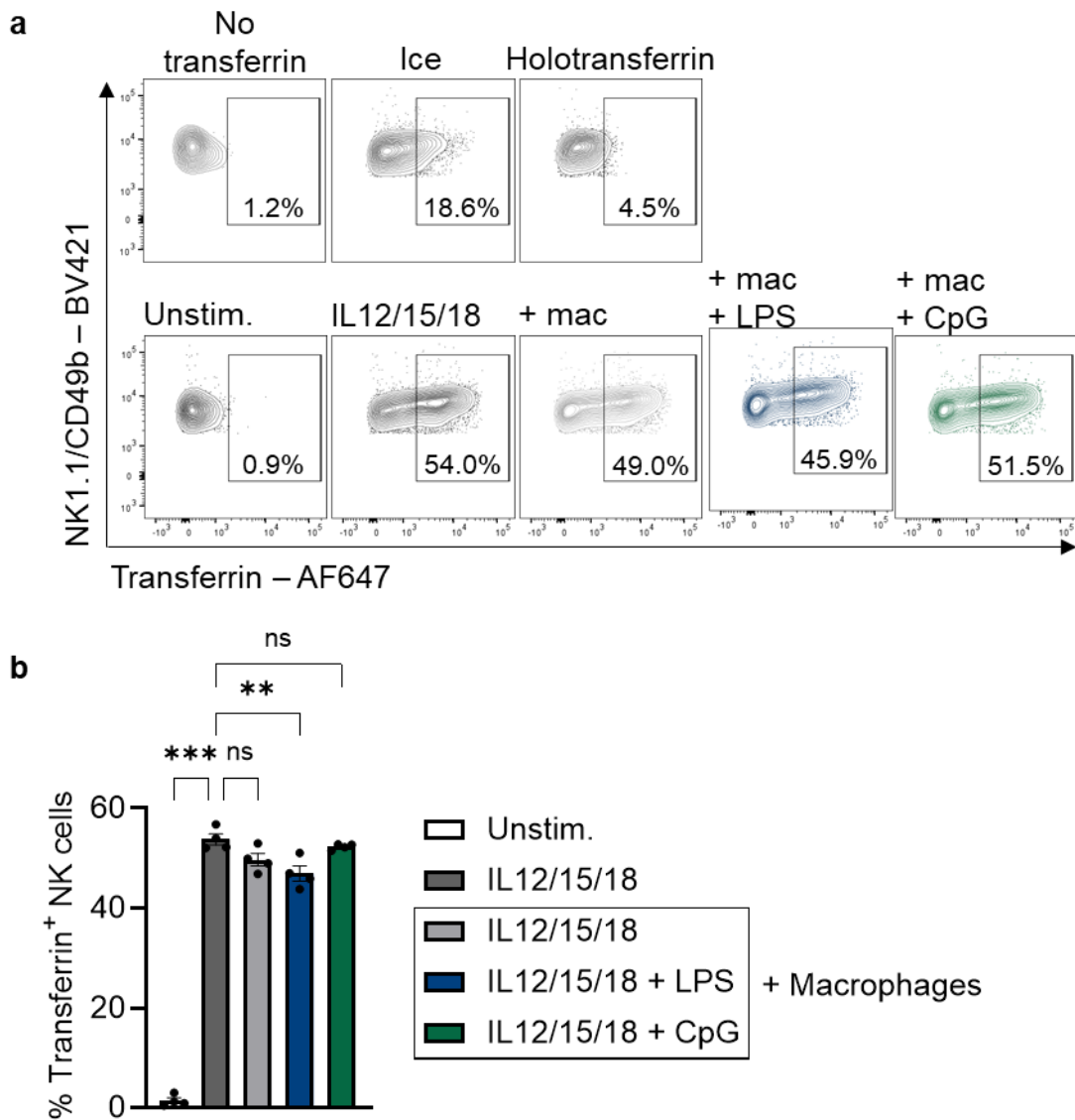


Figure 4.29 Splenic NK cells have modestly reduced holotransferrin uptake in the presence of LPS-stimulated peritoneal macrophages *in vitro*.

NK cells were purified from the spleen and were stimulated with IL12 (10 ng/mL), IL15 (10 ng/mL) and IL18 (50 ng/mL) for 18 hours in the presence or absence of macrophages which were purified from the peritoneal cavity. Macrophages were left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 µg/mL). Holotransferrin uptake by NK cells was determined by flow cytometry using fluorescently labelled transferrin. Unlabelled holotransferrin was used as a competition control using IL12/15/18-stimulated NK cells. Data is representative (a) or mean $\pm$ SEM of 4 biological replicates (b). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (**p<0.001, ***p<0.001, ns=non-significant).

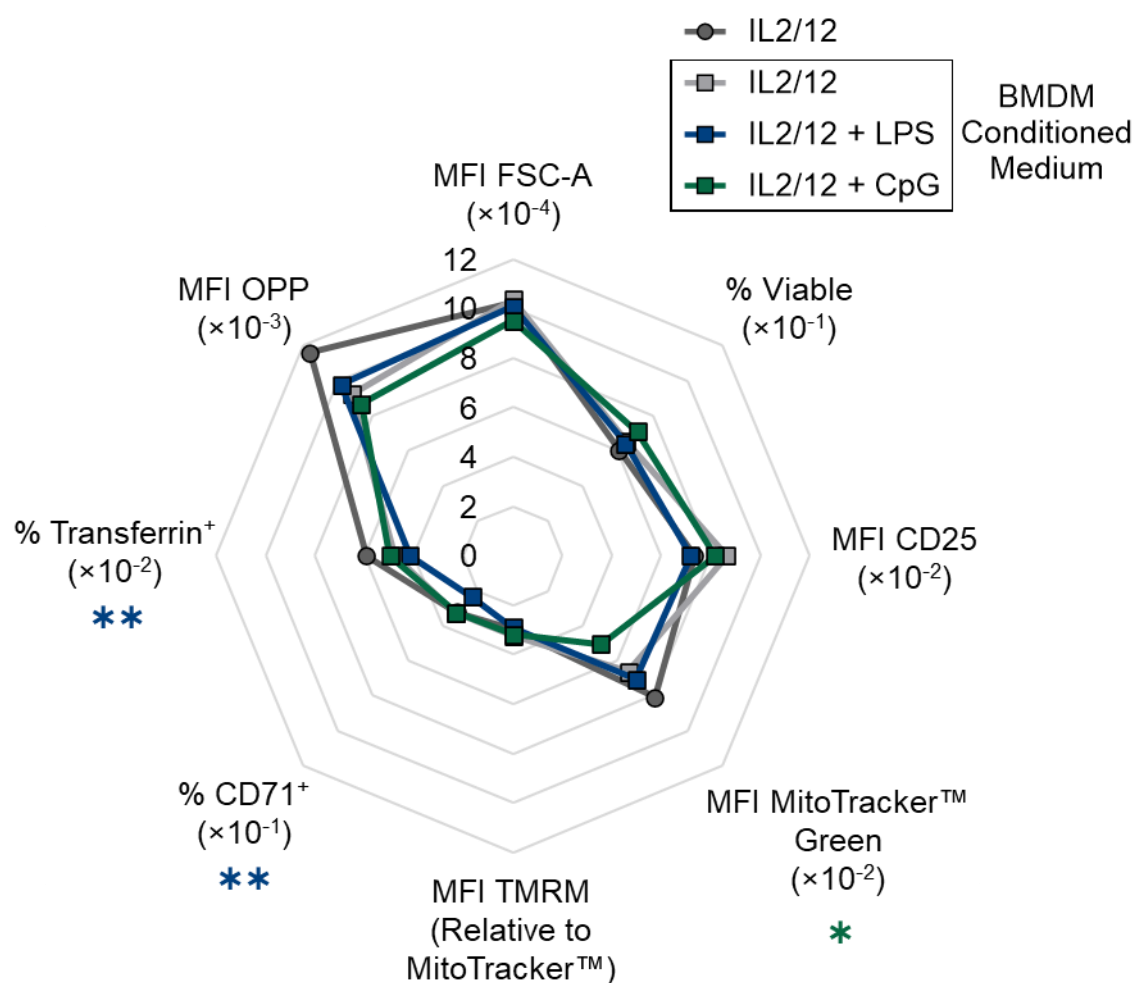


Figure 4.30 BMDM-conditioned medium modestly alters some metabolic parameters of IL2-NK cells.

NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) in the presence or absence of 66% BMDM conditioned medium from BMDMs that were left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL) for 24 h. FSC-A was used as a measure of cell size. Viability was determined by Zombie™ NIR staining. MitoTracker™ Green and TMRM were used as measures of mitochondrial mass and inner membrane potential respectively. OPP incorporation into proteins was used as a measure of protein synthesis. All parameters were measured by flow cytometry. Data is mean of at least 4 biological replicates across at least 2 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. Only statistically significant comparisons were included on the graph and were made versus IL2/12-stimulated NK cells only (*p<0.05, **p<0.001, ns=non-significant).

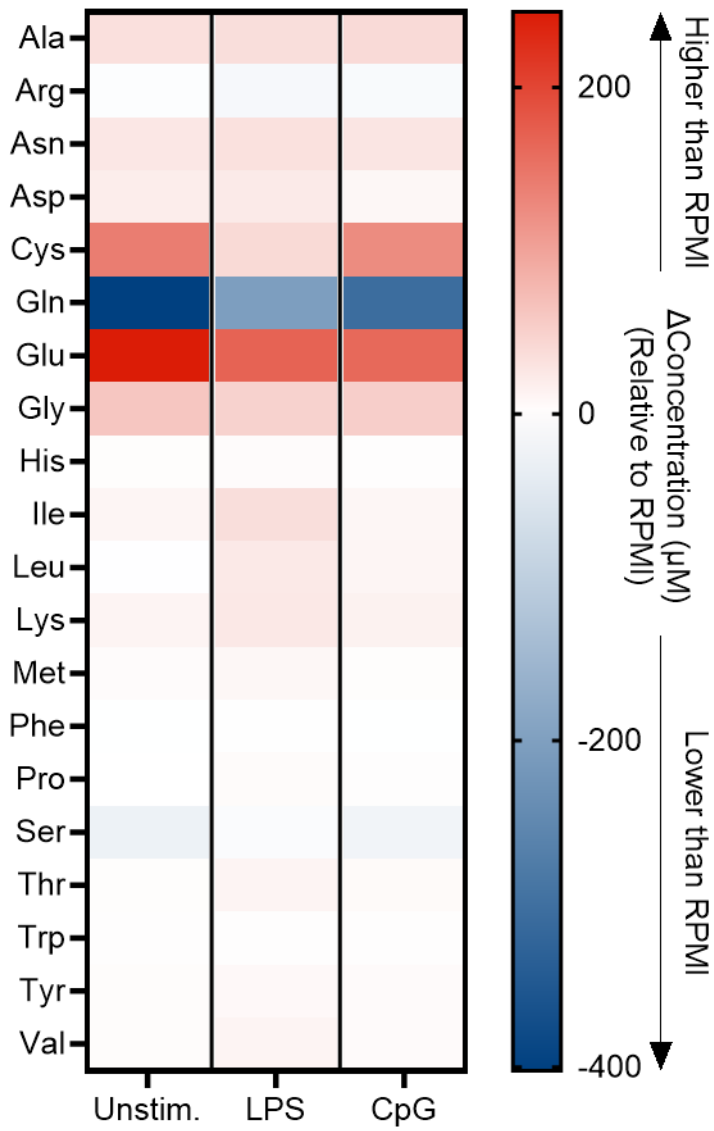
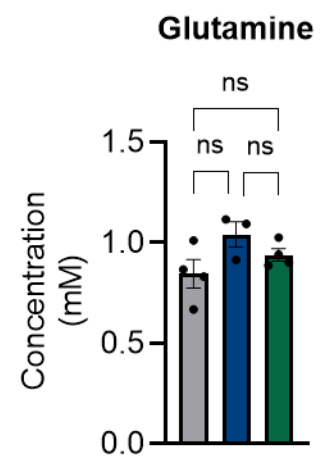
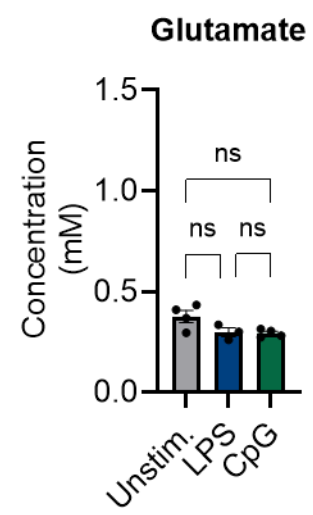
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Figure 4.31 Amino acid availability is not limiting in co-cultures of IL2/12-stimulated NK cells and BMDMs.

BMDMs were left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 µg/mL) for 24 h. IL2-NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 24 h. BMDMs and IL2-NK cells were then co-cultured for 18 h in the absence of FCS. Supernatants were harvested and concentrations of amino acids in the culture medium were measured by LC/MS. Data is corrected for amino acids present in RPMI (a) or is mean +/- SEM of the absolute concentration of glutamine (b) and glutamate (c) for at least 3 biological replicates. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (ns=non-significant). *Metabolomics was performed on samples provided to the Brennan Laboratory, University College Dublin, and was analysed by Cathal Keane.*

4.5 LPS-immunisation increases rates of NK cell metabolism *in vivo*.

Cell size can be interpreted as a measure of NK cell metabolic rate as following activation NK cells are known to undergo blastogenesis, a process that requires increased nutrient uptake and biosynthetic metabolism. FSC-A, measured by flow cytometry can be used as a crude measure of cell size. In mice immunised with LPS there was a significant increase in NK cell size in both the spleen and the peritoneum, with peritoneal NK cells in LPS-immunised mice being slightly bigger than splenic NK cells (Figure 4.32). Protein translation rates, measured by the incorporation of OPP into translated proteins were also used as a surrogate indicator of NK cell anabolic metabolism. In agreement with NK cell functional data in this chapter, peritoneal NK cells also showed higher rates of protein translation than splenic NK cells in mice immunised with LPS (Figure 4.33).

The frequency of CD71 expressing NK cells in the spleen is slightly increased following LPS-immunisation, however, there is a much higher increase in the frequency of CD71-expressing NK cells in the peritoneum indicating that these cells have increased iron requirements (Figure 4.34). Measurements of holotransferrin uptake revealed comparable frequencies of holotransferrin-positive NK cells in both the spleen and peritoneum, although peritoneal NK cells showed a greater amount of holotransferrin uptake following LPS-immunisation (Figure 4.35). This increase in iron uptake can indicate an increase in processes such as proliferation or oxidative metabolism. In fact, most iron-binding proteins in NK cells are found in the mitochondria, where iron forms an important component of Fe-S clusters in the ETC. Assessment of mitochondrial mass using acridine orange revealed that peritoneal and splenic NK cells had comparable mass that does not significantly increase following LPS-immunisation (Figure 4.36a,b). Using TMRM to assess the polarisation of the mitochondrial inner membrane in these NK cells also revealed that there was no significant increase in polarisation in either NK cells from the spleen or peritoneum (Figure 4.36c,d). Furthermore, the peritoneal NK cells had less maximal polarisation than splenic NK cells when looking at oligomycin-induced hyperpolarisation (Figure 4.36c).

To test the relative importance of glycolysis or OxPhos for sustaining NK cell effector function, peritoneal cells were exposed to 2DG or oligomycin for 4 h *in vitro* following LPS-immunisation *in vivo*. While 2DG treatment had no impact on any NK cell effector outputs, treatment with oligomycin resulted in a significant decrease in granzyme B expression (Figure 4.37).

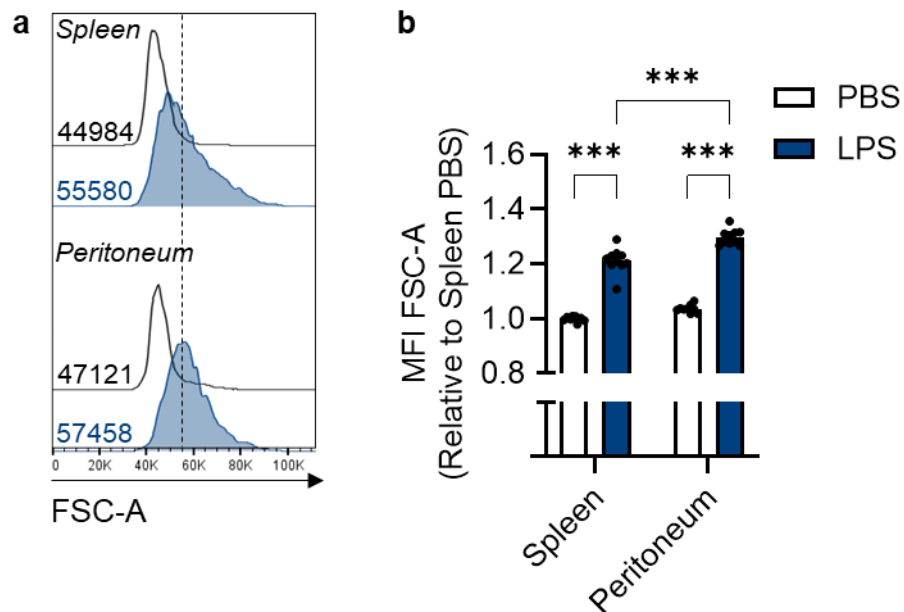


Figure 4.32 Peritoneal and splenic NK cells from LPS-immunised mice increase cell size.

Mice were administered PBS or LPS (2.4 mg/kg) by intraperitoneal injection. Spleens and peritoneal cells were harvested 24 h post-administration. FSC-A was used as an indicator of cell size and was measured in NK cells (CD3⁻, CD19⁻, CD49b⁺, NK1.1⁺) by flow cytometry. A minimum of 9 mice were used per group across 2 independent experiments. Data is representative (a) or mean $\pm$ SEM (b). Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test (** $p < 0.001$).

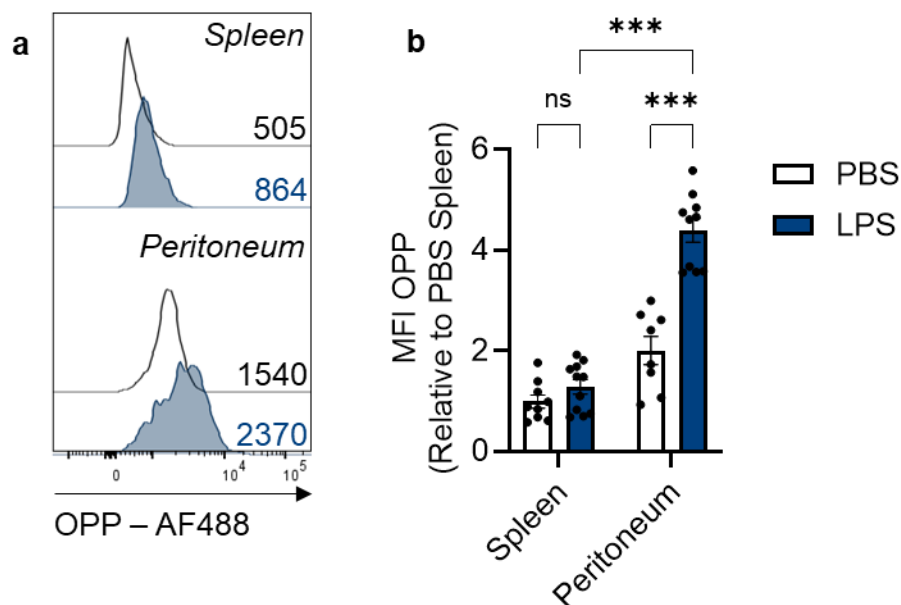


Figure 4.33 Peritoneal NK cells from LPS-immunised mice have increased levels of protein translation.

Mice were administered PBS or LPS (2.4 mg/kg) by intraperitoneal injection. Spleens and peritoneal cells were harvested 24 h post-administration. Incorporation of O-propargyl-puromycin (OPP) into translated proteins of NK cells (CD3⁻, CD19⁻, CD49b⁺, NK1.1⁺) was measured by flow cytometry. A minimum of 8 mice were used per group across 2 independent experiments. Data is representative (a) or mean $\pm$ SEM (b). Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test (**p<0.01, ***p<0.001, ns=non-significant).

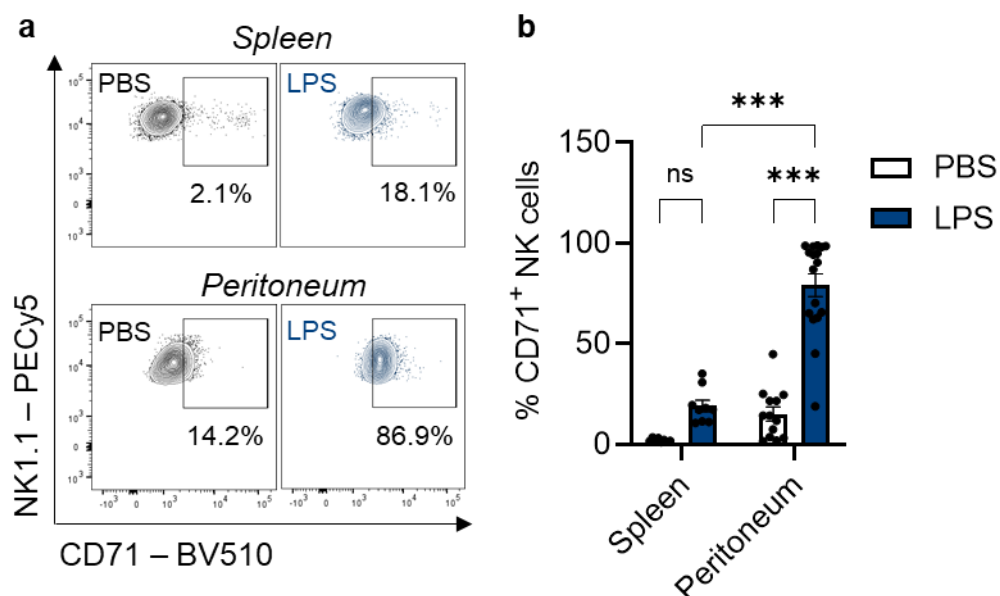


Figure 4.34 Peritoneal NK cells from LPS-immunised mice have elevated expression of CD71.

Mice were administered PBS or LPS (2.4 mg/kg) by intraperitoneal injection. Spleens and peritoneal cells were harvested 24 h post-administration. CD71 expression by NK cells (CD3⁻, CD19⁻, CD49b⁺, NK1.1⁺) was determined by flow cytometry. A minimum of 8 mice were used per group across 4 independent experiments. Data is representative (a) or mean $\pm$ SEM (b). Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test (**p<0.001, ns=non-significant).

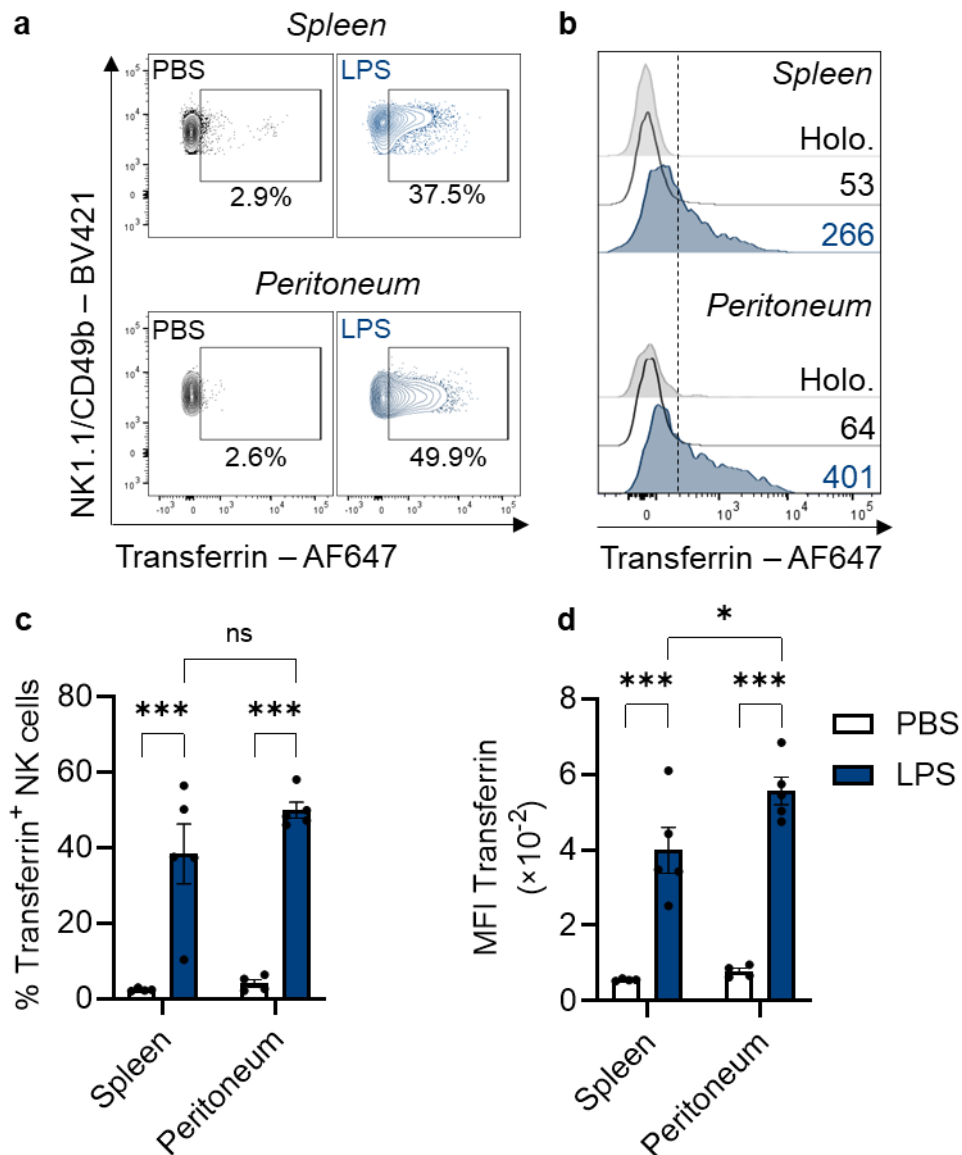


Figure 4.35 Peritoneal NK cells from LPS-immunised mice have elevated levels of holotransferrin uptake.

Mice were administered PBS or LPS (2.4 mg/kg) by intraperitoneal injection. Spleens and peritoneal cells were harvested 24 h post-administration. Transferrin uptake was measured in NK cells (CD3⁻, CD19⁻, CD49b⁺, NK1.1⁺) was determined by flow cytometry. Unlabelled holotransferrin (holo.) was used as a competition control using cells from LPS-immunised mice. A minimum of 4 mice were used per group. Data is representative (a,b) or mean +/- SEM (c,d). Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test (*p<0.05, ***p<0.001, ns=non-significant).

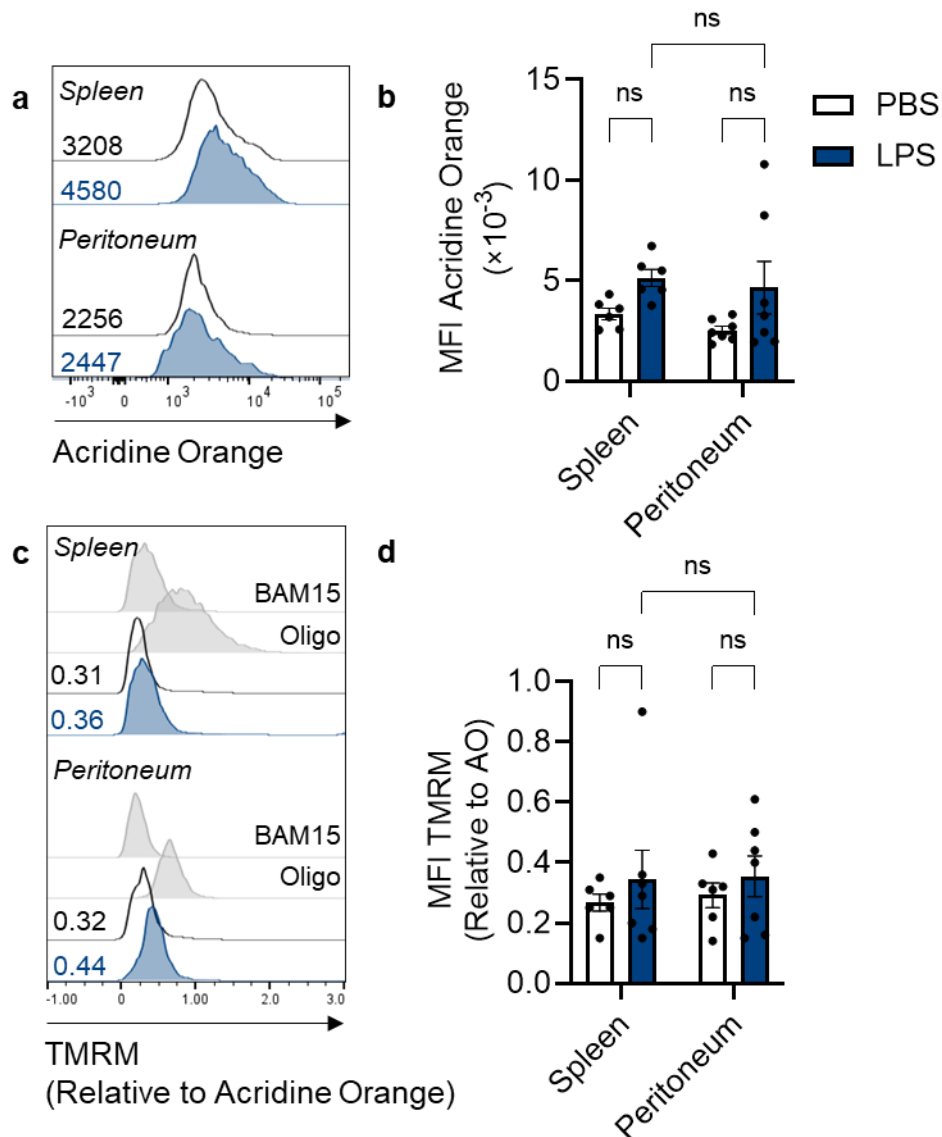


Figure 4.36 Peritoneal NK cells do not increase mitochondrial mass or inner membrane potential following LPS-immunisation.

Mice were administered PBS or LPS (2.4 mg/kg) by intraperitoneal injection. Spleens and peritoneal cells were harvested 24 h post-administration. Acridine orange (AO) and tetramethyl rhodamine methyl ester (TMRM) were used to measure mitochondrial mass (a,b) and mitochondrial inner membrane polarisation (c,d) of NK cells ($CD3^{-}$, $CD19^{-}$, $CD49b^{+}$, $NK1.1^{+}$) respectively by flow cytometry. BAM15 (2.5 μ M) and oligomycin (2 μ M) were administered to control cells from LPS-immunised mice to induce hypopolarisation and hyperpolarisation respectively. A minimum of 6 mice were used per group across 2 independent experiments. Data is representative (a,c) or mean $\pm$ SEM (b,d). Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test (ns=non-significant).

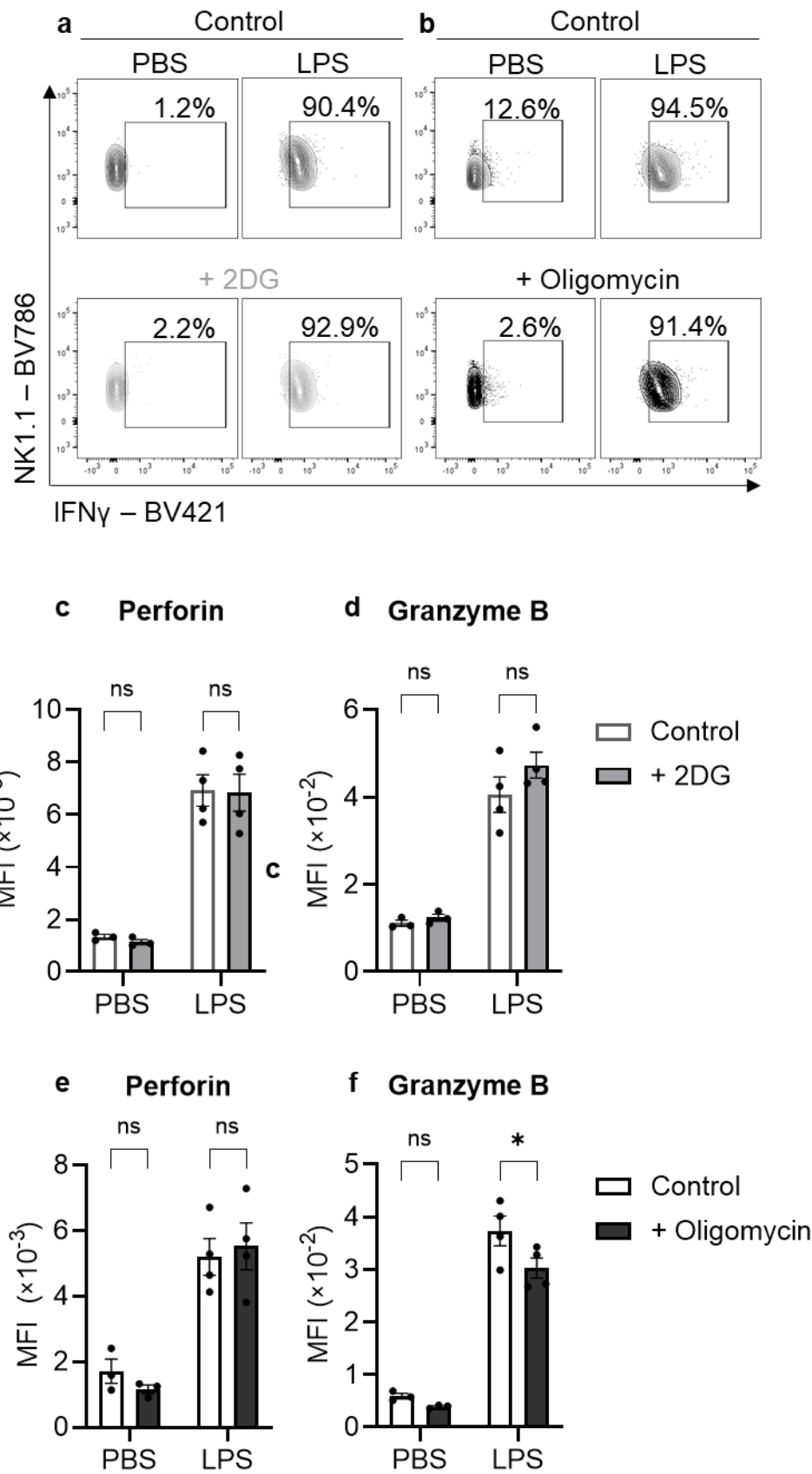


Figure 4.37 Peritoneal NK cells from LPS-immunised mice exhibit plasticity in the metabolic configuration required to support effector molecule production.

Mice were administered PBS or LPS (2.4 mg/kg) by intraperitoneal injection. Spleens and peritoneal cells were harvested 24 h post-administration. Cells were cultured for 4 h in the presence or absence of 2DG (10 mM) (a,c,d) or oligomycin (2 μ M) (b,e,f) before IFN γ (a,b), perforin (c,e) and granzyme B (d,f) expression by NK cells (CD3 $^{-}$, CD19 $^{-}$, CD49b $^{+}$, NK1.1 $^{+}$) was measured by flow cytometry. A minimum of 4 mice were used per group across 2 independent experiments. Data is representative (a, b) or mean $\pm$ SEM (c-f). Statistical analysis was carried out using a two-way ANOVA with Šidák's post-test (* p <0.05, ns=non-significant).

4.6 Discussion

The experiments in this chapter sought to understand the reasons for LPS-stimulated BMDMs dampening IFN γ expression in NK cells in co-culture. The data included in chapter 3 showed how different TLR stimulations provided to BMDMs can elicit different effects on NK cells, suggesting that macrophages may shape NK cell responses depending on the type of infection they are responding to. NK cells are known to play important roles in bacterial infections, though these are not fully characterised. The ability of NK cells to kill virally infected cells is well-described, but they can also kill cells infected with intracellular bacteria such as *Shigella flexneri*, *Legionella pneumophila* and *Mycobacterium avium* (Klimpel *et al.*, 1986; Blanchard *et al.*, 1987; Katz *et al.*, 1990). Furthermore, NK cells can also exert direct bactericidal activity against extracellular bacteria, which has been shown against extracellular *Bacillus anthracis*, *Mycobacterium kansasii* and *Mycobacterium tuberculosis in vitro* (Gonzales *et al.*, 2012; Lu *et al.*, 2014). The production of perforin and granulysin by NK cells is hypothesised to cause interruptions to the outer membrane leading to death of the bacteria (Stenger *et al.*, 1998; Lu *et al.*, 2014). The most well-described role of NK cells in bacterial infection is the production of pro-inflammatory mediators such as IFN γ , MIP-1 and GM-CSF, which are important to support sustained activation of macrophages (Bermudez *et al.*, 1995; Bluman *et al.*, 1996; Lai *et al.*, 2018). IFN γ in particular is known to promote the activation of macrophages in combination with TLR agonists, and can promote macrophage killing of bacteria through the induction of nitric oxide (Liew, Li, *et al.*, 1990; Liew, Millott, *et al.*, 1990). The production of IFN γ by NK cells in bacterial infection has been shown to rely on macrophage activation, through their production of IL12 and IL18 (Nomura *et al.*, 2002). In this way NK cell-macrophage crosstalk can lead to enhanced killing of bacteria, though there are a number of studies which demonstrate that NK cell activation is a significant risk factor for progression to sepsis. Taking this into account, these experiments sought to characterise the communication that exists between pro-inflammatory macrophages and NK cells to understand whether immunoregulatory axes exist between the two cells.

Killing of RMA/S target cells is well characterised as they are engineered to lack expression of MHC class I molecules, making them susceptible to NK cell-

mediated killing (Kärre *et al.*, 1986). The data here shows that in the presence of BMDMs, NK cells do not alter their killing of target cells. However, if they are only exposed to soluble factors produced by BMDMs, they have enhanced killing of their targets. This suggests that direct contacts with macrophages may be limiting NK cell cytotoxicity. Notably, cytotoxicity assays were carried out using NK cells that had been removed from co-culture with BMDMs to prevent engagement of BMDM-derived MHC class I molecules with Ly49 receptors which could otherwise distract the NK cells from killing RMA/S target cells. It is known that macrophages can express some NKG2D ligands which are hypothesised to enhance IFN γ -mediated activation of macrophages in *Bacillus anthracis* infections (Klezovich-Bénard *et al.*, 2012). In tumours, TAMs were shown to express and shed the ligands CD48 and RAE1 which can activate NKG2D and 2B4 on NK cells respectively. While this was beneficial for early activation of NK cells, prolonged engagement led to desensitisation of NK cells through decreasing NKG2D expression, ultimately preventing their recognition of tumour target cells (Deng *et al.*, 2015). It is possible that prolonged interactions between NK cells and BMDMs in the co-cultures in this chapter desensitised NK cells to certain ligands expressed on RMA/S target cells leading to suppressed killing. However, if these ligands are shed by macrophages, it is likely that the desensitisation would have also occurred in both trans-well co-cultures and cultures in BMDM-conditioned medium. Furthermore, macrophages have been shown to express Qa-1, an NKG2A ligand, which will lead to suppression of NK cells, protecting themselves from NK cell-mediated cytotoxicity (Zhou *et al.*, 2012). Similar to chronic exposure to activating ligands, chronic exposure to the inhibitory ligand HLA-E in acute myeloid leukaemia has been shown to cause NK cell exhaustion through sustained NKG2A engagement (Xie *et al.*, 2025). Whether the suppression of NK cell killing is an artefact of overnight co-culture of the cells, or a real biological effect is not yet understood.

In the context of bacterial infection, it is not known whether macrophages can directly enhance NK cell bactericidal functions. This is due in part to insufficient understanding of the mechanism NK cells use to recognise bacteria. It has been suggested that the expression of a range of PRRs by NK cells is sufficient to drive cytotoxicity (Sivori *et al.*, 2004; Hart *et al.*, 2005). Human NK cells are known to

express a wide range of TLRs, which enhanced killing of K562 target cells *in vitro* (Sivori *et al.*, 2004; Lauzon *et al.*, 2006). However, whether TLR signalling serves to prime NK cells or actually induce direct bacterial killing is not known. NKp44 has also been described to bind to ligands on the surface of *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* (Esin *et al.*, 2008). It is difficult to make parallels between killing of mammalian target cells and bacteria given the lack of understanding of how NK cells recognise bacterial targets. Regardless, NK cell killing of bacterially infected cells is another important aspect of host defence to infection. Therefore, this data suggests that macrophages may use soluble factors to enhance this arm of the NK cell response.

Aside from killing, this chapter also investigated whether macrophages were using direct interactions or soluble factors to influence NK cell expression of effector molecules. This data was a lot less clear due to inconsistencies between direct co-cultures, trans-well co-cultures and culture of NK cells in BMDM-conditioned medium. Trans-well co-cultures inhibited IFN γ production by NK cells, though there was no change in IFN γ production when NK cells were cultured in BMDM-conditioned medium. It is possible that there is a short-lived soluble factor that is acutely inhibiting NK cell IFN γ production in the trans-well co-cultures that is not present in the conditioned medium. The differences between trans-well co-cultures and direct co-cultures are more difficult to reconcile. LPS- but not CpG-stimulated BMDMs were shown to consistently inhibit IFN γ production by NK cells in direct co-culture throughout this thesis. Therefore, it is interesting that in trans-well co-cultures there is a stronger inhibition of IFN γ production, regardless of the TLR agonist used to stimulate the BMDMs. It is possible that this is an artefact of the co-culture set-up. In trans-well co-cultures, there are 1×10^6 NK cells present in a much smaller area, which may potentially impact the activation of the NK cells. This is unlikely as studies have shown that pure populations of NK cells have enhanced activation in response to IL2 when cultured at higher cell densities (Kim *et al.*, 2014). It should also be considered that the four biological replicates in the trans-well co-culture were performed as part of one independent experiment and require further replicates to confirm whether this effect is real. As already mentioned, throughout this thesis it was consistently shown that direct interactions with LPS-stimulated

macrophages dampened IFN γ production by NK cells, and this result was further investigated in subsequent experiments. The use of more physiological cells in co-cultures also showed slightly different results to co-cultures between IL2-NK cells and BMDMs. Peritoneal macrophages were also shown to dampen IFN γ production by splenic NK cells regardless of the TLR agonist used. Again, this data strengthens the hypothesis that direct interactions with LPS-stimulated macrophages exerts an inhibitory effect to NK cell IFN γ production.

NK cells have been described to be the primary source of IFN γ in many bacterial infections such as *Listeria monocytogenes*, *Chlamydia pneumoniae* and *Yersinia enterocolitica* (Teixeira and Kaufmann, 1994; Bohn and Autenrieth, 1996; Rottenberg *et al.*, 2000; Berg *et al.*, 2005). Inhibition of NK cell IFN γ production by macrophages seems to contradict the concept of NK cell-macrophage crosstalk being required to enhance immune cell activation and subsequent clearance of bacteria or infected cells. However, there is emerging evidence that NK cells can support the immune response early in infection but can contribute to hyperinflammation and sepsis if their activation remains unregulated (Badgwell *et al.*, 2002; Sherwood *et al.*, 2004; Goldmann *et al.*, 2005; Enoh *et al.*, 2006; Christaki *et al.*, 2015). It is also interesting that direct interactions between macrophages and NK cells reduce the frequency of IFN γ -producing NK cells, but the amount of IFN γ being produced is not significantly altered in this chapter. This suggests that only a proportion of NK cells are responsive to the inhibitory factor produced by macrophages. Assessment of IFN γ production by peritoneal NK cells following injection of LPS, showed slightly different results with almost all NK cells producing IFN γ , but the amount produced being relatively low. Although both *in vitro* and *in vivo* models show slightly different results, it does remain possible that macrophages are preventing further IFN γ production by NK cells. Demonstrating this effect *in vivo* is difficult as inhibiting activation of peritoneal macrophages would also mean NK cells do not receive signals from innate cytokines to initiate their own activation. Some of the macrophage-derived anti-inflammatory mechanisms that NK cells are likely exposed to in bacterial infections include IL10, TGF β , prostaglandin E $_2$ and prostaglandin D $_2$ (Lauzon and Lemaire, 1994; Yingying Chen *et al.*, 2007; Chiu *et al.*, 2008). These studies have shown that these soluble factors can be produced by macrophages and can

inhibit NK cells *in vitro*. However, the mechanisms of crosstalk between NK cells and macrophages in more complex immune environments, such as active infections are likely more complicated.

This thesis shows the use of LPS-induced peritonitis to model a complex immune environment where NK cells and macrophages are interacting. The dose of LPS administered to mice in this chapter does mimic sepsis in mice. LPS is a bacterial endotoxin, meaning it does not mimic later stages in sepsis, as there is no active infection occurring. However, it is described to be a good model for acute phase sepsis, following infection with Gram-negative bacteria (Aydın and Bekmez, 2023). This is reflected in the cytokine measurements in the peritoneal lavage in this context. Production of IL12 and IFN γ is associated with the early stages of this model, which may explain why they were not detected in the lavage fluid. Furthermore, the collection of the peritoneal lavage involves a large dilution of the small amount of peritoneal fluid found *in vivo*, meaning the lack of detection of certain cytokines in this chapter does not necessarily mean that they are not found at higher concentrations physiologically. The fact that IL2 is detected highlights how abundant it is at this time point. Again, the detection of IL2 highlights how transient the levels of inflammation are in this model, as IL2 production is associated with later stages of infection (Charley *et al.*, 2024). It is also possible that the large amount of IL2 present is associated with expansion of T_{reg} cells in the peritoneal cavity which are associated with resolving inflammation (Churlaud *et al.*, 2014).

Peritoneal macrophages are a well-studied macrophage population, and their activation is well characterised. Following LPS-immunisation it is known that the frequency of LPMs is significantly reduced due to the formation of clots within the peritoneum, leaving SPMs to carry out their pro-inflammatory functions (Zhang *et al.*, 2019). In this chapter, SPMs were shown to produce IL12 as late as 24 h following LPS-immunisation, across all doses used. Other pro-inflammatory cytokines produced by SPMs were shown to be associated with early response to infection. It has been shown that IL12 production by macrophages is necessary, but not sufficient to drive NK cell IFN γ production (Lapaque *et al.*, 2009). This same study showed that both IL12 and IL18 production by *Salmonella enterica*-infected monocyte-derived macrophages acted

synergistically to enhance IFN γ production by NK cells. Therefore, it is not surprising that there is considerable NK cell activation even at this late time point correlating with the high levels of IL12 production by SPMs observed in the peritoneum. The activation of NK cells in LPS-induced peritonitis is not as well-described, though it is known that they are the primary source of IFN γ (Emoto *et al.*, 2002). This feed-forward loop between NK cells and macrophages can lead to uncontrolled inflammation, leading to organ damage and septic shock (Souza-Fonseca-Guimaraes *et al.*, 2012). It is not known what exact mechanisms macrophages can use to directly inhibit NK cell IFN γ production to prevent progression to sepsis. While it has been shown that anti-inflammatory soluble mediators can alter NK cell production, this chapter suggests that direct interactions with macrophages could limit NK cell IFN γ production, and act to resolve inflammation.

IFN γ expression by NK cells has been linked with cellular metabolism in numerous studies (Marçais *et al.*, 2014; Keppel *et al.*, 2015; Keating *et al.*, 2016; Assmann *et al.*, 2017; Marçais *et al.*, 2017; Loftus *et al.*, 2018). Given that direct interactions with macrophages were dampening IFN γ production by NK cells, metabolic parameters were investigated in NK cell-macrophage co-cultures. As previously described, the rate of protein translation in NK cells was used as a surrogate measurement of ATP production (Buttgereit and Brand, 1995; Argüello *et al.*, 2020). Considering NK cell cytotoxicity and production of cytotoxic molecules were broadly preserved in co-cultures with BMDMs, the extent of the decrease in rates of protein translation was surprising. It suggests that NK cells may be able to direct protein translation and rates of ATP production to essential processes such as cytotoxicity. There are a number of ways that eukaryotic cells have been described to selectively translate certain proteins. For example, activation of translation factors has been described to control expression of certain mRNAs in immune cells. While they are often thought to non-specifically control protein translation, certain translation factors are required to support translation of a specific subset of mRNAs (Zimmer *et al.*, 2000; Dever, 2002). eIF4E is one such translation factor that has been shown to enhance expression of IRF7 as it has an eIF4E-sensitivity element in its 3' untranslated region (UTR). In this way eIF4E has been shown to control the type I interferon response in

plasmacytoid DCs following viral infection in mice (Colina *et al.*, 2008). Activation of eIF5A in metabolically inactive NK cells was shown to enhance IFN γ production and cytotoxicity, while expression of metabolic proteins such as CD71 were unchanged (O'Brien, 2019; O'Brien *et al.*, 2021). This suggests that under times of metabolic stress, cells may be able to control translation of proteins that are more essential to their functions. There is also evidence that NK cells can spatially control where ATP production occurs to support their effector function. It is known that NK cells also polarise their mitochondria to the immunological synapse, and this is thought to increase ATP production locally to support degranulation (Carpen *et al.*, 1982; Abarca-Rojano *et al.*, 2009).

Protein translation was used as an indicator of energy metabolism throughout this thesis. This meant it was important to understand which metabolic pathways were being used to provide ATP to the ribosomes. In IL2-NK cells, it was very clear that the cells were highly glycolytic and had little requirement for OxPhos. While increased rates of glycolysis have been shown to be essential to support NK cell biosynthesis (Sheppard *et al.*, 2021; Kern Coquillat *et al.*, 2025), oxidative metabolism is also important to support NK cell functions, especially in the context of viral infection (Littwitz-Salomon *et al.*, 2021; Schimmer *et al.*, 2023; Sheppard *et al.*, 2024). The use of these cultured NK cells to investigate metabolism should be interpreted carefully, as their dependence on glycolysis for ATP production may be due to glucose addiction following the 6-day culture in excess glucose. This data shows that following cytokine stimulation they enhance this highly glycolytic metabolic configuration. Regardless, the NK cells did not have any change in expression of glycolytic machinery in the presence of BMDMs, suggesting that mTORc1 and cMyc activation were not affected (Donnelly *et al.*, 2014; Loftus *et al.*, 2018). Furthermore, SREBP activation was not affected, suggesting no change in SREBP-controlled glucose metabolism. In these cells the low rate of OxPhos may be required to support homeostatic ATP demands, as shown in NK cells in other contexts (Marçais *et al.*, 2014; Keppel *et al.*, 2015; Kern Coquillat *et al.*, 2025). Although cytokine stimulation did not significantly increase mitochondrial mass or inner membrane potential, comparison of TMRM staining in these samples to FCCP and oligomycin controls shows that they do have a considerable baseline level of mitochondrial

polarisation which may be more important to support ATP production in co-culture with macrophages compared to NK cells alone. Due to the impaired rates of protein translation in NK cells co-cultured with macrophages, a SCENITH-based approach to assess metabolic requirements would be difficult to interpret. In this context assessment of OCR could give a clearer picture of the dependence on OxPhos for ATP production. With reference to the metabolism of NK cells in the peritoneum, inhibition of glycolysis had no impact on effector molecule production by peritoneal NK cells, whereas inhibition of OxPhos only slightly impaired expression of granzyme B. This suggests that NK cells from these mice are very plastic and can alter their metabolism to support ATP production, potentially through using alternative fuels or metabolic configurations. This has previously been shown to occur in cultured NK cells (Walls *et al.*, 2020; Sheppard *et al.*, 2021), and may also be occurring here due to the strength of NK cell activation in the peritoneum.

Iron deprivation has been shown to acutely impact NK cells antiviral responses, causing impaired cytotoxicity and IFN γ production (Littwitz-Salomon *et al.*, 2021; Schimmer *et al.*, 2025). Expression of the transferrin receptor (CD71) at both mRNA and protein level was impaired when NK cells were co-cultured with BMDMs. This was associated with a decrease in holotransferrin uptake in the NK cells and was mirrored in co-cultures of splenic NK cells with peritoneal macrophages. *Tfrc* is a cMyc target gene which implies that there may be a decrease in cMyc expression. cMyc is a transiently expressed protein with a half-life of 30 mins (Hann and Eisenman, 1984). This has also been demonstrated in NK cells, where its expression is highly dependent on amino acid availability (Loftus *et al.*, 2018). Data in this chapter showed that amino acid availability was not limiting in the supernatants of NK cells co-cultured with macrophages, which supports the argument that a loss of cMyc is not responsible for the loss of CD71 expression or holotransferrin uptake. Ultimately there was no significant change in intracellular iron content in NK cells co-cultured with BMDMs. The discrepancy between this result and the holotransferrin uptake assay could be because the holotransferrin uptake assay measures the capacity of a cell to take up iron, whereas iron measurements were performed using NK cells in holotransferrin

replete medium. Due to the lack of expression of ferroportin, NK cells are unlikely to continually take up large amounts of iron which could lead to ferroptosis.

In lymphocytes, most iron is bound to proteins related to oxidative metabolism and are found primarily in the mitochondria (Andreini *et al.*, 2018; Teh *et al.*, 2021; Brenes *et al.*, 2023). Here it is maintained in iron-sulfur clusters where it can facilitate electron transfer in the complexes of the ETC. However, this is also essential to prevent iron toxicity, as excess iron can lead to increased ROS accumulation due to the Fenton reaction (Winterbourn, 1995). Although NK cells do express the ferritin heavy and light chains (Sottile *et al.*, 2019), which keep iron sequestered in the cell, it is recently being appreciated that cells can also use mitochondria as intracellular iron stores which can buffer them against iron deprivation (Hara *et al.*, 2020; Waters *et al.*, 2022; Long *et al.*, 2022). Through increasing mitophagy the cells can replenish the labile iron pool in times of reduced iron uptake and in doing so maintain other iron-dependent cellular processes such as DNA replication and histone demethylation. The trend towards decreased mitochondrial mass, could be replenishing the labile iron pool in the cell and preserving NK cell functions. It is unlikely that iron is limiting in the culture despite the fact that M1-polarised macrophages have been described to sequester iron (Birgegård and Caro, 1984; Cairo *et al.*, 2011). Iron deprivation would induce a response in NK cells that leads to increased CD71 expression, via increased CD71 translation. This is facilitated through the activation of iron regulatory protein (IRP) 1 and 2. However, CD71 expression was in fact decreased at both mRNA and protein level, arguing against iron deprivation as a mechanism for IFN γ suppression.

Chapter 5

NK cells obtain macrophage-derived lipids through direct interactions.

5.1 Introduction

It is now well-established that immune cells undergo significant metabolic changes depending on their activation but also the signals that they receive in their niche. So far, this thesis has shown that simply adding macrophages to cultures with NK cells resulted in significant changes to metabolic and functional parameters. The fact that direct interactions with macrophages seem to be limiting cytotoxicity and IFN γ production suggested that macrophages were either using receptor-ligand interactions or directly transferring molecules into NK cells to dampen these functions.

Lipid homeostasis has been shown to be essential for correct immune cell functions (Garcia *et al.*, 2023). In the case of NK cells, increased fatty acid uptake has been shown to support OxPhos in viral infections (Schimmer *et al.*, 2023; Sheppard *et al.*, 2024). However, dysregulated lipid uptake in diseases such as cancer or obesity, is associated with impaired NK cell responses (Michelet *et al.*, 2018; Slattery *et al.*, 2025). Because of the association between lipid uptake and viral infections, it was considered whether macrophages control NK cell lipid metabolism and storage, potentially influencing the subsequent immune response. This chapter investigates whether lipids play an important role in communication between macrophages and NK cells, specifically looking at the potential mechanisms these cells may use to facilitate the exchange or sensing of lipids as signalling molecules.

5.2 Direct interactions with macrophages cause lipid accumulation in NK cells.

Initial experiments characterising the metabolism of NK cells in co-culture with macrophages used IL2-NK cells and BMDMs and showed that regardless of the TLR agonist used there was a significant increase in intracellular lipid content of IL2-NK cells (Figure 5.1). LipidTOX™ neutral lipid stains were used in this

analysis. As they specifically stain neutral lipids, this will exclusively stain lipid species such as diacylglycerides (DAGs), triacylglycerides (TAGs) and cholesteryl esters (CEs). This is a novel finding that has potential implications for NK cell functions. To characterise the interaction between NK cells and macrophages leading to increased lipid accumulation, three different experiments were carried out: IL2-NK cells were in direct co-culture with BMDMs, IL2-NK cells were in trans-well co-cultures with BMDMs and, IL2-NK cells were cultured in BMDM-conditioned medium. This revealed that lipid accumulation in NK cells is a process that requires direct interactions with macrophages (Figure 5.2). Furthermore, it is not an immediate process, requiring overnight co-culture before detectable lipid accumulation was observed. IL2-NK cells in the presence of LPS- and CpG-stimulated BMDMs exhibited a continual increase in lipid content 48 h after the cells were initially cultured together (Figure 5.3). This informed all subsequent *in vitro* experiments investigating lipid accumulation in NK cells which were done using direct co-cultures with macrophages.

The accumulation of lipids within NK cells could be attributed to *de novo* biosynthesis or uptake of lipid species from the cell culture medium, among other mechanisms. Metabolomics of NK cell-BMDM co-culture medium revealed very modest changes to the metabolome. Looking more specifically at the lipid species detected in the supernatants, the change in their concentration versus their concentration in RPMI revealed small changes in the nanomolar range (Figure 5.4). The absolute concentration of all detected lipid species was also measured showing that they are all present in the co-culture medium at concentrations $<0.5 \mu\text{M}$ (Figure 5.5). To further assess whether the lipids were coming from extracellular sources, uptake of the fatty acid: oleic acid was assessed using oleic acid alkyne and click chemistry. This bioorthogonal molecule was used as an indicator of oleic acid uptake in this study due to its similarity in structure to oleic acid, and the fact that its uptake can be competed with sodium oleate (Figure 5.6a). This revealed that in the presence of BMDMs, IL2-NK cells have decreased uptake of oleic acid (Figure 5.6).

To obtain an overview of how NK cells were handling the intracellular lipids being accumulated, gene expression of lipid transporters and binding proteins was performed revealing that there was an increase in mRNA expression of *Cd36*,

Fabp5 and *Plin2* in IL2-NK cells that were co-cultured with LPS-stimulated BMDMs (Figure 5.7). This data suggests that CD36-mediated lipid uptake may be important for lipid accumulation in these NK cells. Furthermore, it suggests that the lipids being accumulated are fatty acids. Fatty acid binding protein (FABP) 5 is important for solubilising intracellular fatty acids. Furthermore, perilipin (PLIN) 2 is an essential protein for maintaining integrity of lipid droplets consisting of DAGs and TAGs. To assess whether the lipids in the IL2-NK cells were being stored as lipid droplets, changes in SSC-A were used as a surrogate measurement of changes in internal complexity. Indeed, there was an increase in SSC-A in these NK cells, independently of any changes to cell size, measured by FSC-A (Figure 5.8). Lipids within the NK cells were imaged by confocal microscopy, revealing that in the presence of stimulated macrophages there was an increase in lipid droplet number in IL2-NK cells (Figure 5.9). Lipid droplets in immune cells can vary greatly in size, with smaller lipid droplets requiring more sensitive techniques such as electron microscopy for detection. Nonetheless, analysis of these images revealed significant increases in frequency of IL2-NK cells containing lipid droplets greater than 100 nm in diameter.

Similar to other data shown in this thesis, this work has been carried out on IL2-NK cells in co-culture with BMDMs. In order to assess whether this effect is also observed using more physiological cells *in vitro*, peritoneal macrophages were directly co-cultured with splenic NK cells for 20 h. In agreement with previous data shown, NK cells showed significant increases in intracellular neutral lipid content when co-cultured with LPS- and CpG-stimulated macrophages (Figure 5.10). The other model of acute infection used in this study is the induction of LPS-induced peritonitis. 24 h following intraperitoneal injection of LPS (2.4 mg/kg), peritoneal NK cells, but not splenic NK cells, showed significant increases in intracellular neutral lipid content (Figure 5.11). The fact that NK cells showed increased lipid accumulation at the site of inflammation: the peritoneum, but not the spleen, could indicate that direct interactions with pro-inflammatory macrophages are necessary for lipid accumulation. The lipid scavenging receptor CD36 has been shown to be important for lipid accumulation in many cell types and it also has higher expression in peritoneal NK cells from LPS-immunised mice compared to PBS controls or splenic NK cells (Figure 5.12). Following the

same trends as LipidTOX™ staining in peritoneal NK cells, uptake of oleic acid was shown to increase in peritoneal NK cells following LPS-immunisation (Figure 5.13), highlighting that *in vivo* it is possible that NK cells are obtaining lipids from exogenous sources.

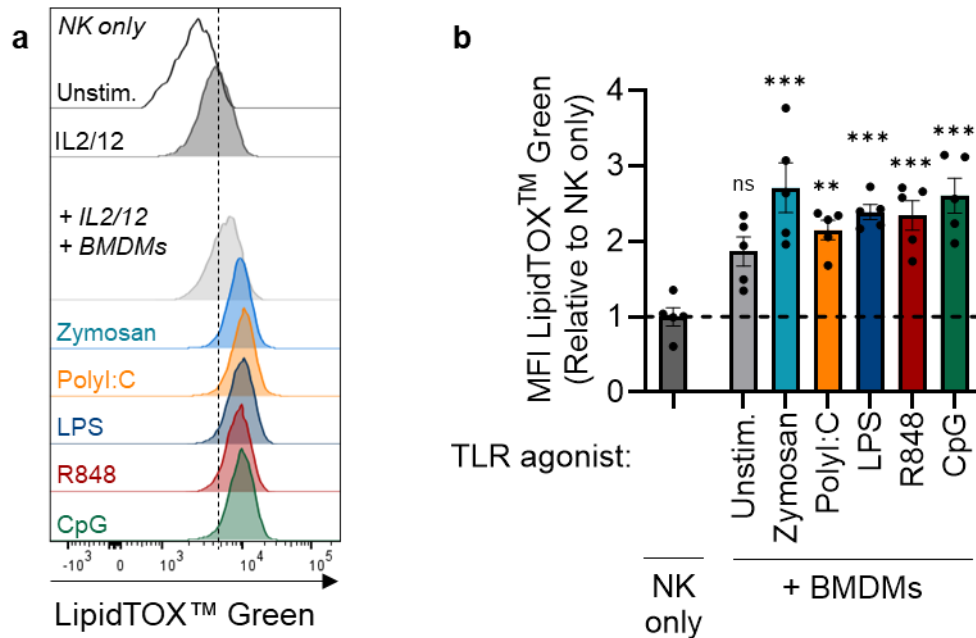


Figure 5.1 IL2-NK cells accumulate intracellular neutral lipids in the presence of TLR-stimulated BMDMs.

BMDMs were left unstimulated or pre-treated with zymosan (100 µg/mL), polyI:C (10 µg/mL), LPS (10 ng/mL), R848 (1 µg/mL) or CpG (4 µg/mL) for 6 hours. IL2-NK cells were activated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and co-cultured with BMDMs for 18 h. Neutral lipid content of NK cells was measured using LipidTOX™ Green by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 5 biological replicates across 2 independent experiments (b). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. All comparisons are made to NK cells only (** $p < 0.01$, *** $p < 0.001$, ns=non-significant).

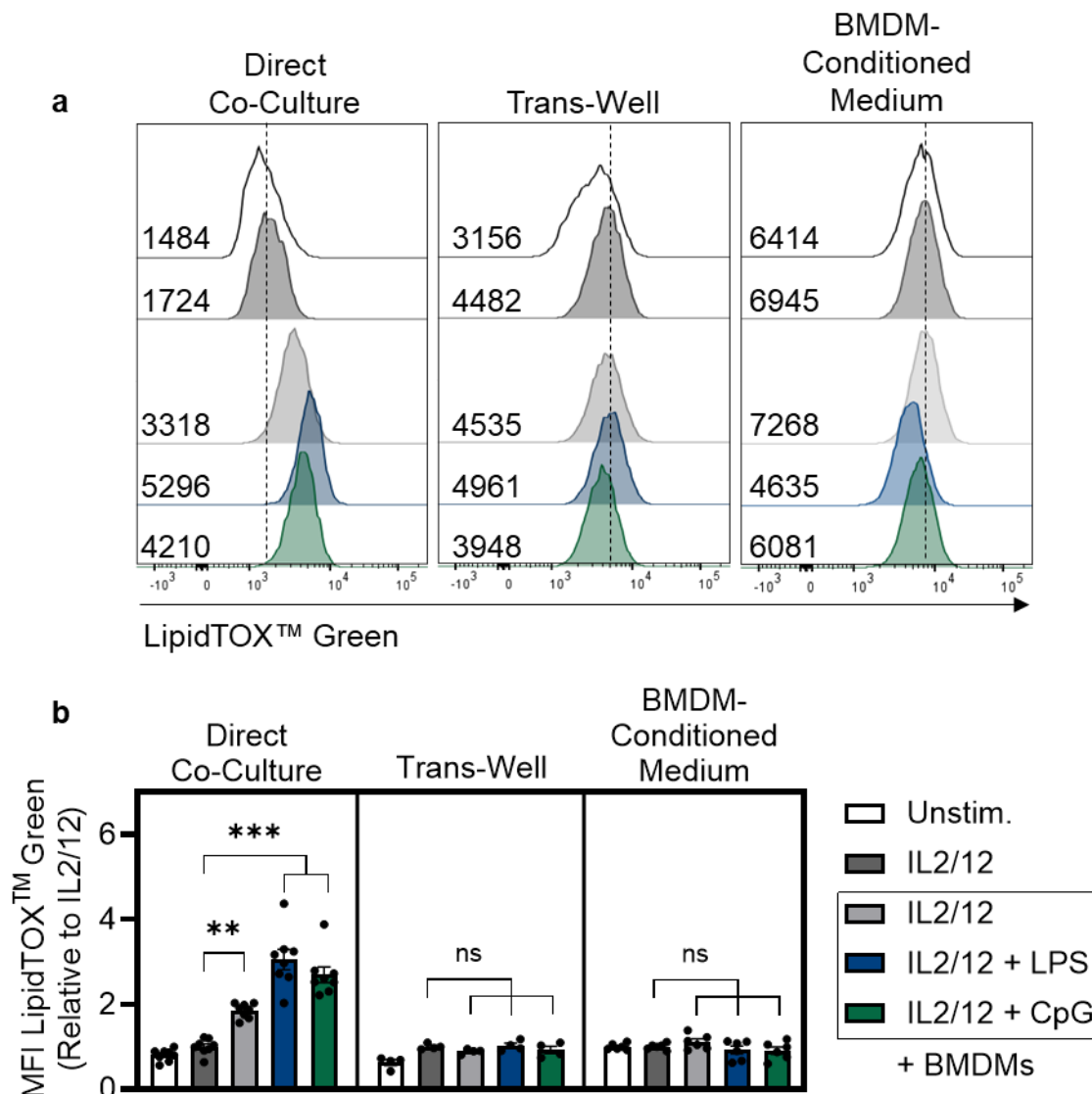


Figure 5.2 Lipid accumulation requires direct interactions between IL2-NK cells and BMDMs.

BMDMs were stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL) or left unstimulated for 6 h. Then IL2-NK cells were either directly co-cultured with BMDMs or co-cultured in a trans-well for 18 h in the presence of NK cell-activating cytokines IL2 (20 ng/mL) and IL12 (10 ng/mL). Alternatively, NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h in 70% conditioned medium collected from BMDMs previously stimulated with LPS (10 ng/mL), CpG (4 μ g/mL) or unstimulated for 24 h. Neutral lipid content of NK cells was measured using LipidTOX™ Green by flow cytometry. Data is representative (a) or mean $\pm$ SEM of at least 4 biological replicates (b). Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test (** $p < 0.01$, *** $p < 0.001$, ns=non-significant).

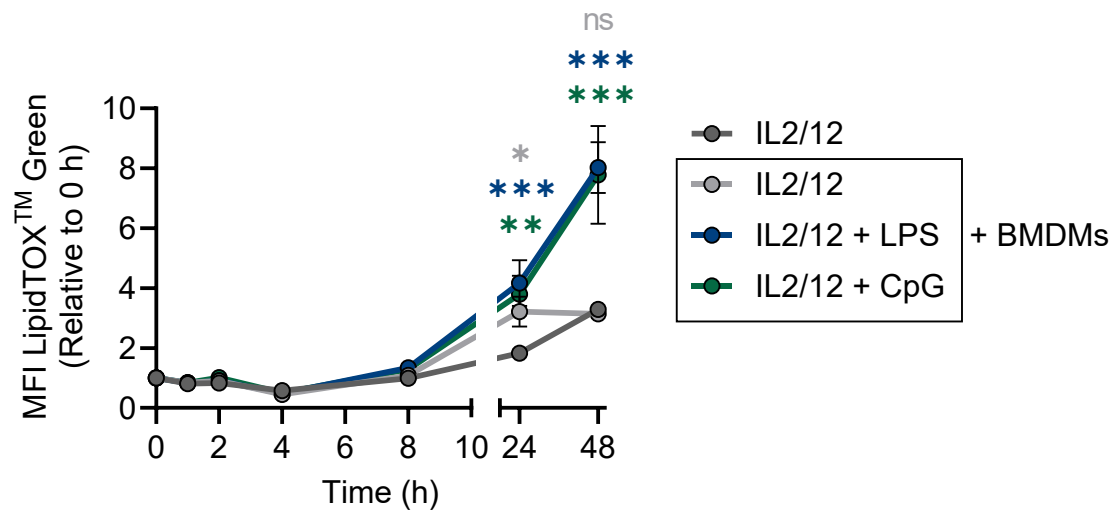


Figure 5.3 Lipid accumulation is not an early effect following co-culture of IL2-NK cells with BMDMs.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 μ g/mL) for 6 hours. IL2-NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and were co-cultured with BMDMs for 18 h. Neutral lipid content of NK cells was measured using LipidTOX™ Green by flow cytometry. Data is mean $\pm$ SEM of at least 4 biological replicates across 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's post-test. All comparisons shown on the graph are versus IL2/12-stimulated NK cells alone (**p<0.01, ***p<0.001).

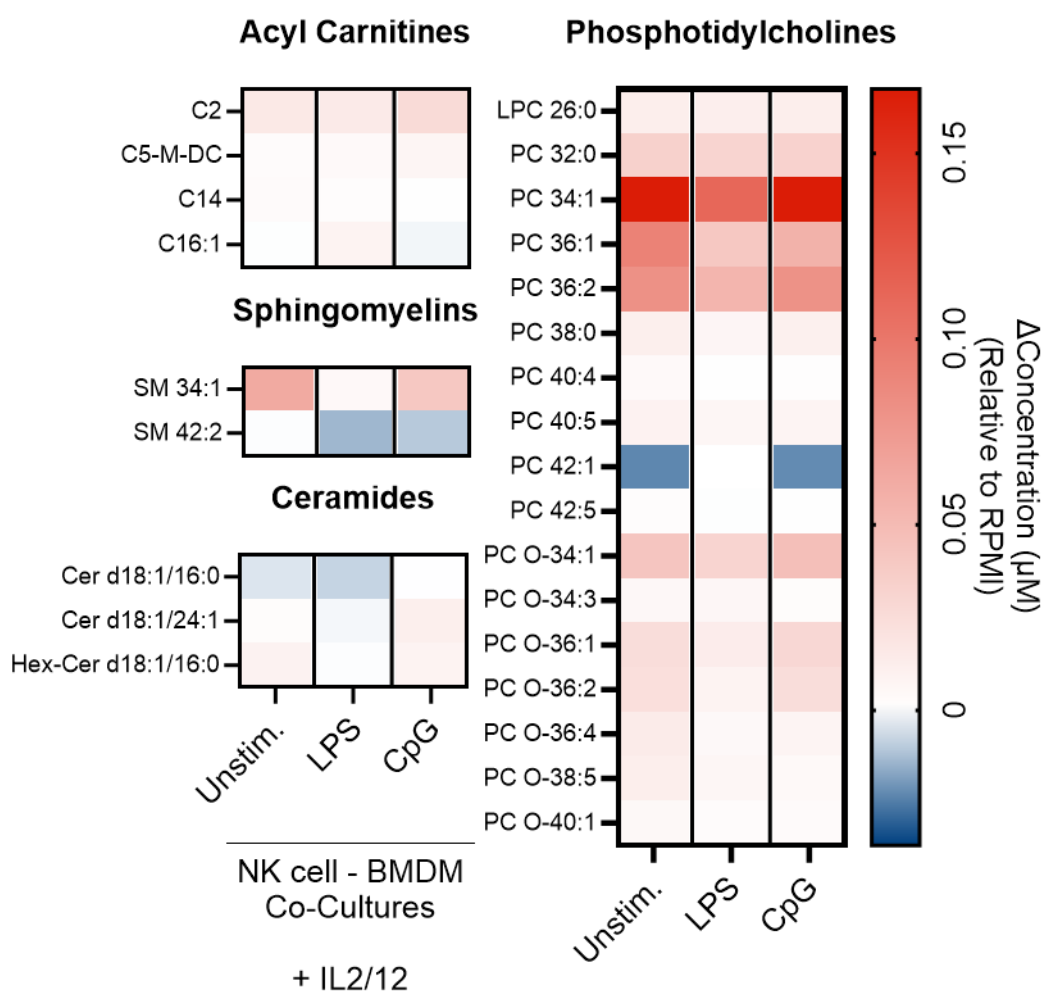


Figure 5.4 There are modest changes in concentration of lipid species in the medium of NK cell-BMDM co-cultures.

BMDMs were left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μg/mL) for 24 h. IL2-NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 24 h before co-culture with BMDMs for 18 h. Supernatants were harvested, and metabolomics was performed by FIA/MS. Data is mean of at least 3 biological replicates. *Metabolomics was performed on samples provided to the Brennan Laboratory, University College Dublin, and was analysed by Cathal Keane.*

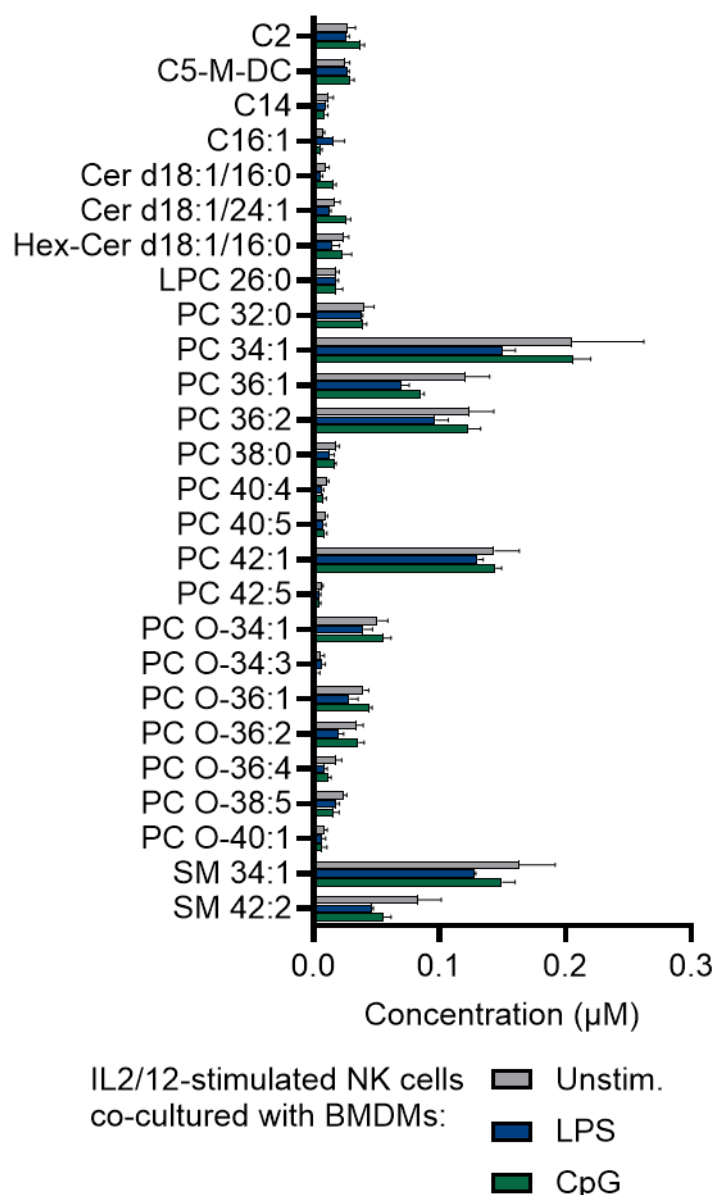


Figure 5.5 Absolute concentrations of lipid species in the medium of NK cell-BMDM co-cultures.

BMDMs were left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μg/mL) for 24 h. IL2-NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 24 h before co-culture with BMDMs for 18 h in the absence of FCS. Supernatants were harvested, and metabolomics was performed by FIA/MS. Data is mean of at least 3 biological replicates. *Metabolomics was performed on samples provided to the Brennan Laboratory, University College Dublin, and was analysed by Cathal Keane.*

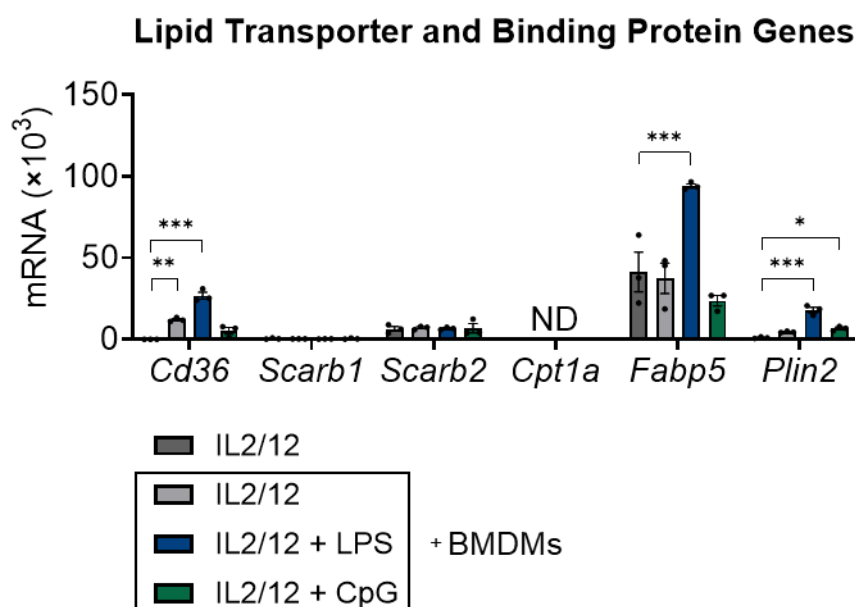


Figure 5.7 IL2-NK cells increase expression of some lipid transporters and binding protein mRNA in the presence of LPS-stimulated BMDMs.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 µg/mL) for 6 hours. IL2-NK cells were either activated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and co-cultured with BMDMs for 18 h. Cells were lysed and analysed by RT-qPCR for expression of *Cd36*, *Scarb1*, *Scarb2*, *Cpt1a*, *Fabp5* and *Plin2* mRNA. Data is normalised to levels of control genes *Gapdh* and *Rplp0*. Data is mean $\pm$ SEM of 3 biological replicates across 2 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. Only statistically significant comparisons are plotted on the graph (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (ND=not detected)

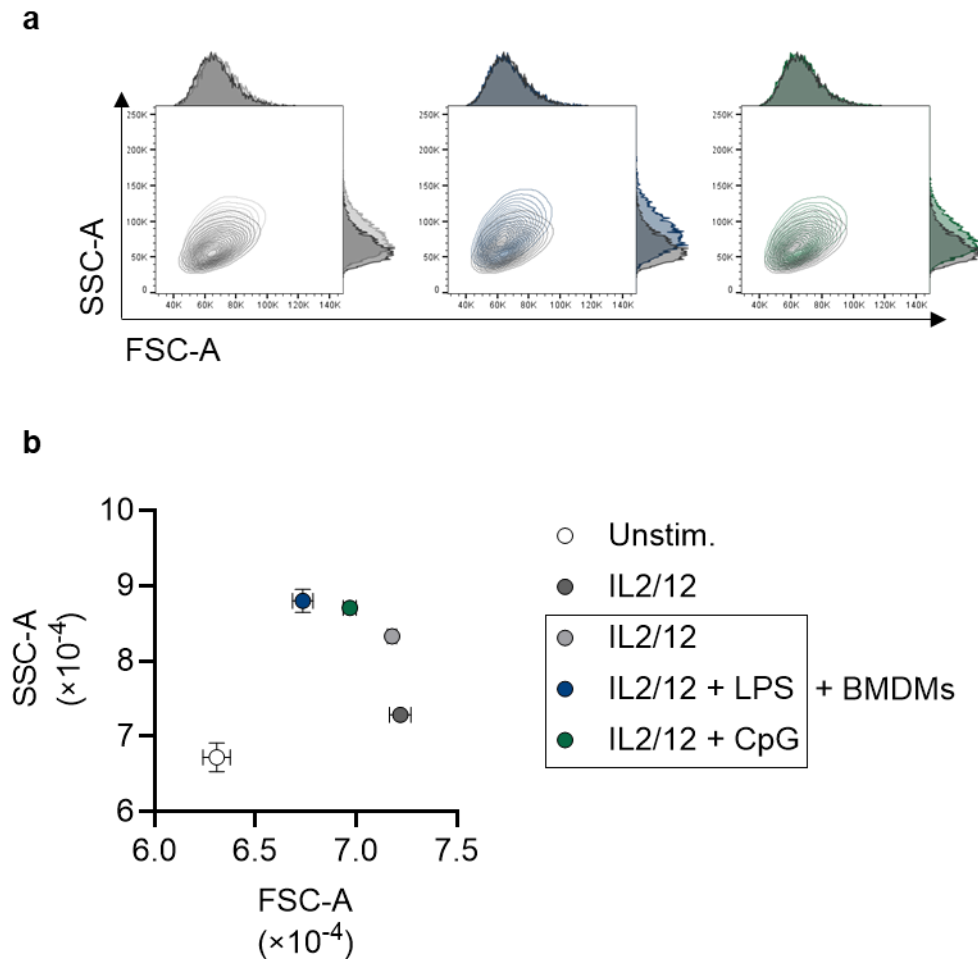


Figure 5.8 IL2-NK cells increase internal complexity in the presence of BMDMs.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 μ g/mL) for 6 h. IL2-NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and co-cultured with BMDMs for 18 h. FSC-A and SSC-A were used as measures of cell size and granularity respectively and were measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 7 biological replicates across 3 independent experiments (b).

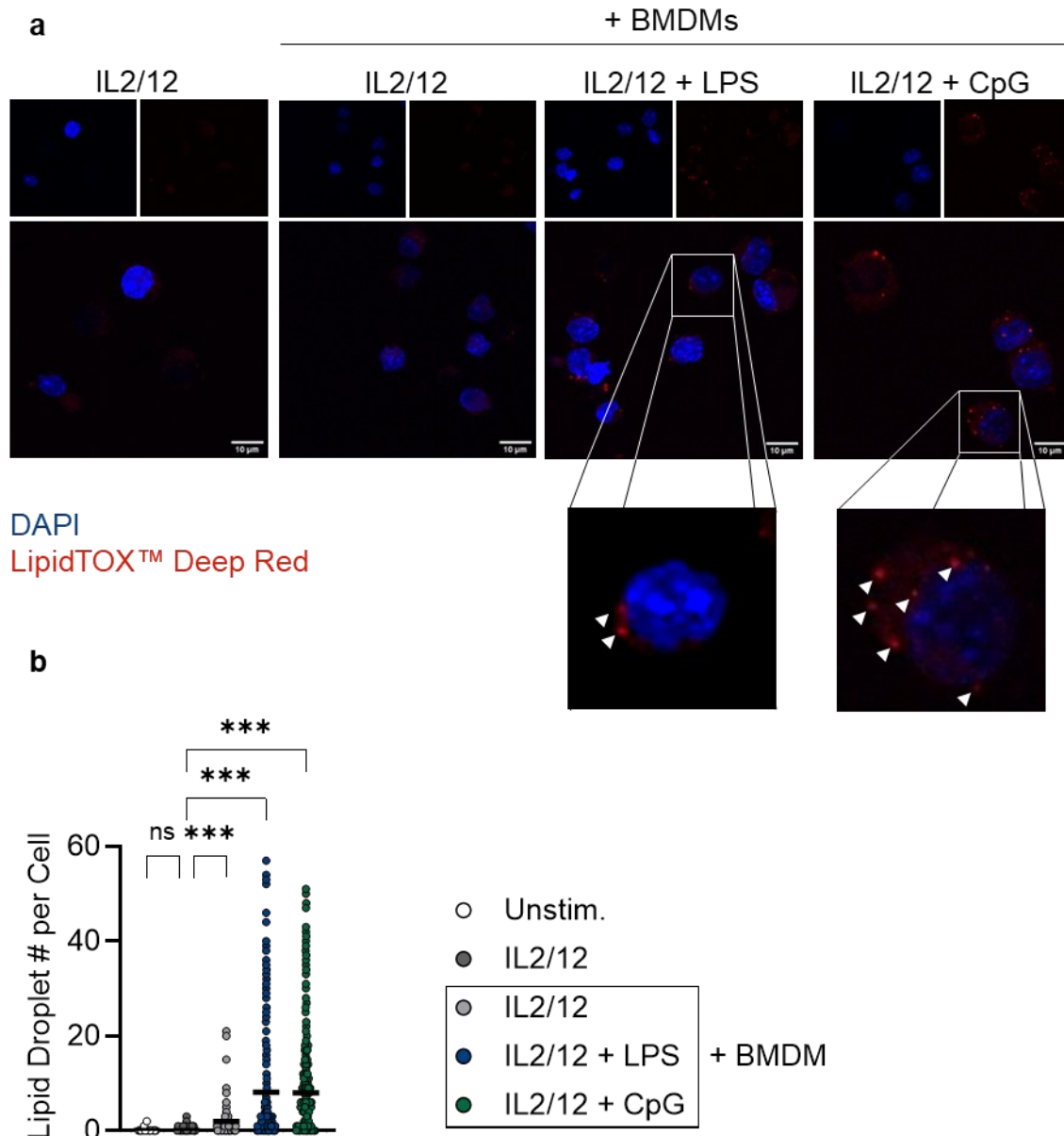


Figure 5.9 IL2-NK cells have increased numbers of lipid droplets in the presence of LPS- and CpG-stimulated BMDMs.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 μg/mL) for 6 hours. IL2-NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and co-cultured with BMDMs for 18 h. Lipid droplets were quantified using LipidTOX™ Deep Red by confocal microscopy. Lipid droplets were defined as particulate staining >100 nm in diameter. Data is representative (a) or median of at least 172 cells from 3 biological replicates across 3 independent experiments (b). Statistical analysis was performed using a Kruskal-Wallis test with Dunn's post-test (**p<0.01, ***p<0.001, ns=non-significant)

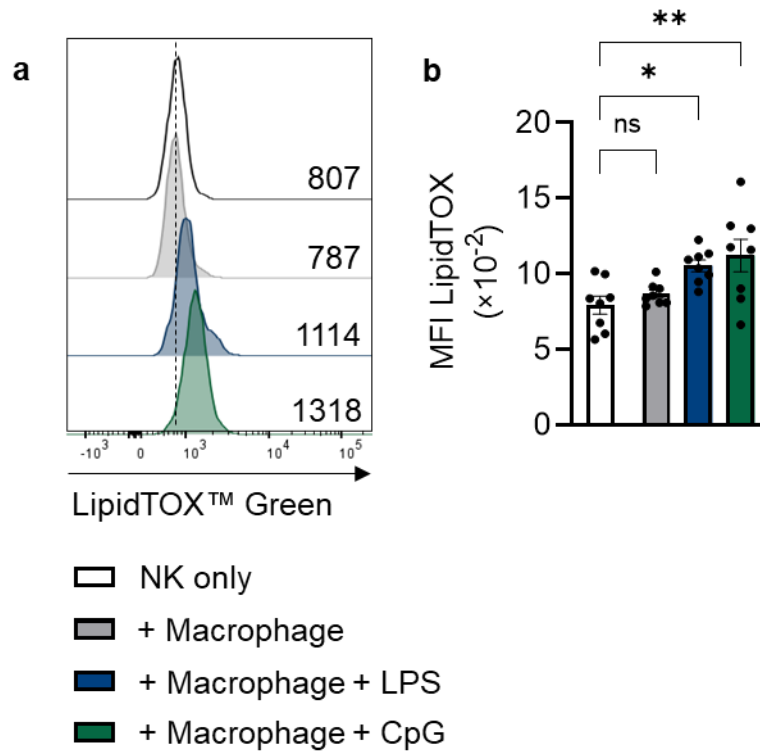


Figure 5.10 Splenic NK cells accumulate neutral lipids in the presence of stimulated peritoneal macrophages *in vitro*.

Splenic NK cells were purified and cultured alone or in the presence of purified peritoneal macrophages 20 h. All NK cells were treated with low dose murine IL15 (2 ng/mL) as a survival factor. Macrophages were either left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL). Neutral lipid content of NK cells was determined using LipidTOX™ Green by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 8 biological replicates across 2 independent experiments (b). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (* p <0.05, ** p <0.01, ns=non-significant).

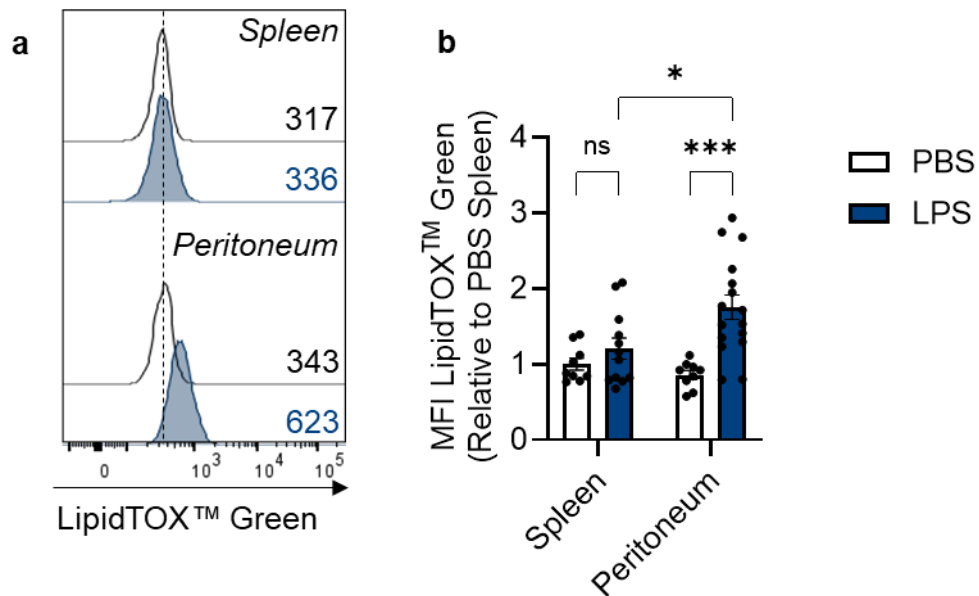


Figure 5.11 Peritoneal NK cells from LPS-immunised mice have increased levels of intracellular neutral lipids.

Mice were administered LPS (2.4 mg/kg) by peritoneal injection. Spleens and peritoneal cells were harvested 24 hours post-administration. LipidTOX™ Green was used to measure intracellular neutral lipid content of NK cells ($CD3^{-}$, $CD19^{-}$, $CD49b^{+}$, $NK1.1^{+}$) by flow cytometry. A minimum of 9 mice were used per group across 2 independent experiments. Data is representative (a) or mean $\pm$ SEM (b). Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test (* $p < 0.05$, *** $p < 0.001$, ns=non-significant).

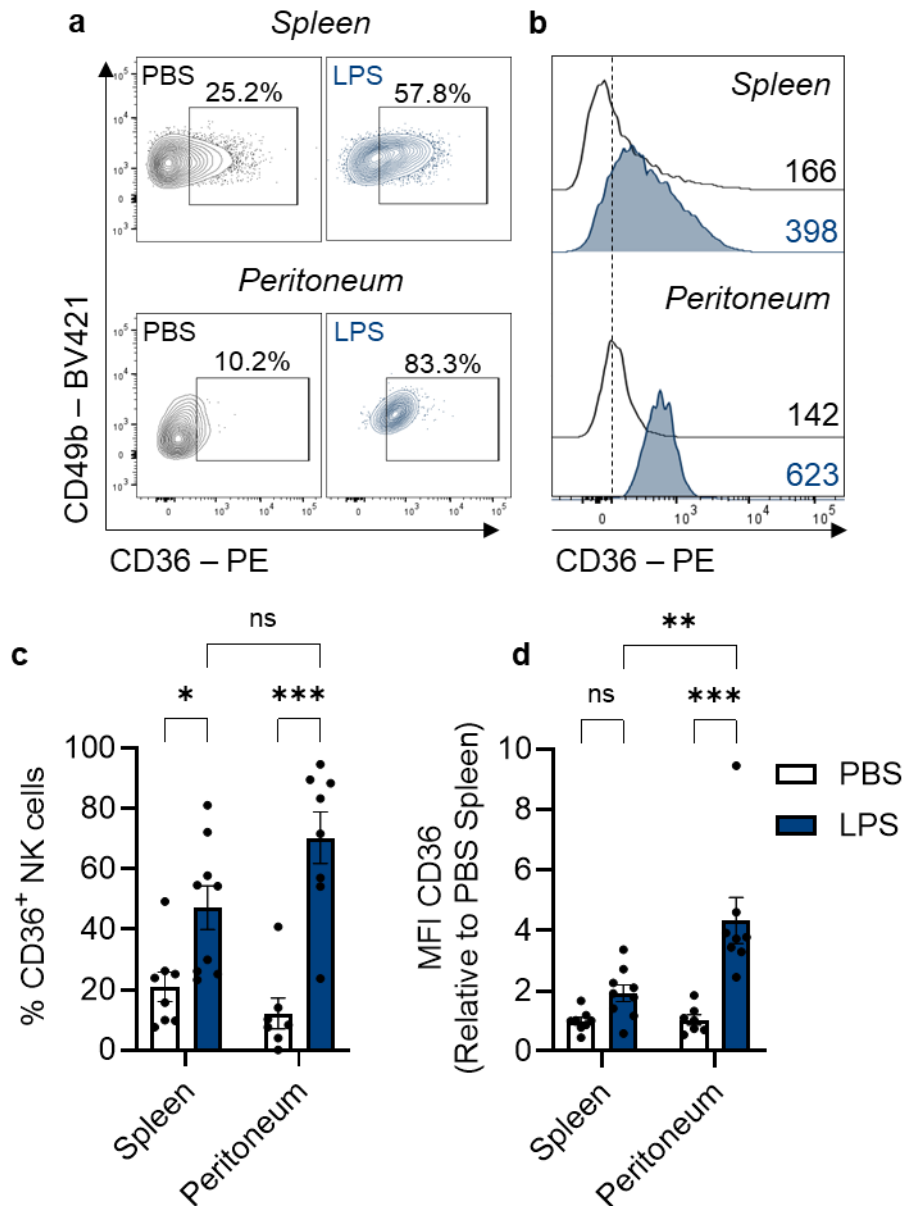


Figure 5.12 Peritoneal NK cells from LPS-immunised mice have increased expression of CD36.

Mice were administered LPS (2.4 mg/kg) by peritoneal injection. Spleens and peritoneal cells were harvested 24 hours post-administration. CD36 expression by NK cells (CD3⁻, CD19⁻, CD49b⁺, NK1.1⁺) was measured by flow cytometry. A minimum of 7 mice were used per group across 2 independent experiments. Data is representative (a,b) or mean +/- SEM (c,d). Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test (*p<0.05, **p<0.01, ***p<0.001, ns=non-significant).

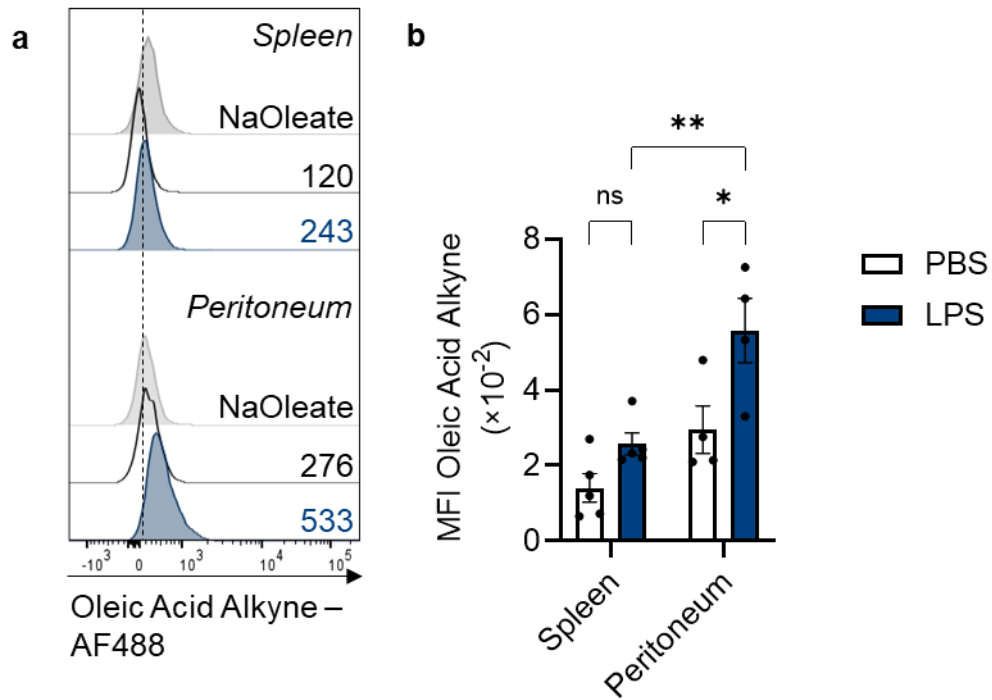


Figure 5.13 Peritoneal NK cells from LPS-immunised mice have increased uptake of oleic acid.

Mice were administered LPS (2.4 mg/kg) by peritoneal injection. Spleens and peritoneal cells were harvested 24 hours post-administration. Uptake of oleic acid was measured in NK cells ($CD3^{-}$, $CD19^{-}$, $CD49b^{+}$, $NK1.1^{+}$) using click chemistry and flow cytometry. Sodium oleate (NaOleate) was used as a competition control using cells from LPS-immunised mice. A minimum of 4 mice were used per group. Data is representative (a) or mean $\pm$ SEM (b). Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test (* $p < 0.05$, ** $p < 0.01$, ns=non-significant).

5.3 NK cells trogocytose CD36 from macrophages.

There are many different mechanisms that cells can use to increase their lipid content, either through *de novo* synthesis or through increasing expression of scavenging receptors and transporters. Analysis of expression of lipid binding proteins and transporters by RT-qPCR revealed that among the scavenging receptors expressed by NK cells following co-culture with TLR-stimulated macrophages, *Cd36* was the only significantly increased gene measured (Figure 5.7). Further to this, peritoneal NK cells from LPS-immunised mice showed elevated CD36 expression (Figure 5.12). Consequently, the expression of CD36 at protein level was measured and showed that in the presence of BMDMs, IL2-NK cells showed significant increase in the frequency of CD36-expressing NK cells following co-culture, regardless of the TLR-agonist present (Figure 5.14). Similar to the lipid accumulation observed in the NK cells, the interaction required to induce CD36 expression on IL2-NK cells required cell-cell interactions in direct co-culture with BMDMs (Figure 5.15). No CD36 expression was seen in NK cells co-cultured with BMDMs in trans-wells or NK cells cultured in BMDM-conditioned medium (Figure 5.15). Because of this, all subsequent *in vitro* experiments investigating the role of CD36 expression on NK cells were done in direct co-cultures with macrophages. The kinetics of CD36 mRNA and protein expression were also evaluated, revealing expression of CD36 protein on IL2-NK cells within 1 h (Figure 5.16b), preceding any accumulation of intracellular lipids (Figure 5.3). CD36 protein expression, measured by flow cytometry, rapidly increased when IL2-NK cells were cultured in the presence of BMDMs, regardless of their activation status. The frequency of CD36-expressing IL2-NK cells also appeared to plateau after 2 h. Interestingly, comparison of *Cd36* gene expression (Figure 5.16a) to protein expression shows that the increase in CD36 protein precedes any significant increase in expression of the gene's mRNA. The increase in *Cd36* mRNA was not detected until 4 h following co-culture. This rapid increase in CD36 expression by IL2-NK cells in co-culture with BMDMs was further investigated to determine whether it was an important precursor to lipid accumulation in NK cells.

CD36^{KO} (B6.129S1-*Cd36*^{tm1Mfe}/J) mice lack expression of CD36 in all tissues and can be used as a useful tool to interrogate the role of CD36 in this *in vitro* system. Flow cytometry was used to confirm that the splenic immune cell populations had

in fact lost expression of CD36 in these mice (Figure 5.17). CD36^{KO} IL2-NK cells were then co-cultured with wild-type BMDMs, so that CD36 deletion was specific to NK cells. This data surprisingly revealed that IL2-NK cells from CD36^{KO} mice had comparable expression of CD36 to IL2-NK cells from CD36^{WT} mice following co-culture with BMDMs (Figure 5.18). Because the only possible source of CD36 expressing cells in the co-culture were CD36^{WT} BMDMs, co-cultures were performed using BMDMs cultured from CD36^{KO} mice. CD36^{WT} IL2-NK cells in co-cultures with CD36^{KO} BMDMs lost expression of CD36 (Figure 5.19). These experiments revealed that the source of rapid CD36 protein expression on NK cells must be macrophages. This agrees with data showing that NK cells have increased expression of CD36 protein before there is an increase in *CD36* gene expression (Figure 5.16). Indeed, NK cells have been known to express proteins synthesised by other cell types through a selective process called trogocytosis (Figure 5.20). It is known that trogocytosis requires the formation of a synapse, followed by re-organisation of the actin cytoskeleton in order to facilitate clathrin-mediated endocytosis and re-expression of the obtained proteins. Therefore, in order to target the process of trogocytosis more specifically, latrunculin A, an actin-binding molecule was used to prevent polymerisation and therefore, prevent trogocytosis. When wild-type IL2-NK cells and BMDMs were co-cultured in the presence of latrunculin A for 8 h, there was a significant decrease in expression of CD36 by NK cells (Figure 5.21). The cells were co-cultured for 8 h to prevent any issues with latrunculin A-mediated cytotoxicity. This mimicked the experiments where CD36^{KO} BMDMs and CD36^{WT} IL2-NK cells were co-cultured. Taken together this data shows that NK cells and macrophages have the ability to share biomolecules and that NK cells can re-express macrophage-derived CD36.

The expression of macrophage-derived CD36 by NK cells is a novel finding, but this data does not show whether the CD36 being re-expressed by NK cells is functional. Because CD36-mediated lipid uptake could be responsible for lipid droplet accumulation in NK cells, LipidTOX™ staining was carried out. Unsurprisingly, co-culture of CD36^{KO} IL2-NK cells with CD36^{WT} BMDMs did not reveal any changes in intracellular lipid accumulation as data has shown that NK cells can still express CD36 in these co-cultures (Figure 5.22). Co-cultures were

again, performed using CD36^{WT} IL2-NK cells with CD36^{KO} BMDMs, showing no changes in intracellular lipid accumulation by NK cells (Figure 5.23). This data suggests that the accumulation of lipids by NK cells occurs independently of CD36 expression. Uptake of oleic acid, measured using oleic acid alkyne, revealed that CD36 was also not required for this process (Figure 5.24). Nonetheless, this data does not exclude the possibility that macrophage-derived CD36 re-expression by NK cells is not regulating other processes.

Using measures of protein translation as a surrogate for cellular energy metabolism showed that macrophage-derived CD36 was not responsible for the defect in translation rate observed by IL2-NK cells in the presence of BMDMs (Figure 5.25). However, IL2-NK cells co-cultured with CD36^{KO} BMDMs stimulated with LPS, did show a rescue in IFN γ expression (Figure 5.26) with no changes observed in expression of cytolytic molecules (Figure 5.27). In accordance with this there was no enhancement of NK cell killing of RMA/S target cells by IL2-NK cells co-cultured with CD36^{KO} BMDMs (Figure 5.28).

Whether this effect was occurring using more physiological cells *in vitro* was investigated using peritoneal macrophages in co-culture with splenic NK cells. This showed that there was a very modest increase in expression of CD36 protein by splenic NK cells following co-culture with peritoneal macrophages, with the frequency of CD36-expressing cells being <6% (Figure 5.29). To determine whether these NK cells were also carrying out trogocytosis to obtain CD36, CD36^{WT} splenic NK cells were co-cultured with CD36^{WT} peritoneal macrophages or CD36^{KO} peritoneal macrophages. In the presence of CD36^{KO} peritoneal macrophages, NK cells had significantly reduced CD36 expression in comparison with CD36^{WT} controls, revealing that macrophage expression of CD36 was essential for the NK cells to acquire CD36 in the co-culture (Figure 5.30). This model differs significantly to the other *in vitro* system where highly activated IL2-NK cells are directly co-cultured in a tissue culture dish with macrophages at 90-100% confluency. These splenic NK cells do not receive any supplementary cytokines, and any activation can be attributed to signals from the low number of TLR-stimulated macrophages that survive in the culture. To better understand why there were such a low frequency of NK cells expressing CD36, peritoneal macrophages were stimulated with LPS or CpG for 16 h, before

splenic NK cells were purified and co-cultures were performed in an Incucyte Cell Imager, which took photos every 5 mins for 4 h (Figure 5.31). This analysis showed that the splenic NK cells were slow-moving in a tissue culture dish that had very small macrophage numbers. Furthermore, there was no clustering of the NK cells around the static macrophages. NK cell-macrophage interactions appeared to continually occur as NK cells moved around the dish, however, many interactions that did occur were long lasting and could be detected for the remaining duration of the co-culture (Figure 5.31a). Taken together this data suggests that the NK cells are using trogocytosis to obtain CD36 from macrophages, but the low frequency of interactions occurring in this *in vitro* system leads to a low frequency of CD36-expressing NK cells.

Whether the CD36 obtained from macrophages was responsible for the increased lipid content in this co-culture was again assessed using LipidTOX™ staining of CD36^{WT} splenic NK cells co-cultured with either CD36^{WT} or CD36^{KO} peritoneal macrophages. This mimicked the data from the co-cultures of IL2-NK cells with BMDMs (Figure 5.23), with CD36^{WT} splenic NK cells in co-culture with CD36^{KO} peritoneal NK cells showing no changes in lipid content in comparison with NK cells co-cultured with CD36^{WT} peritoneal macrophages (Figure 5.32). Although there were no changes in lipid content in these experiments, whether the macrophage-derived CD36 was in fact functional was assessed. This showed that following co-culture with peritoneal macrophages, CD36⁺ splenic NK cells did not increase their lipid content (Figure 5.33a), but if exogenous cytokines were added to the co-culture the activated NK cells did increase their intracellular neutral lipid content (Figure 5.33b).

In order to assess whether this acquisition of CD36 from macrophages was important for NK cell responses *in vivo*, whole-body CD36^{KO} mice were used. These mice showed no differences in intracellular lipid content in either splenic or peritoneal NK cells from naïve or LPS-immunised mice, compared to CD36^{WT} controls (Figure 5.34). Furthermore, there were no detected changes in expression of the NK cell activation markers: CD69 or CD71 (Figure 3.35) following LPS-immunisation, nor were there any changes in expression of IFN γ or cytolytic molecules, in CD36^{KO} mice compared to CD36^{WT} mice (Figure 3.36).

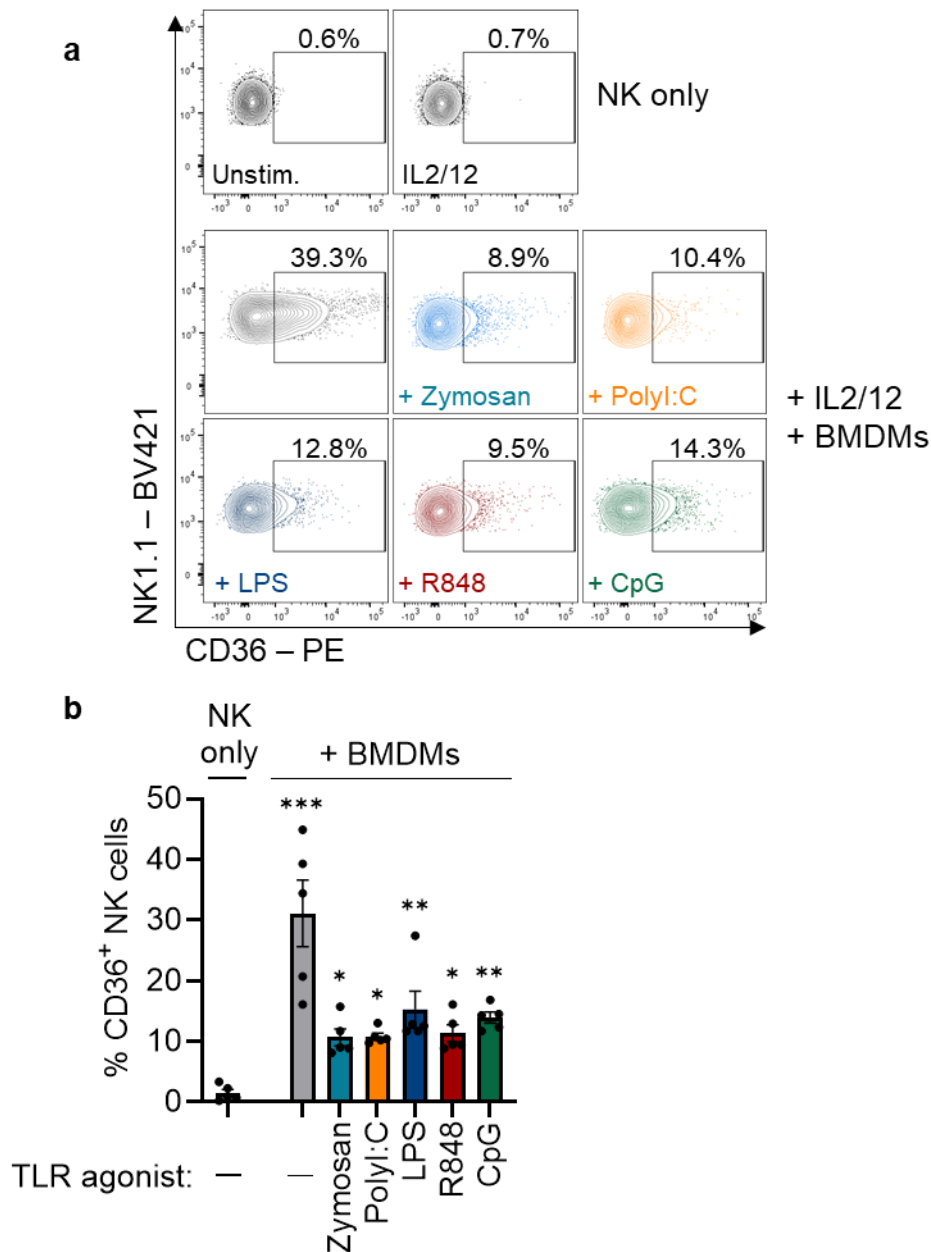


Figure 5.14 IL2-NK cells increase expression of CD36 in the presence of BMDMs.

BMDMs were left unstimulated or pre-treated with zymosan (100 $\mu\text{g/mL}$), polyI:C (10 $\mu\text{g/mL}$), LPS (10 ng/mL), R848 (1 $\mu\text{g/mL}$) or CpG (4 $\mu\text{g/mL}$) for 6 hours. IL2-NK cells were activated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and co-cultured with BMDMs for 18 h. CD36 expression was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 5 biological replicates across 2 independent experiments (b). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. All comparisons are made to NK cells only (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

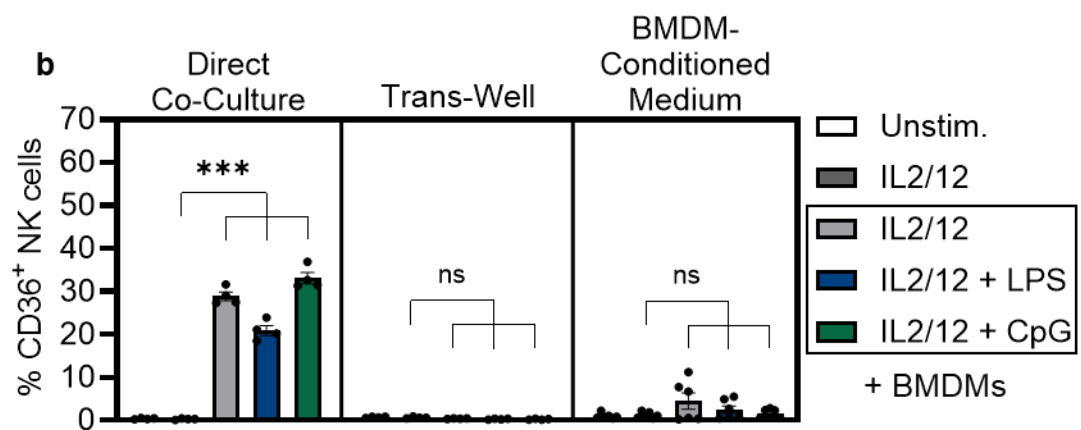
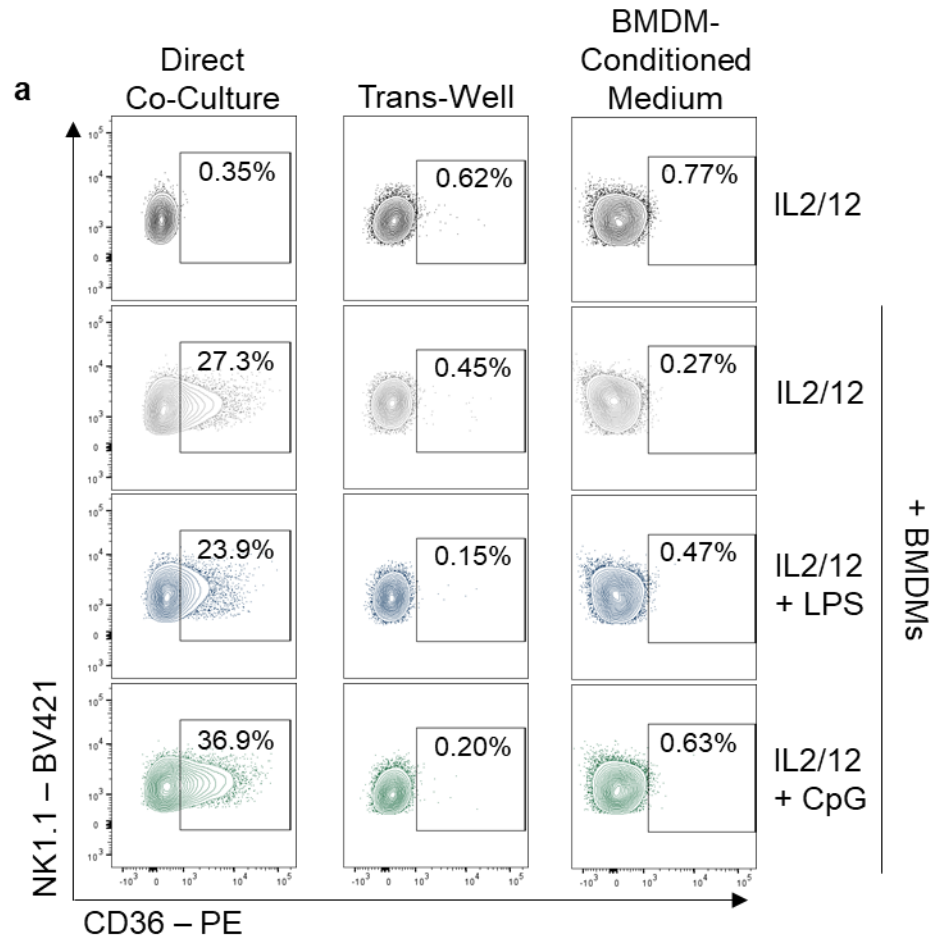


Figure 5.15 CD36 protein expression requires direct interactions between IL2-NK cells and BMDMs.

BMDMs were stimulated with LPS (10 ng/mL) or CpG (4 µg/mL) or left unstimulated for 6 h. Then IL2-NK cells were either directly co-cultured with BMDMs or co-cultured in a trans-well for 18 h in the presence of NK cell-activating cytokines IL2 (20 ng/mL) and IL12 (10 ng/mL). Alternatively, NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for 18 h in 70% conditioned medium collected from BMDMs previously stimulated with LPS (10 ng/mL), CpG (4 µg/mL) or unstimulated for 24 h. CD36 expression was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of at least 4 biological replicates (b). Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test. Comparisons plotted on the graph are versus IL2/12-stimulated NK cells only (** $p < 0.001$, ns=non-significant).

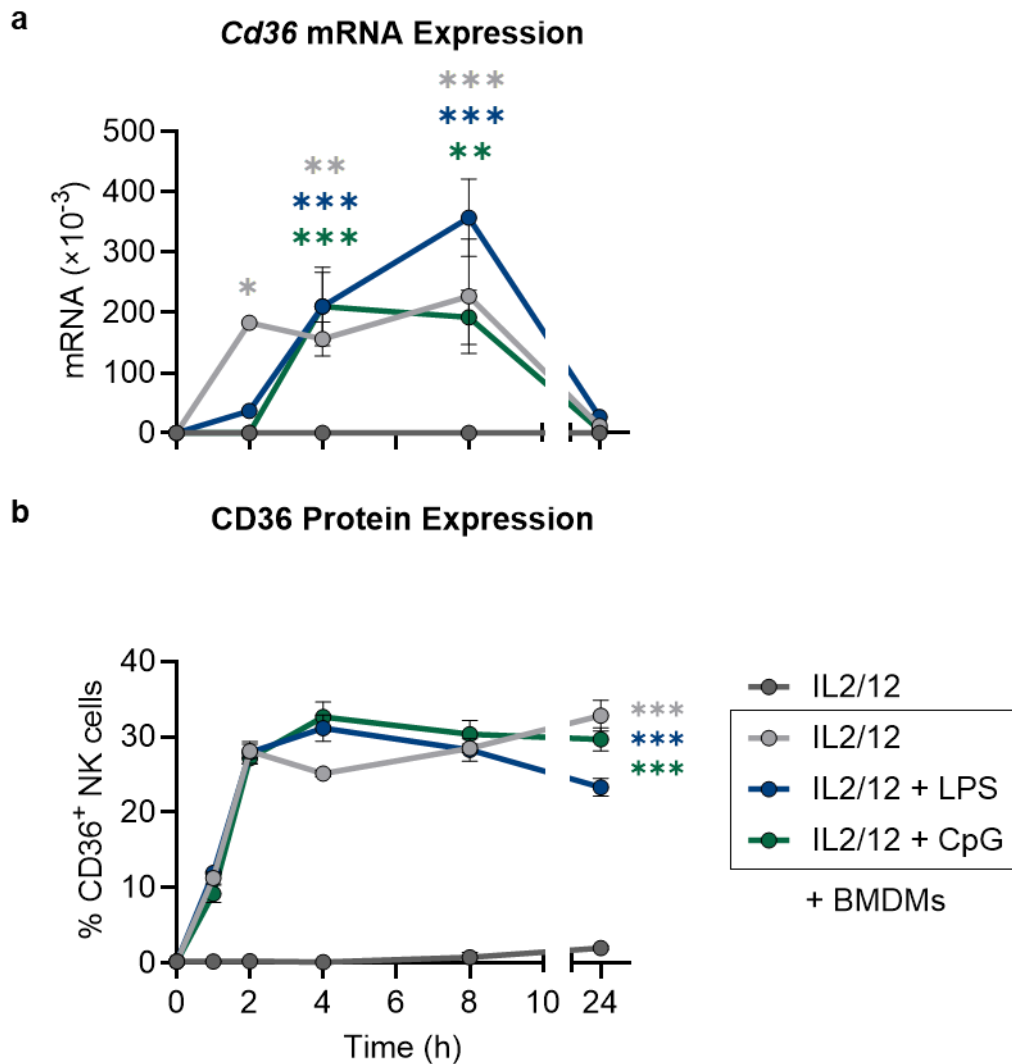


Figure 5.16 IL2-NK cells rapidly increase expression of CD36 at protein level following co-culture with BMDMs.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 µg/mL) for 6 hours. IL2-NK cells were either left unstimulated or activated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and were co-cultured with BMDMs. Cells were lysed and analysed by RT-qPCR for expression of *Cd36* mRNA (a). Data is normalised to levels of control genes *Gapdh* and *Rplp0*. Protein expression of CD36 was measured by flow cytometry (b). Data is mean $\pm$ SEM of 3 biological replicates across 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's post-test. All comparisons shown are versus IL2/12-stimulated NK cells only (* p <0.05, ** p <0.01, *** p <0.001). In (b) p <0.001 for all time points.

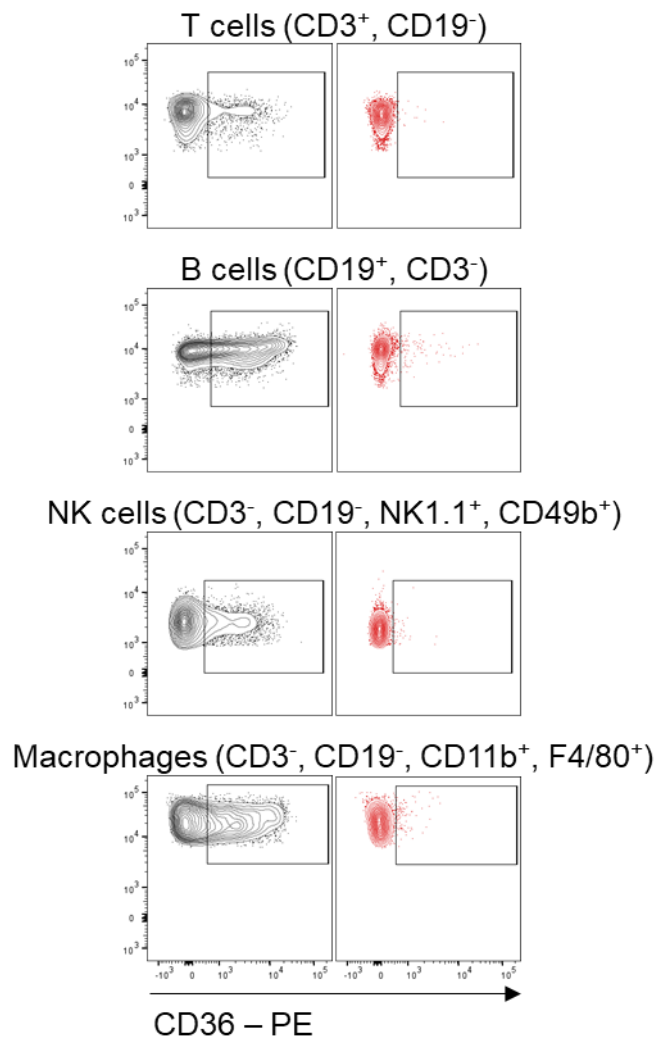


Figure 5.17 Loss of CD36 expression by splenocytes in CD36^{KO} mice.

Spleens were harvested from CD36^{WT} (grey) or CD36^{KO} (red) mice. CD36 expression was measured by flow cytometry. Data is representative of three biological replicates.

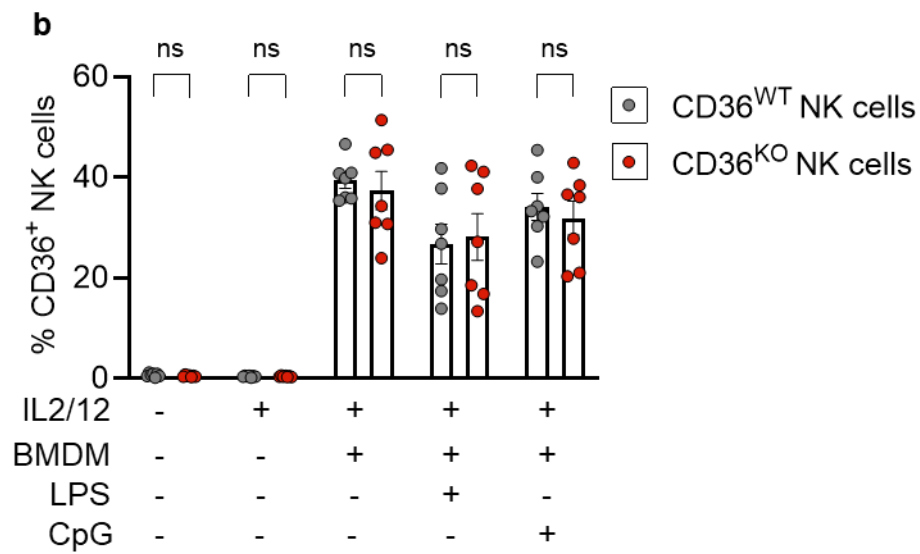
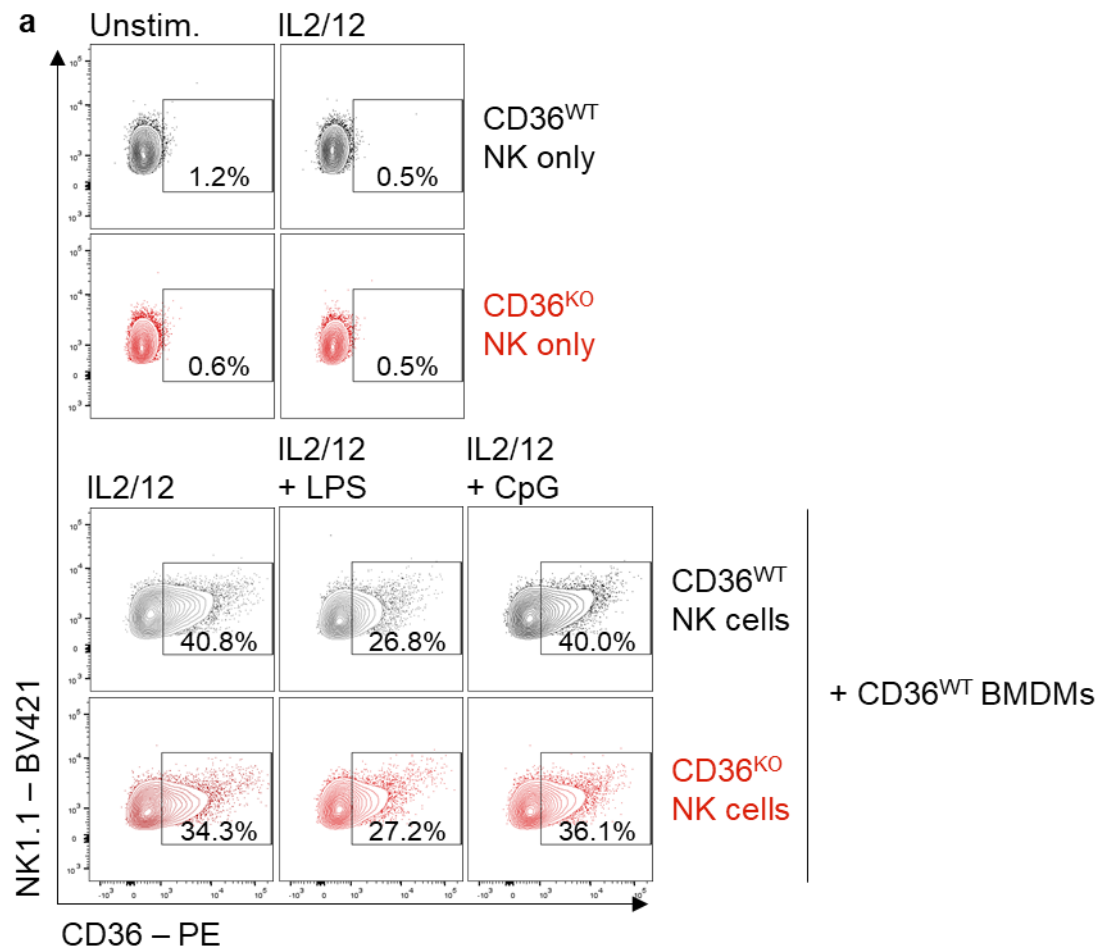


Figure 5.18 CD36^{KO} IL2-NK cells in co-culture with CD36^{WT} BMDMs increase expression of CD36.

CD36^{WT} BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 µg/mL) for 6 hours. CD36^{WT} or CD36^{KO} IL2-NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and co-cultured with CD36^{WT} BMDMs for 18 h. CD36 expression was measured by flow cytometry. Data is representative (a) or mean +/- SEM of 7 biological replicates across 2 independent experiments (b). Statistical analysis was carried out using a two-way ANOVA with Šidák's post-test (ns=non-significant).

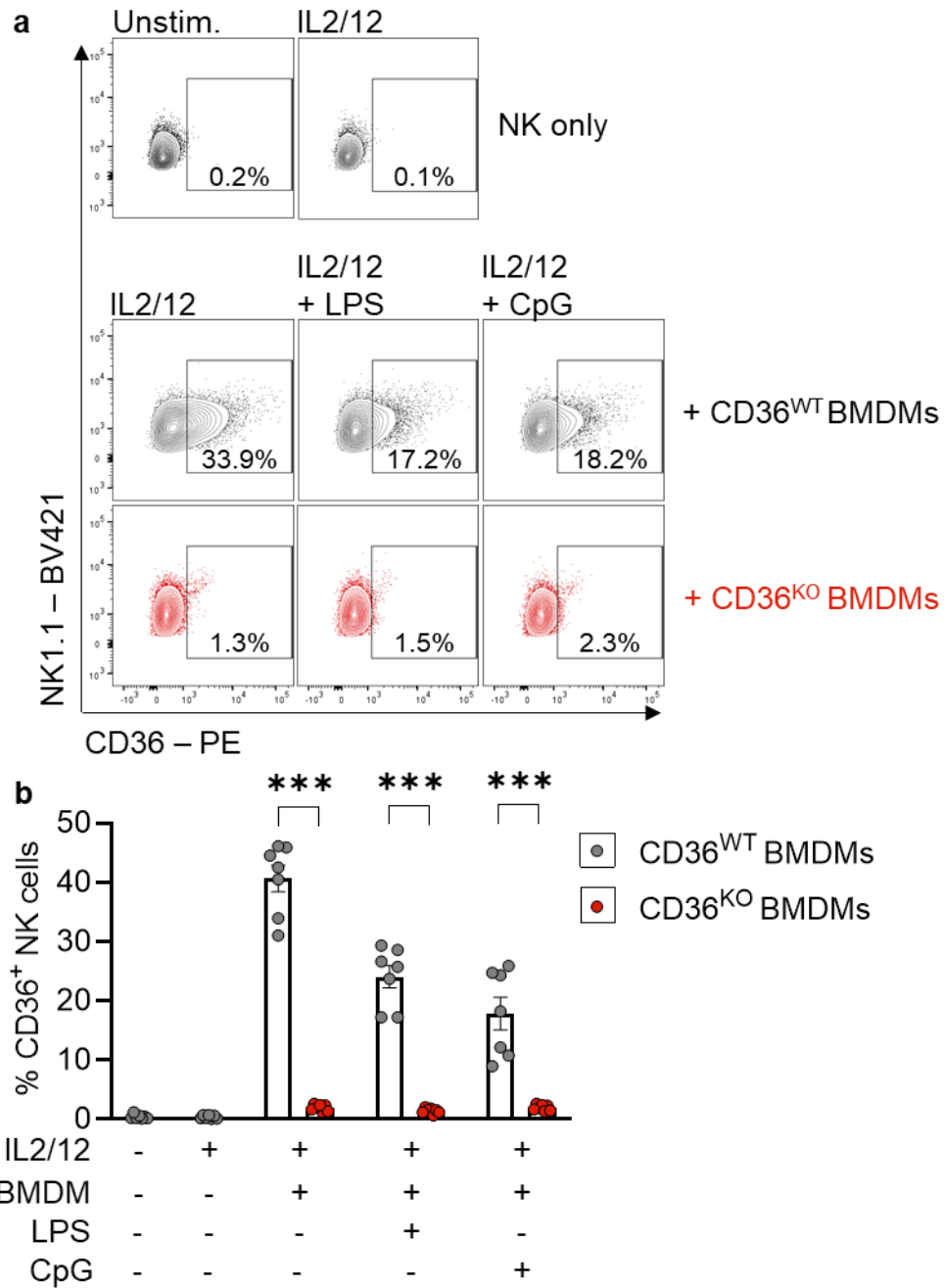


Figure 5.19 CD36^{WT} IL2-NK cells in co-culture with CD36^{KO} BMDMs do not increase expression of CD36.

CD36^{WT} or CD36^{KO} BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 µg/mL) for 6 hours. CD36^{WT} IL2-NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and co-cultured with BMDMs for 18 h. CD36 expression was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 7 biological replicates across 2 independent experiments (b). Statistical analysis was carried out using a two-way ANOVA with Šidák's post-test (** $p < 0.001$).

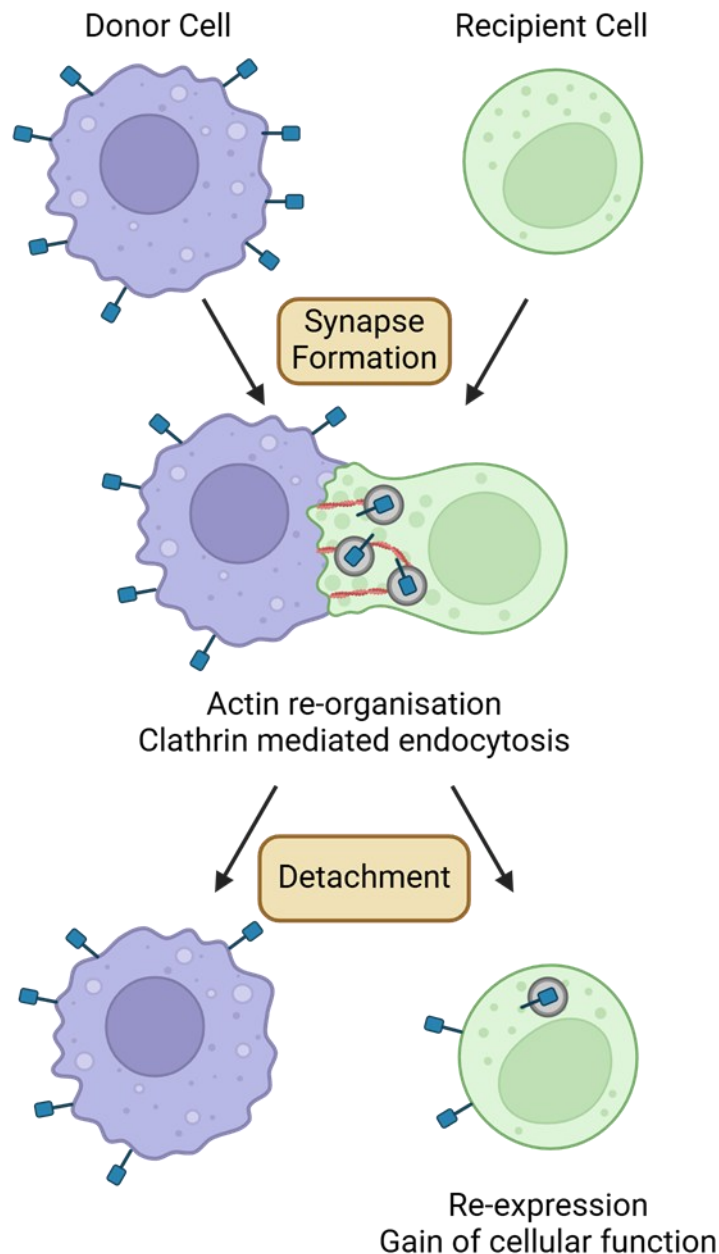


Figure 5.20 Trogocytosis of proteins by recipient cells from donor cells.

The process of trogocytosis has been described by many cell types, including lymphocytes. This process requires the initial formation of a synapse between donor (purple) and recipient (green) cells. This is followed by the gain of donor cell proteins by recipient cells through clathrin-mediated endocytosis, a process which is reliant on reorganisation of the actin cytoskeleton. Following detachment, the recipient cells can express the gained proteins on their own plasma membranes and gain a new biological function. *This figure was created using Biorender.com.*

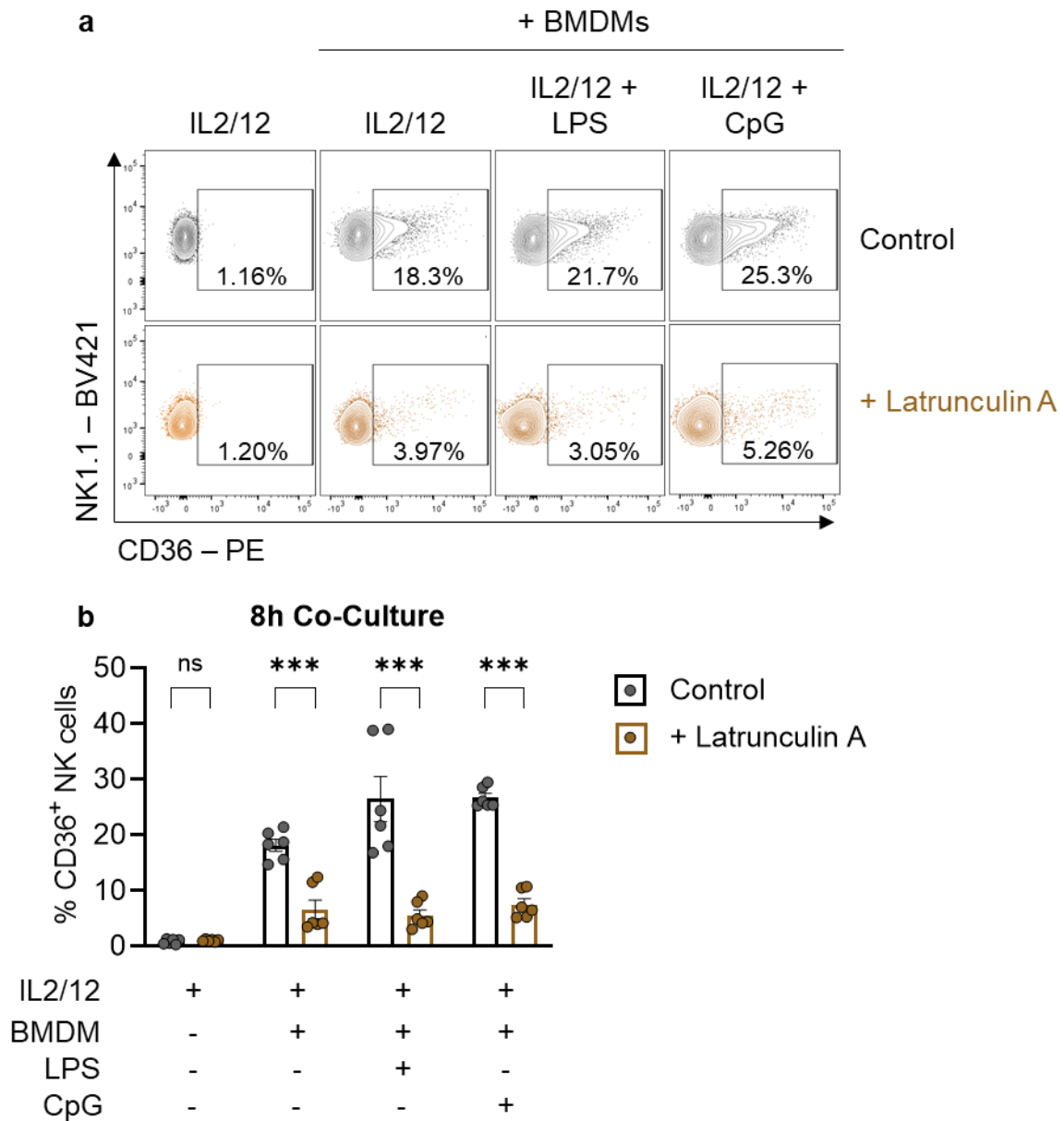


Figure 5.21 Actin polymerisation is required for CD36 expression on IL2-NK cells co-cultured with BMDMs.

IL2-NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) in the presence or absence of BMDMs that were left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 µg/mL) for 8 h. Cells were co-cultured in the presence or absence of latrunculin A (10 µM). CD36 expression on NK cells was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 6 biological replicates across 2 independent experiments (b). Statistical analysis was carried out using a two-way ANOVA with Šidák's post-test (** $p < 0.001$, ns=non-significant).

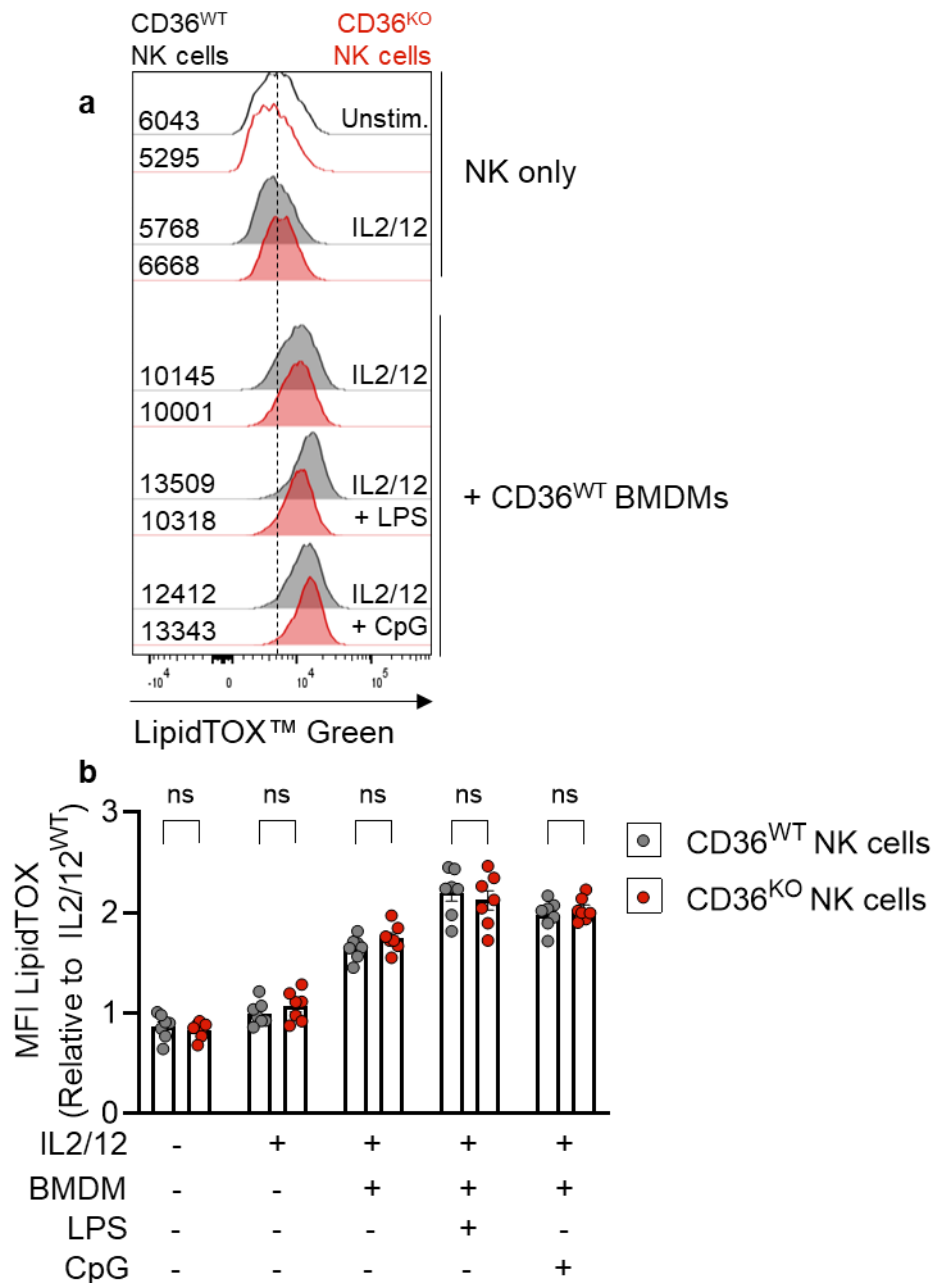


Figure 5.22 CD36^{KO} IL2-NK cells in co-culture with CD36^{WT} BMDMs accumulate intracellular neutral lipids.

CD36^{WT} BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 µg/mL) for 6 hours. CD36^{WT} or CD36^{KO} IL2-NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and co-cultured with CD36^{WT} BMDMs for 18 h. LipidTOXTM Green was used to determine intracellular lipid content of NK cells by flow cytometry. Data is representative (a) or mean +/- SEM of 7 biological replicates across 2 independent experiments (b). Statistical analysis was carried out using a two-way ANOVA with Šidák's post-test (ns=non-significant).

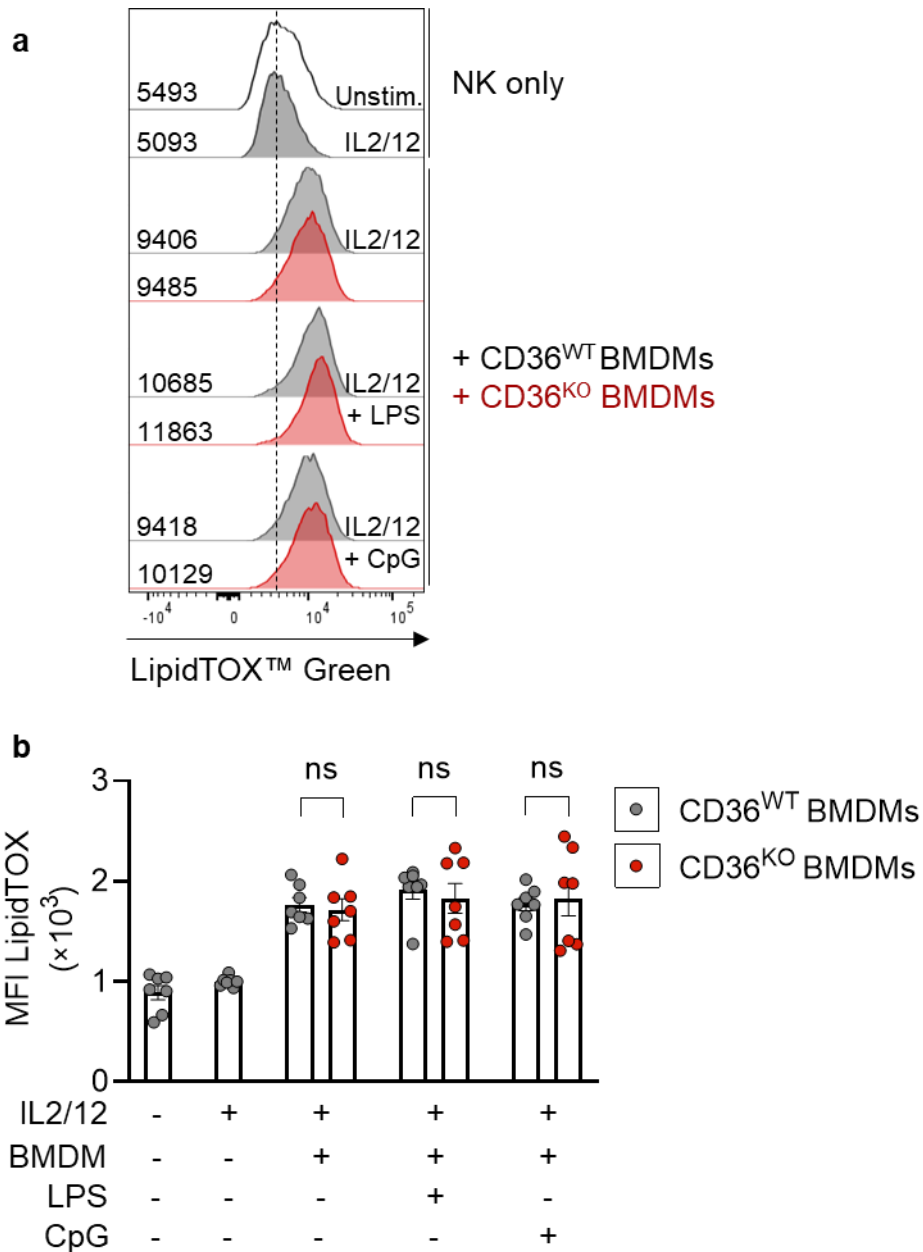


Figure 5.23 CD36^{WT} IL2-NK cells in co-culture with CD36^{KO} BMDMs accumulate intracellular neutral lipids.

CD36^{WT} or CD36^{KO} BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 μ g/mL) for 6 hours. CD36^{WT} IL2-NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and co-cultured with BMDMs for 18 h. LipidTOX™ Green was used to determine the intracellular lipid content of NK cells by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 7 biological replicates across 2 independent experiments (b). Statistical analysis was carried out using a two-way ANOVA with Šidák's post-test (ns=non-significant).

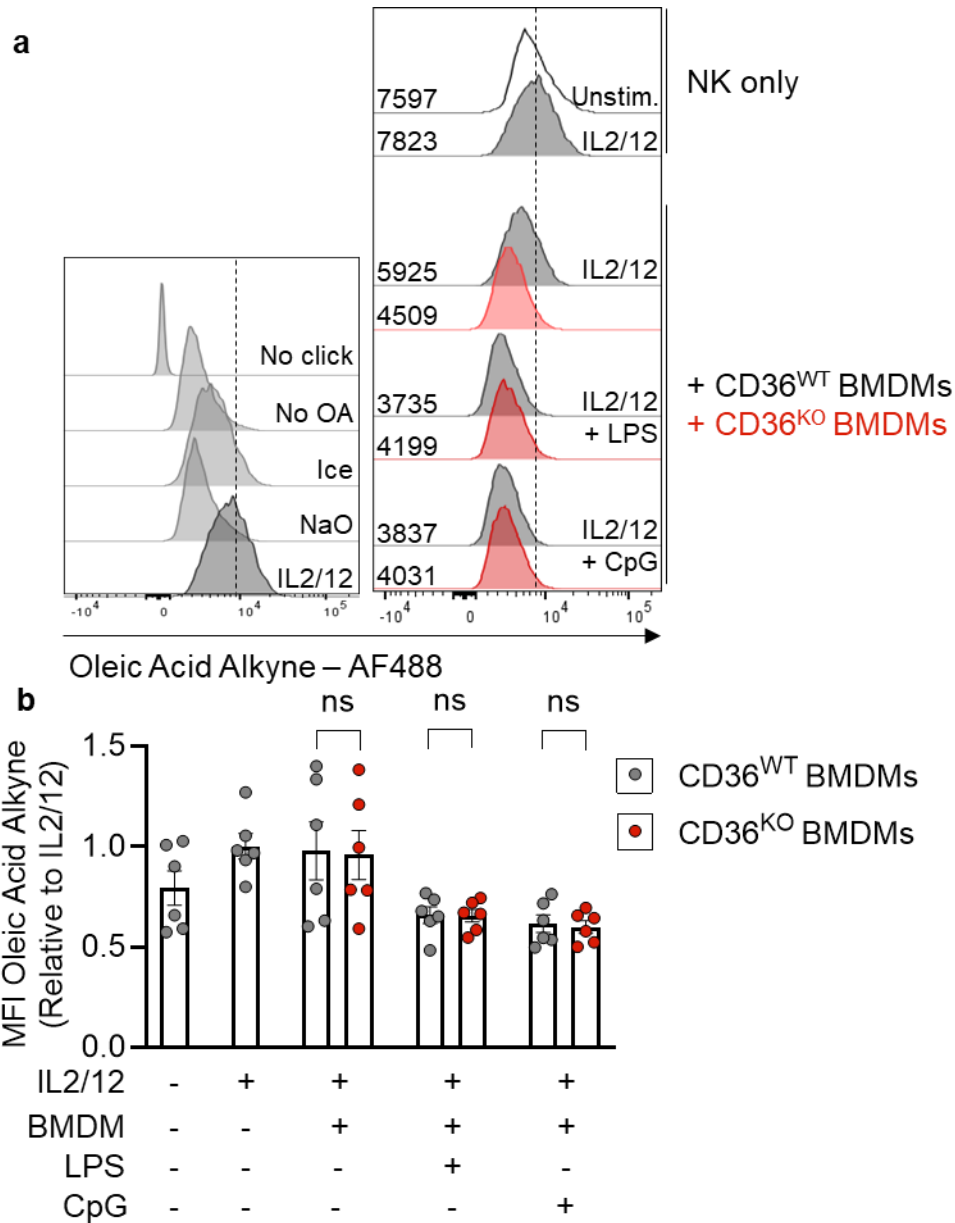


Figure 5.24 CD36^{WT} IL2-NK cells in co-culture with CD36^{KO} BMDMs do not have altered uptake of oleic acid.

CD36^{WT} or CD36^{KO} BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 µg/mL) for 6 hours. CD36^{WT} IL2-NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and co-cultured with BMDMs for 18 h. Oleic acid uptake was determined using oleic acid alkyne (OA) by click chemistry and flow cytometry. Sodium oleate (NaO) was used as a competition control using. Data is representative (a) or mean $\pm$ SEM of at least 6 biological replicates across 2 independent experiments (b). Statistical analysis was carried out using a two-way ANOVA with Šidák's post-test (ns=non-significant).

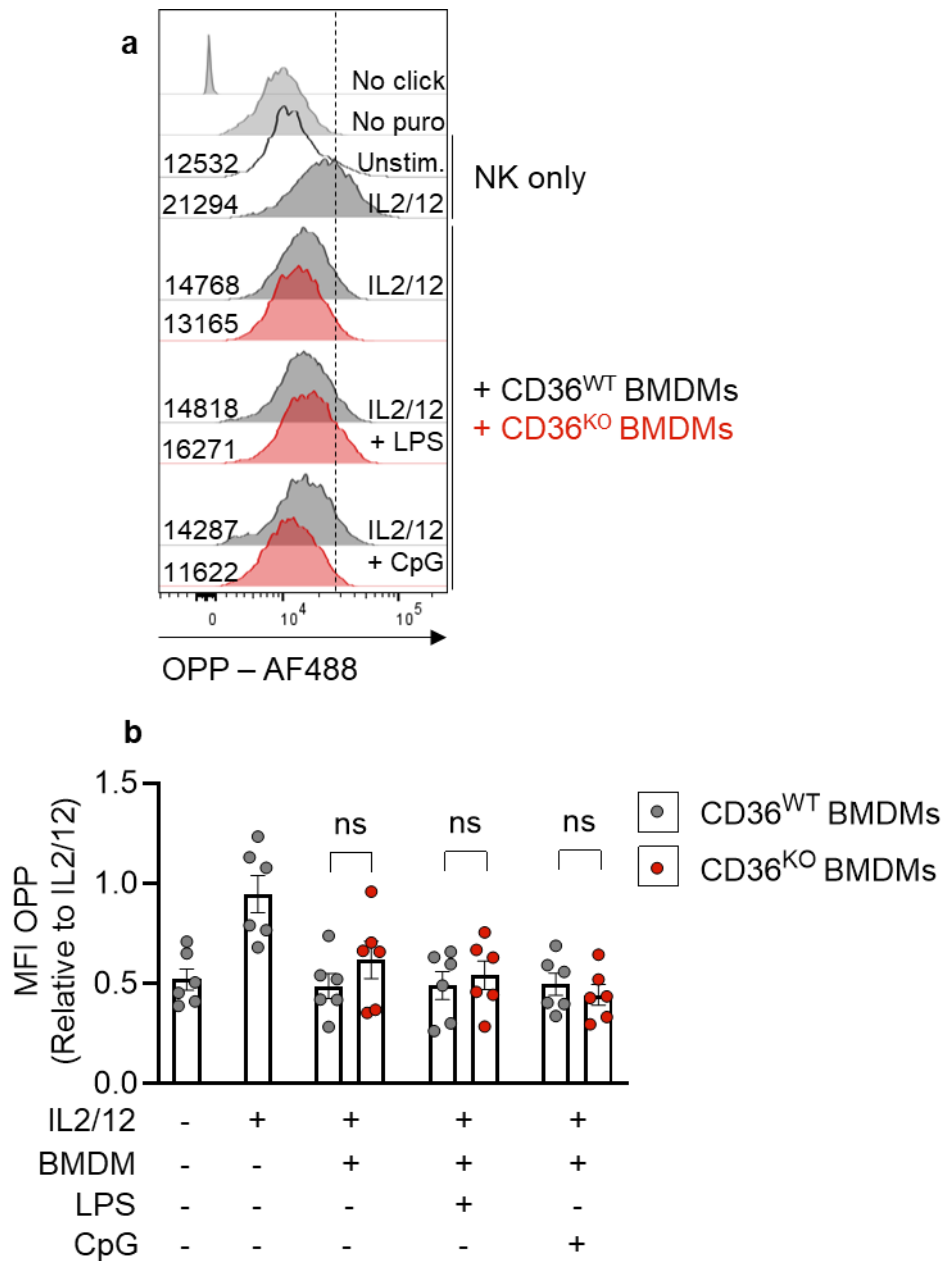


Figure 5.25 CD36^{WT} IL2-NK cells in co-culture with CD36^{KO} BMDMs do not have altered rates of protein synthesis.

CD36^{WT} or CD36^{KO} BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 µg/mL) for 6 hours. CD36^{WT} IL2-NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and co-cultured with BMDMs for 18 h. Incorporation of O-propargyl-puromycin (OPP) into translated proteins of NK cells was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 6 biological replicates across 2 independent experiments (b). Statistical analysis was carried out using a two-way ANOVA with Šidák's post-test (ns=non-significant).

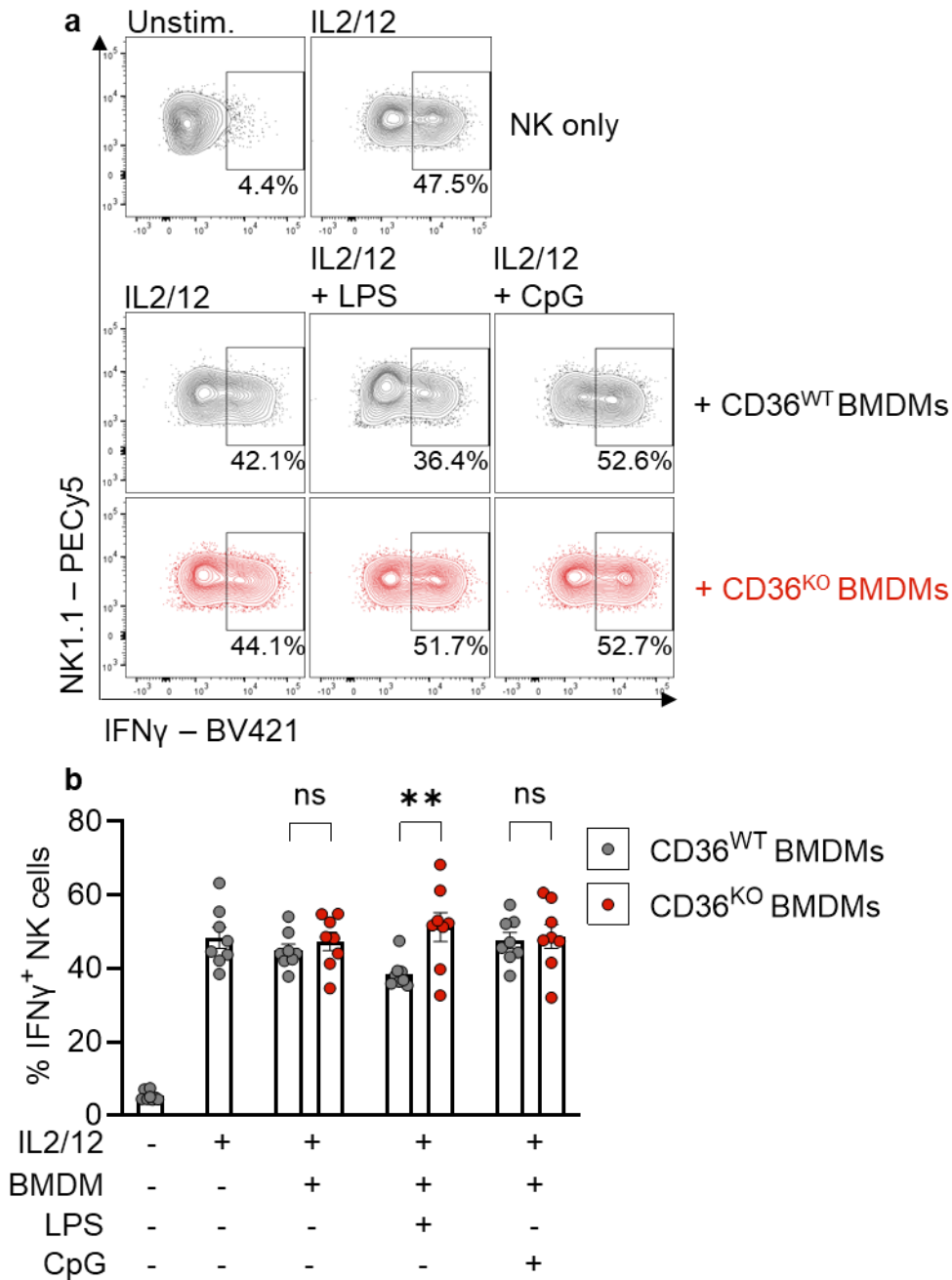


Figure 5.26 CD36^{WT} IL2-NK cells in co-culture with CD36^{KO} BMDMs have rescued IFN γ expression.

CD36^{WT} or CD36^{KO} BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 μ g/mL) for 6 hours. CD36^{WT} IL2-NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and co-cultured with BMDMs for 18 h. IFN γ was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 8 biological replicates across 3 independent experiments (b). Statistical analysis was carried out using a two-way ANOVA with Šidák's post-test (** $p < 0.01$, ns=non-significant).

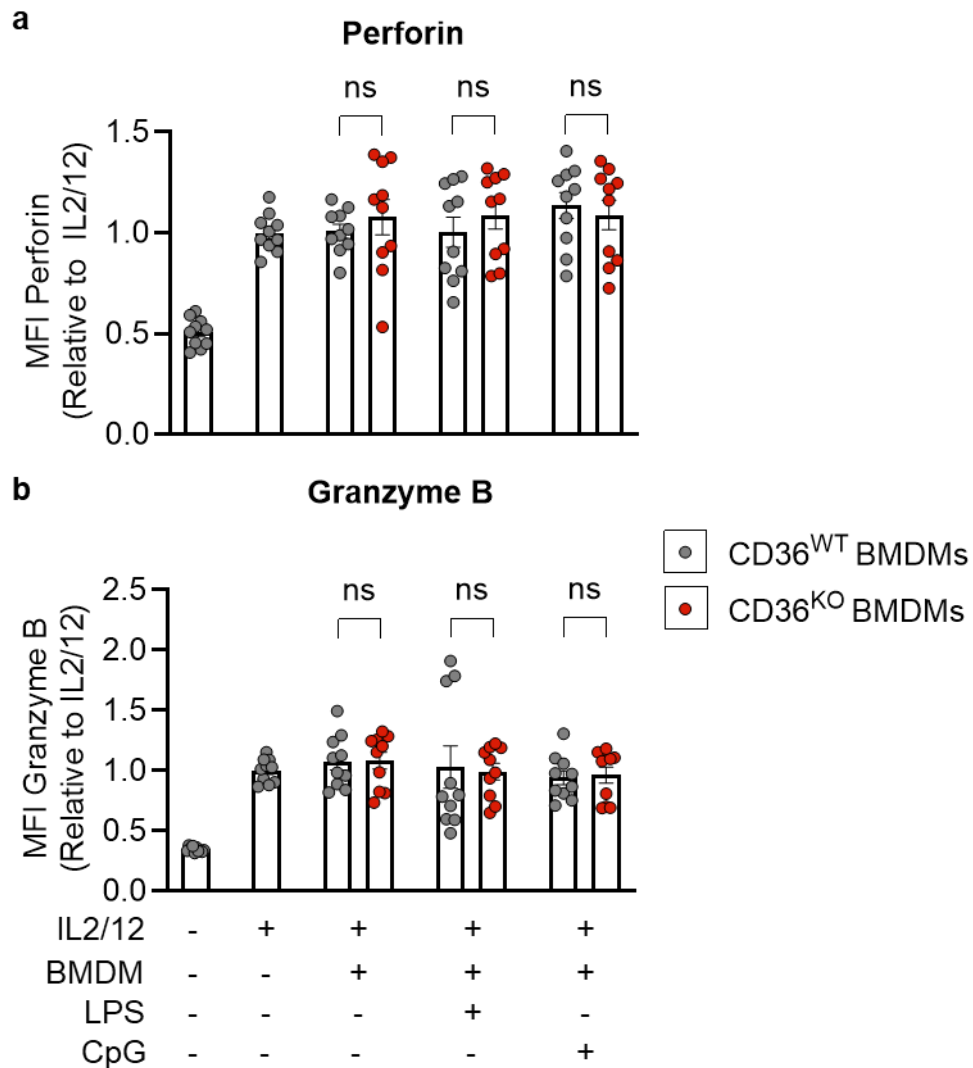


Figure 5.27 CD36^{WT} IL2-NK cells in co-culture with CD36^{KO} BMDMs do not have altered expression of perforin and granzyme B.

CD36^{WT} or CD36^{KO} BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 µg/mL) for 6 hours. CD36^{WT} IL2-NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) and co-cultured with BMDMs for 18 h. Perforin and granzyme B were measured by flow cytometry. Data is representative mean +/- SEM of 10 biological replicates across 3 independent experiments (b). Statistical analysis was carried out using a two-way ANOVA with Šidák's post-test (ns=non-significant).

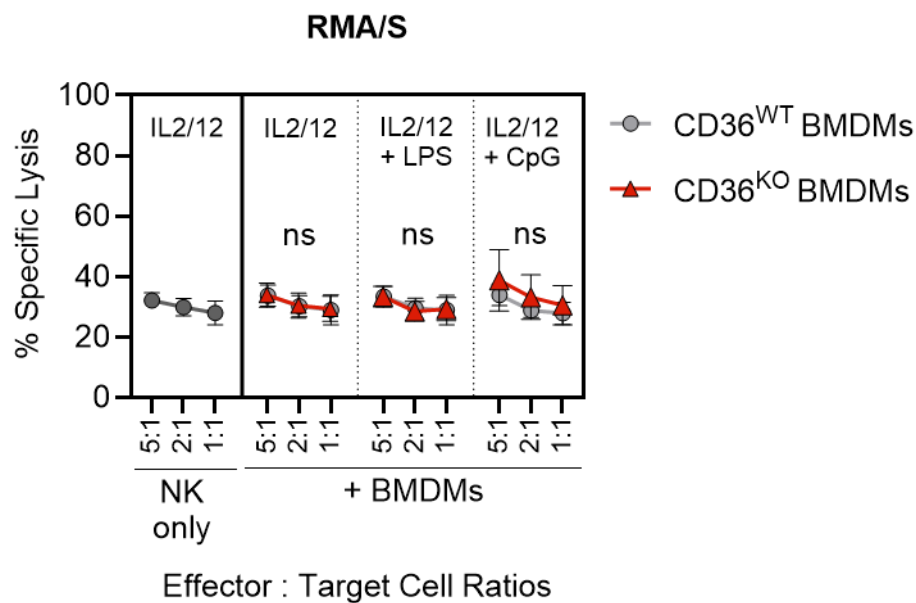


Figure 5.28 CD36^{WT} IL2-NK cells co-cultured with CD36^{KO} BMDMs do not have altered cytotoxicity.

CD36^{WT} IL2-NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) in the presence or absence of either CD36^{WT} or CD36^{KO} BMDMs, that were left unstimulated or pre-stimulated with LPS (10 ng/mL) or CpG (4 µg/mL) for 6 h. Cells were co-cultured for 18 h. NK cell cytotoxicity against RMA/S target cells was determined by fluorescence release assay. Data is mean +/- SEM of 3 biological replicates. Statistical analysis was performed using a two-way ANOVA (ns=non-significant).

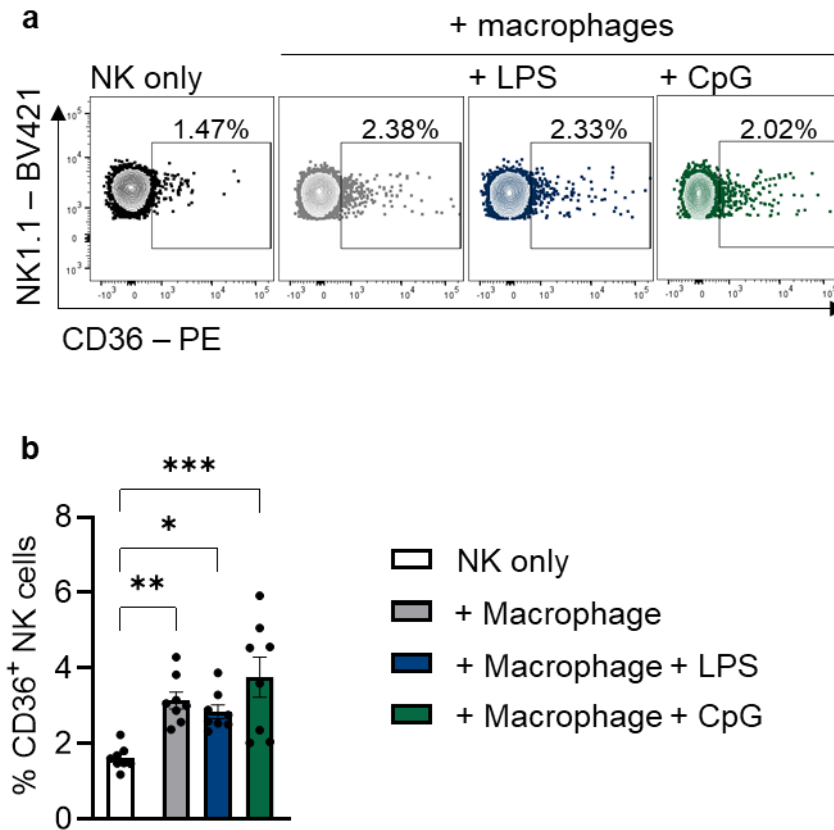


Figure 5.29 Splenic NK cells co-cultured with peritoneal macrophages have modestly increased expression of CD36.

NK cells were purified from the spleen and were cultured in the presence or absence of macrophages which were purified from the peritoneal cavity, for 18 h. All NK cells were treated with low dose murine IL15 (2 ng/mL) as a survival factor. Macrophages were left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL). CD36 expression was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 8 biological replicates across 2 independent experiments (b). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test (* p <0.05, ** p <0.01, *** p <0.001).

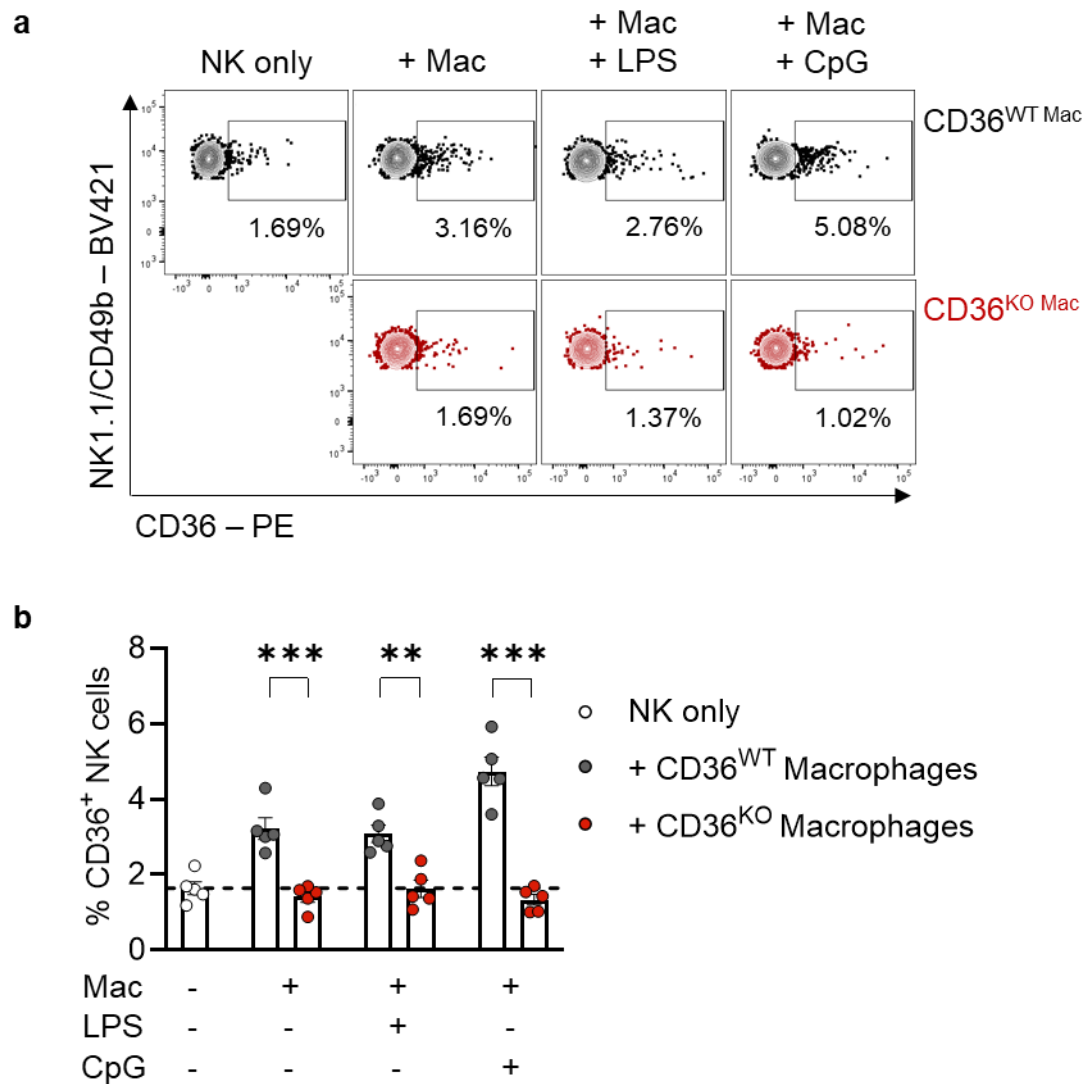


Figure 5.30 CD36^{WT} splenic NK cells co-cultured with CD36^{KO} peritoneal macrophages do not increase expression of CD36.

CD36^{WT} splenic NK cells were purified and cultured alone or in the presence of purified peritoneal macrophages (mac) from either CD36^{WT} or CD36^{KO} mice for 18 h. All NK cells were treated with low dose murine IL15 (2 ng/mL) as a survival factor. CD36^{WT} or CD36^{KO} macrophages were either left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 µg/mL). CD36 expression was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 5 biological replicates across 2 independent experiments (b). Statistical analysis was performed using a two-way ANOVA with Šidák's post-test (** $p < 0.01$, *** $p < 0.001$).

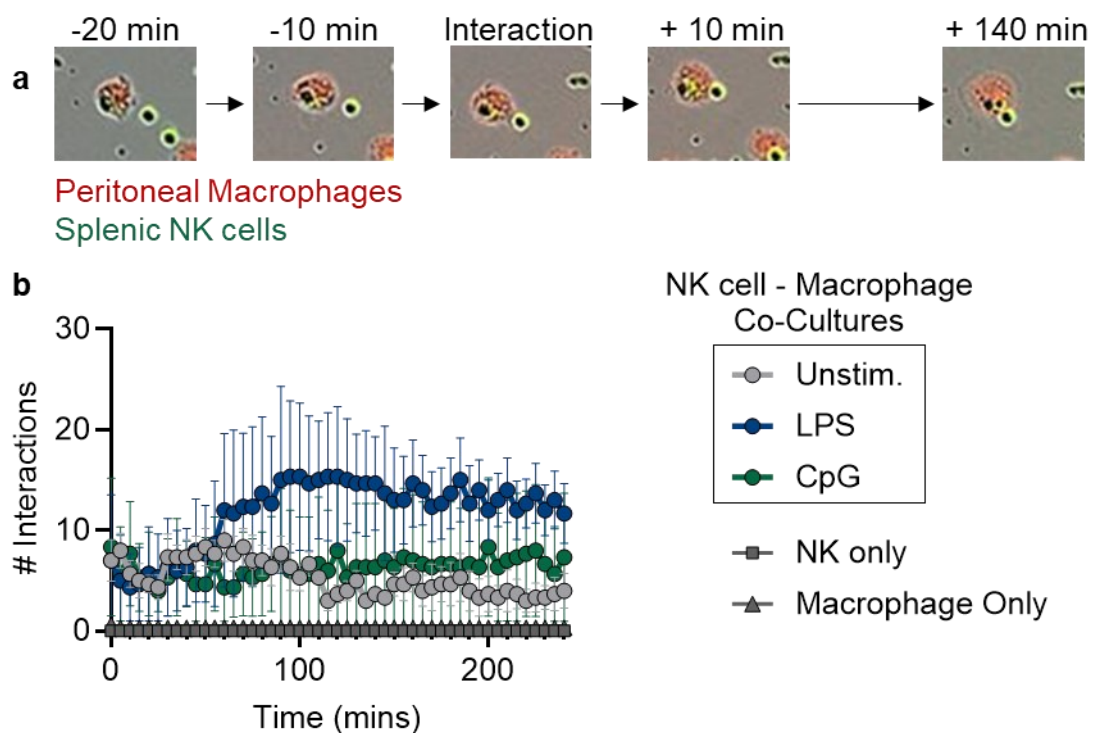


Figure 5.31 Splenic NK cells and peritoneal macrophages can form long-lasting interactions *in vitro*.

Macrophages were purified from the peritoneal cavity and were left unstimulated or were stimulated for 24 h with LPS (10 ng/mL) or CpG (4 μ g/mL). NK cells were purified from the spleen and were cultured with the macrophages for 4 h. NK cells were stained with CFSE. Macrophages were stained with CellTrace™ Far Red. The co-culture was imaged using Incucyte live cell imaging every 5 mins for 4 h. NK cell-macrophage interactions were identified as regions of the image that were stained with both CFSE and CellTrace™ Far Red. Data is representative (a) or mean $\pm$ SEM of 3 biological replicates across 3 independent experiments (b). Representative data is from co-cultures with LPS-stimulated macrophages.

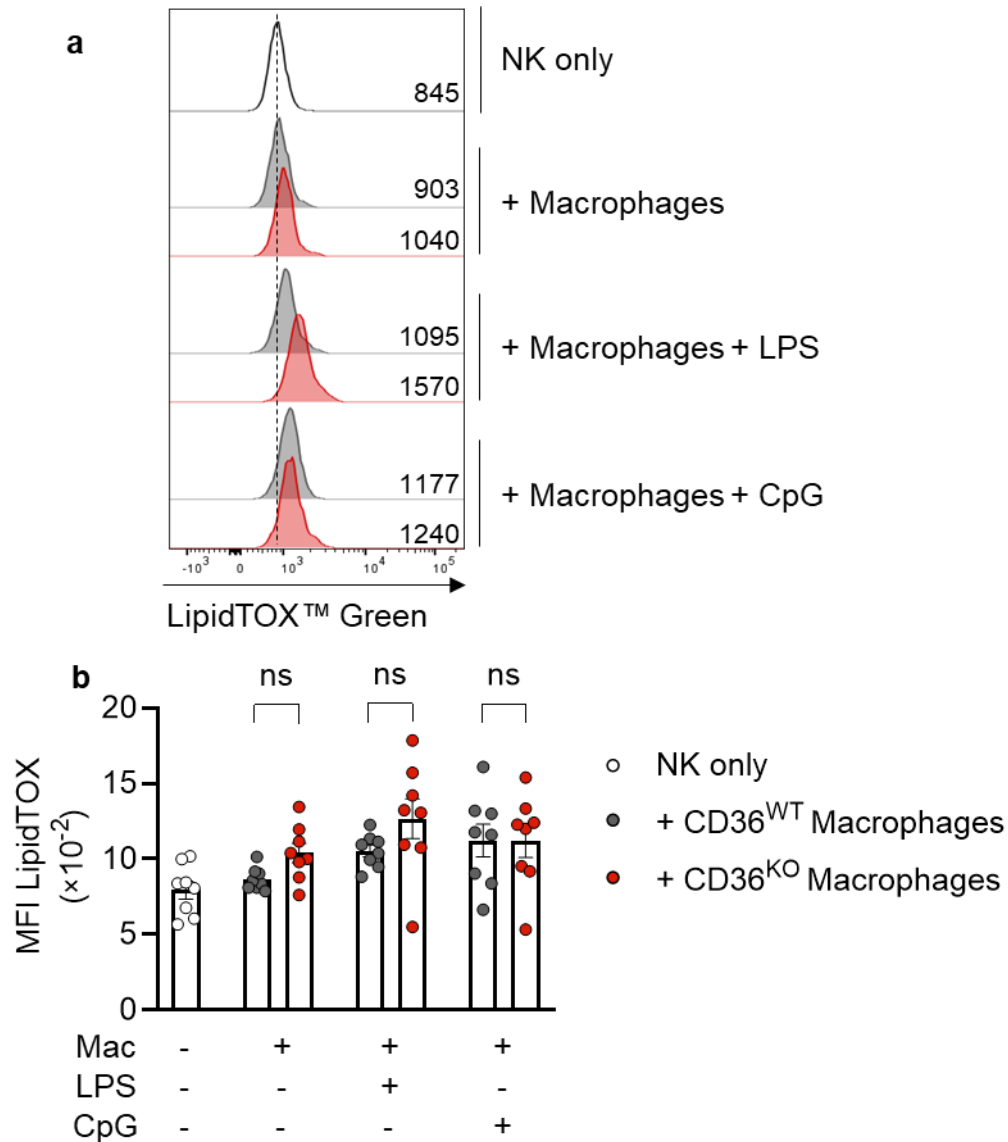


Figure 5.32 CD36^{WT} splenic NK cells co-cultured with CD36^{KO} peritoneal macrophages accumulate intracellular neutral lipids.

Wild-type splenic NK cells were purified and cultured alone or in the presence of purified peritoneal macrophages from either CD36^{WT} or CD36^{KO} mice for 18 h. All NK cells were treated with low dose murine IL15 (2 ng/mL) as a survival factor. CD36^{WT} or CD36^{KO} macrophages were either left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL). Intracellular neutral lipid content was measured using LipidTOX™ Green by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 8 biological replicates across 2 independent experiments (b). Statistical analysis was performed using a two-way ANOVA with Šidák's post-test (ns=non-significant).

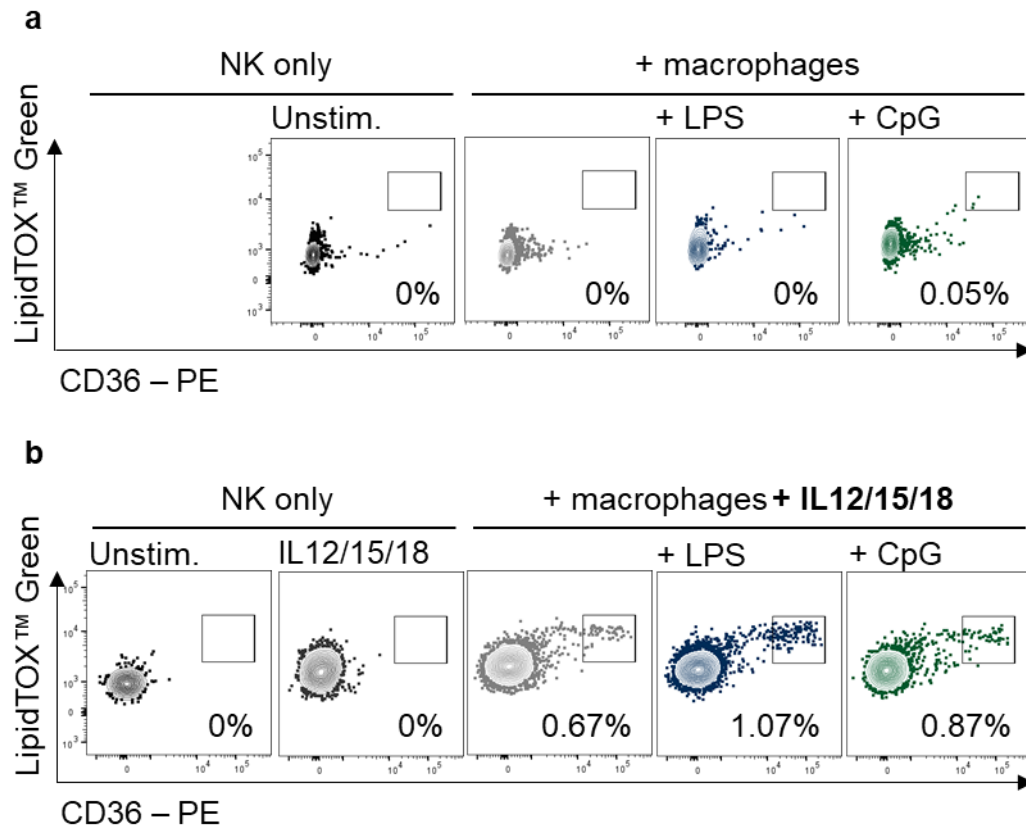


Figure 5.33 CD36⁺ splenic NK cells in co-culture with peritoneal macrophages accumulate neutral lipids following IL12/15/18 stimulation.

Splenic NK cells were purified and left unstimulated (a) or stimulated with IL12 (10 ng/mL), IL15 (10 ng/mL) and IL18 (50 ng/mL) (b) for 18 hours in the presence or absence of purified peritoneal macrophages. Macrophages were left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL). LipidTOX™ Green was used as an indicator of neutral lipid content. CD36 and LipidTOX™ Green were measured by flow cytometry. Data is representative of 8 biological replicates across 3 independent experiments. *Some replicates were performed with Shauna Mayock, Finlay Lab, Trinity College Dublin.*

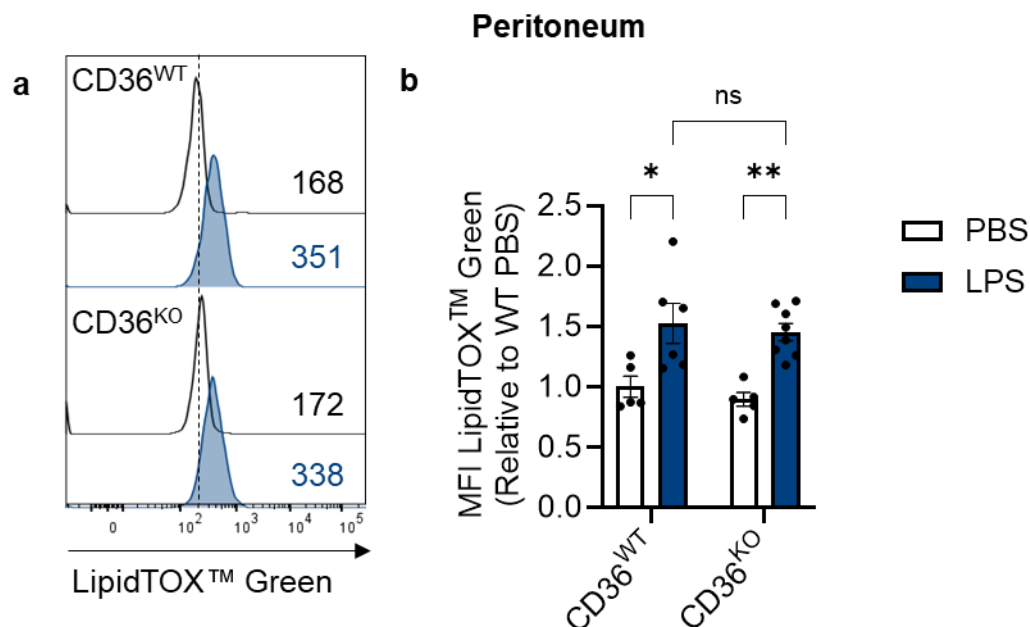


Figure 5.34 CD36 is not required for intracellular neutral lipid accumulation in peritoneal NK cells from LPS-immunised mice.

Wild-type and CD36^{KO} mice were administered PBS or LPS (2.4 mg/kg) by peritoneal injection. Peritoneal cells were harvested 24 hours post-administration. LipidTOXTM Green was used to measure intracellular neutral lipid content of NK cells (CD3⁻, CD19⁻, CD49b⁺, NK1.1⁺) by flow cytometry. A minimum of 5 mice were used per group across 2 independent experiments. Data is representative (a) or mean $\pm$ SEM (b). Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test (ns=non-significant, * p <0.05, ** p <0.01).

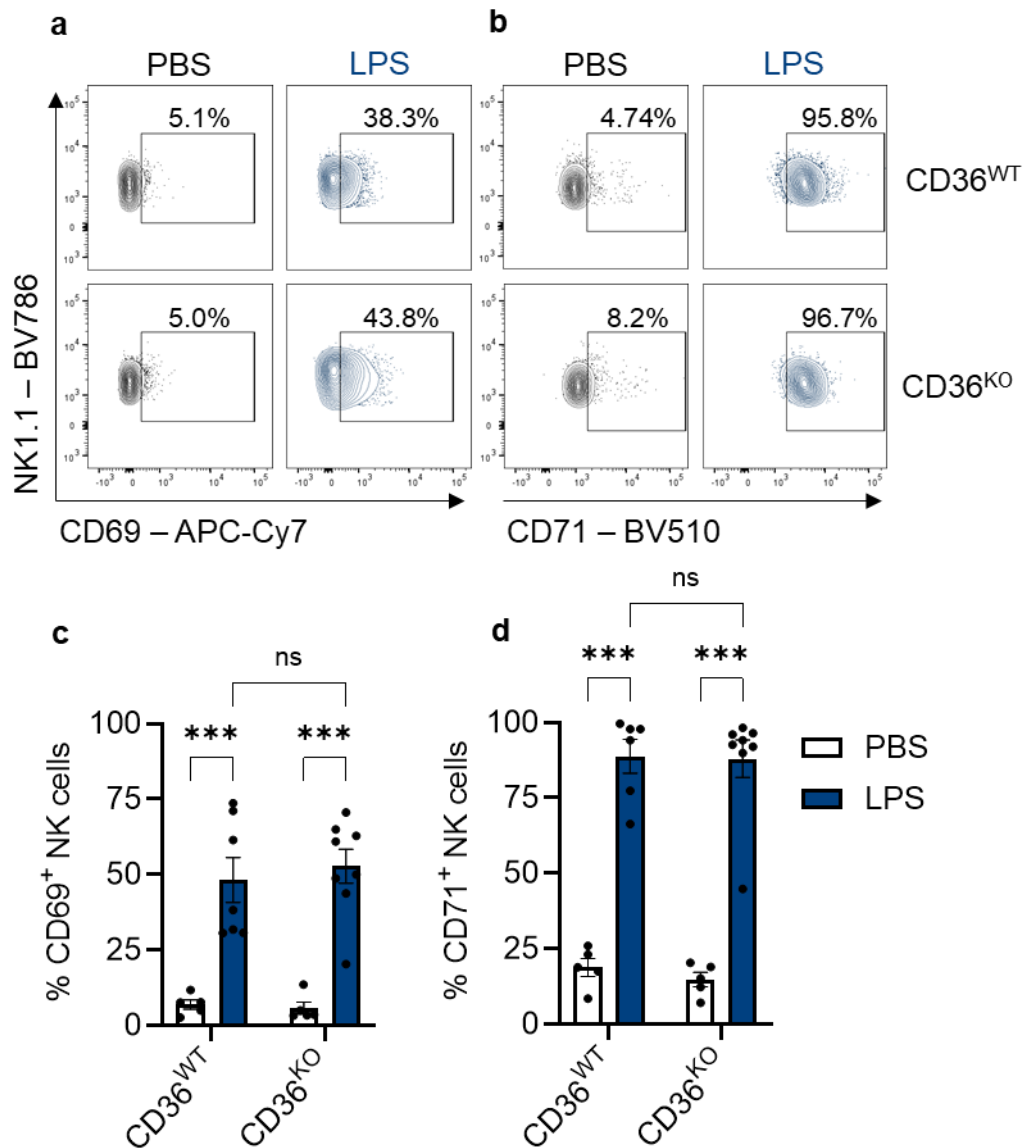


Figure 5.35 CD36 is not required for expression of activation markers by peritoneal NK cells following LPS-immunisation.

Wild-type and CD36^{KO} mice were administered PBS or LPS (2.4 mg/kg) by peritoneal injection. Peritoneal cells were harvested 24 hours post-administration. CD69 (a,c) and CD71 (b,d) expression by NK cells (CD3⁻, CD19⁻, CD49b⁺, NK1.1⁺) was measured by flow cytometry. A minimum of 5 mice were used per group across 2 independent experiments. Data is representative (a,b) or mean +/- SEM (c,d). Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test (**p<0.001, ns=non-significant).

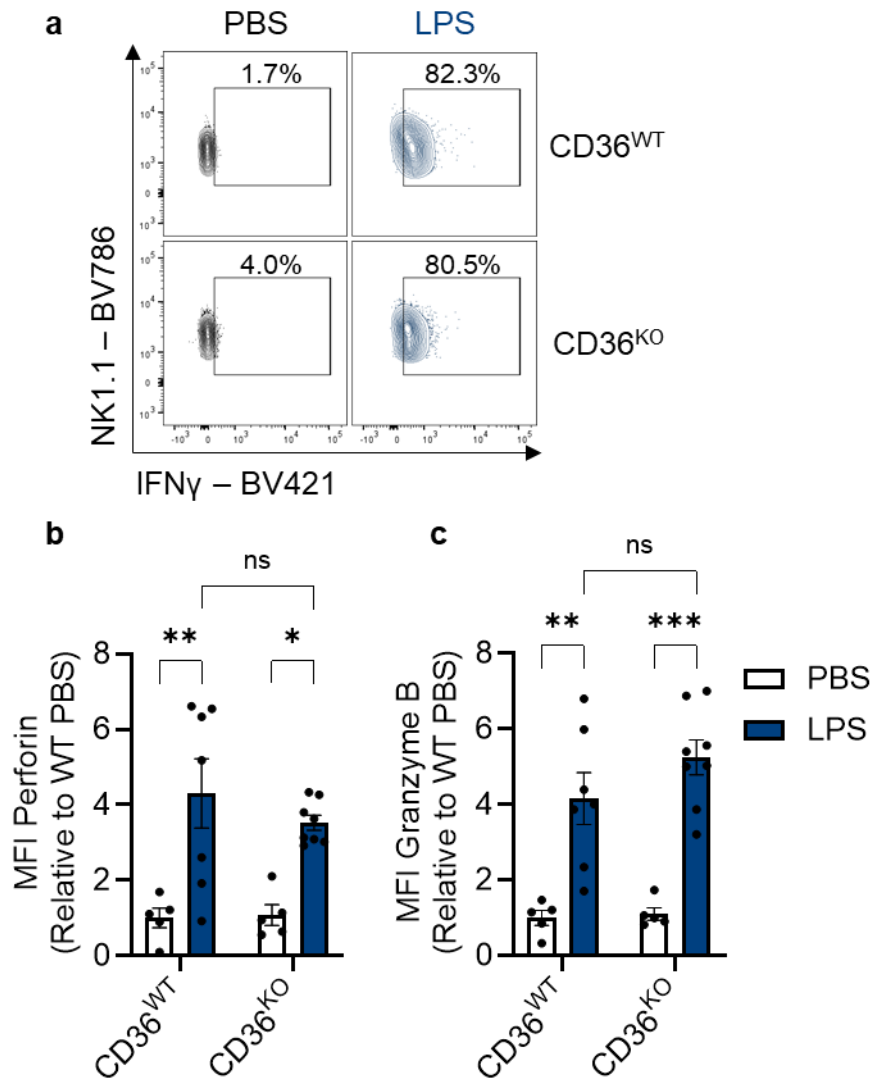


Figure 5.36 CD36 is not required for effector molecule expression by peritoneal NK cells following LPS-immunisation.

Wild-type and CD36^{KO} mice were administered PBS or LPS (2.4 mg/kg) by peritoneal injection. Peritoneal cells were harvested 24 hours post-administration. IFN γ (a), perforin (b) and granzyme B (c) expression by NK cells (CD3⁻, CD19⁻, CD49b⁺, NK1.1⁺) was measured by flow cytometry. A minimum of 5 mice were used per group across 2 independent experiments. Data is representative (a) or mean $\pm$ SEM (b,c). Statistical analysis was carried out using a two-way ANOVA with Tukey's post-test (* p <0.05, ** p <0.01, *** p <0.001, ns=non-significant).

5.4 Lipid accumulation in NK cells requires *de novo* fatty acid synthesis by macrophages.

Data in this chapter has shown that NK cells in co-culture with macrophages accumulate intracellular lipids. Since these lipids do not appear to be coming from the extracellular environment, it was investigated whether *de novo* lipid synthesis was required for lipid droplet accumulation in NK cells. To investigate this further, co-cultures were carried out with IL2-NK cells and BMDMs using a variety of different inhibitors of *de novo* lipid synthesis. How these pharmacological inhibitors target the *de novo* lipid synthesis pathway are detailed in figure 5.37. TOFA is an inhibitor of *de novo* fatty acid synthesis through competitive reversible inhibition of acetyl CoA carboxylase (ACC1) 1. C75 is an inhibitor of *de novo* fatty acid synthesis through competitive irreversible inhibition of fatty acid synthase (FASN). Zaragozic acid is an inhibitor of *de novo* cholesterol synthesis through competitive reversible inhibition of squalene synthase (SQS). These inhibitors can be used as pharmacological tools to determine which lipid species are accumulating in the NK cells and which cells are making them.

Firstly, *de novo* fatty acid synthesis was investigated as the source of lipids in NK cells using TOFA, an inhibitor of ACC1. The data showed that the accumulation of lipids in IL2-NK cells co-cultured with BMDMs was dependent on ACC1 activity, as TOFA significantly reduced lipid accumulation in NK cells (Figure 5.38). Further analysis showed that targeting this pathway can disrupt NK cell viability and function, with IL2-NK cells stimulated in the presence of TOFA exhibiting decreased viability, metabolism and expression of effector molecules (Figure 5.39). This data highlights that any functional analysis of NK cells in co-culture in the presence of TOFA should be interpreted with caution. Zaragozic acid, an inhibitor of SQS was used to determine whether the lipids accumulating in IL2-NK cells co-cultured with BMDMs relied on *de novo* cholesterol synthesis. The only NK cells that appeared to require SQS activity to support lipid accumulation were those cultured in the presence of CpG-stimulated BMDMs (Figure 5.40). Notably, the decrease in lipid accumulation was still higher than NK cells alone suggesting that some CEs may be accumulating in these cells along with fatty acid-derived DAGs and TAGs. Inhibition of *de novo* cholesterol synthesis also

showed no changes in any functional or metabolic parameters assessed in IL2-NK cells (Figure 3.41).

Both of these pharmacological inhibitors are reversible, meaning that both NK cells and macrophages were exposed to them in these experiments. This means that they cannot be used to provide information on which cell type is responsible for lipid synthesis. This chapter has shown that NK cells can obtain CD36 protein from macrophages, leading to the hypothesis that lipids are being derived from macrophages in a similar manner. Other data presented in this chapter has shown that IL2-NK cells continually accumulate neutral lipids for at least 48 h following co-culture with BMDMs (Figure 5.3). Although not statistically significant there was a trend towards increased lipid accumulation as early as 8 h following co-culture. Therefore, in order to assess expression of acetyl CoA synthesising genes in IL2-NK cells, mRNA was measured across the initial 8 h of the co-culture. Acetyl CoA, generated from either endogenous mitochondrial citrate through the activity of ACLY, or exogenous acetate through the activity of ACSS2 is the essential substrate for *de novo* lipid synthesis (Figure 5.36). Expression of both *Acly* and *Acss2* mRNA by IL2-NK cells did not change in the presence of BMDMs (Figure 5.42). This data supports the hypothesis that macrophages are the source of lipids in the co-cultures.

In order to investigate this further another pharmacological inhibitor of *de novo* fatty acid synthesis, C75, was used. C75 inhibits FASN (Figure 5.36), downstream of ACC1 and is an irreversible inhibitor, meaning that macrophages can be pre-treated with C75 before co-culture with NK cells leading to specific inhibition of *de novo* fatty acid synthesis in the macrophages. The experimental design for the use of this inhibitor is outlined in figure 5.43. BMDMs were left untreated or pre-treated with C75 for 6 hours before being washed and co-cultured with IL2-NK cells. IL2-NK cells in co-culture with C75-pre-treated, LPS-stimulated BMDMs showed significantly reduced intracellular lipid content in comparison with those co-cultured with untreated BMDMs (Figure 5.44). Although not significant there were modest decreases in IL2-NK cell lipid content in the presence of unstimulated BMDMs and CpG-stimulated BMDMs pre-treated with C75, in comparison to untreated controls. This supports the hypothesis that fatty acids are accumulating in IL2-NK cells co-cultured with LPS-stimulated

BMDMs while other lipid species may be accumulating in the presence of CpG-stimulated BMDMs. Using SSC-A as a surrogate marker of granularity showed that there was reduced internal complexity of IL2-NK cells co-cultured with C75-pre-treated, LPS-stimulated BMDMs in comparison with untreated controls, suggesting that there is also a reduction in lipid droplet number in these cells (Figure 5.45).

In order to investigate whether this was happening using a more physiological co-culture, peritoneal macrophages were left untreated or pre-treated for 2 h with C75 at the same concentration. These cells were pre-treated for a shorter time because this *in vitro* model did not involve any pre-stimulation of macrophages with TLR agonists. To confirm that lipid content was actually decreased in cultured peritoneal macrophages in the presence of C75, macrophages were removed from the co-culture and stained with LipidTOX™ Green. This revealed that C75 did reduce lipid content in macrophages but also showed that macrophages co-cultured with splenic NK cells also had significantly lower intracellular lipids than unstimulated peritoneal macrophages cultured in isolation (Figure 5.46). NK cells from these co-cultures were also stained with LipidTOX™ Green, revealing that in the presence of C75-pre-treated, LPS-stimulated and CpG-stimulated macrophages, NK cells had reduced lipid accumulation in comparison with those cultured with untreated control macrophages (Figure 5.47). Although the effects in this co-culture are more discreet than co-cultures of IL2-NK cells and BMDMs, it still suggests that macrophage-derived lipids are being transferred to NK cells. Just as the macrophage-derived CD36 was functional in cytokine-stimulated NK cells, these lipids may also have a role in modulating NK cell functions.

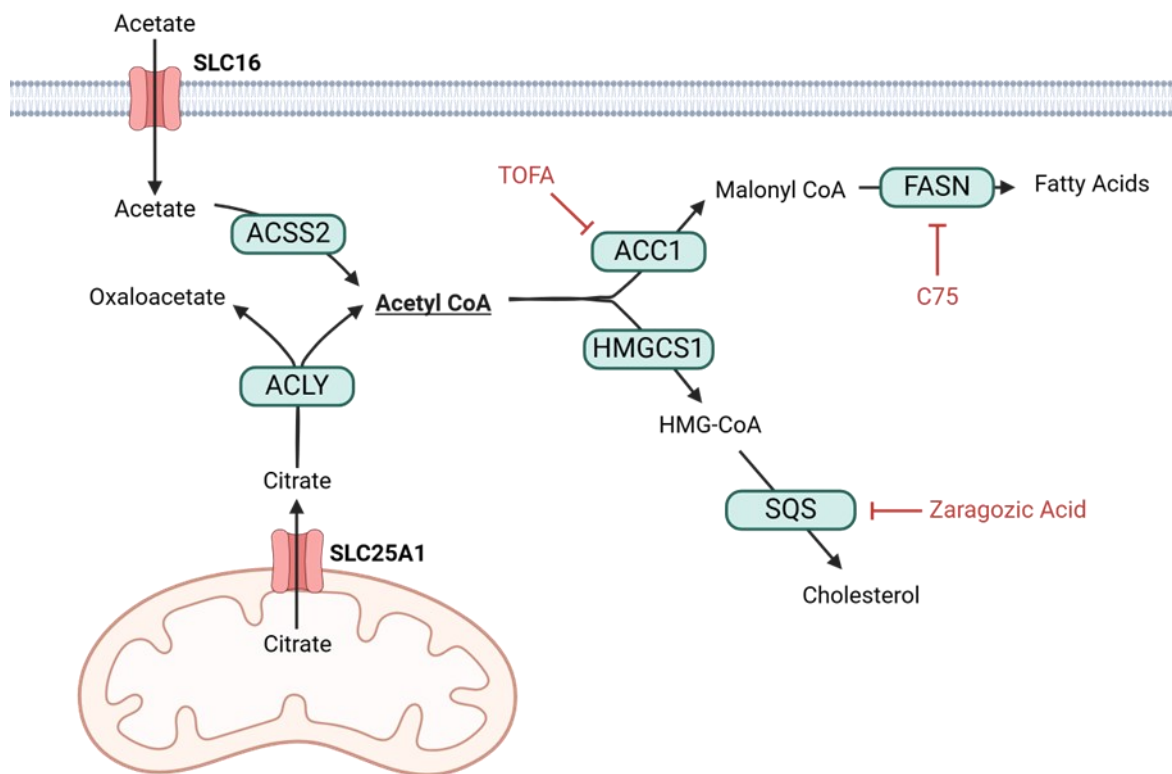


Figure 5.37 *De novo* lipid synthesis and the pharmacological tools used to inhibit synthesis of different lipid species.

De novo lipid synthesis involves the production of either fatty acids or cholesterol which can enter downstream pathways to produce a wide range of lipid species. Acetyl CoA, an essential metabolite for lipid synthesis can be derived from mitochondrial citrate, produced in the TCA cycle through the action of ATP citrate lyase (ACLY). Alternatively, acetyl CoA can be produced from exogenous acetate taken up by the SLC16 family of transporters and enzymatically produced by acetyl CoA synthetases (ACSS). Fatty acid synthesis from acetyl CoA involves the activity of the enzymes acetyl CoA carboxylase (ACC) 1 and fatty acid synthase (FASN) which can be inhibited with TOFA and C75 respectively. 3-hydroxy-3-methylglutaryl CoA synthase (HMGCS) 1 is the first enzyme in the cholesterol biosynthetic pathway. This pathway was inhibited using zaragozic acid A, which blocks the activity of squalene synthase (SQS). *This figure was made using Biorender.com.*

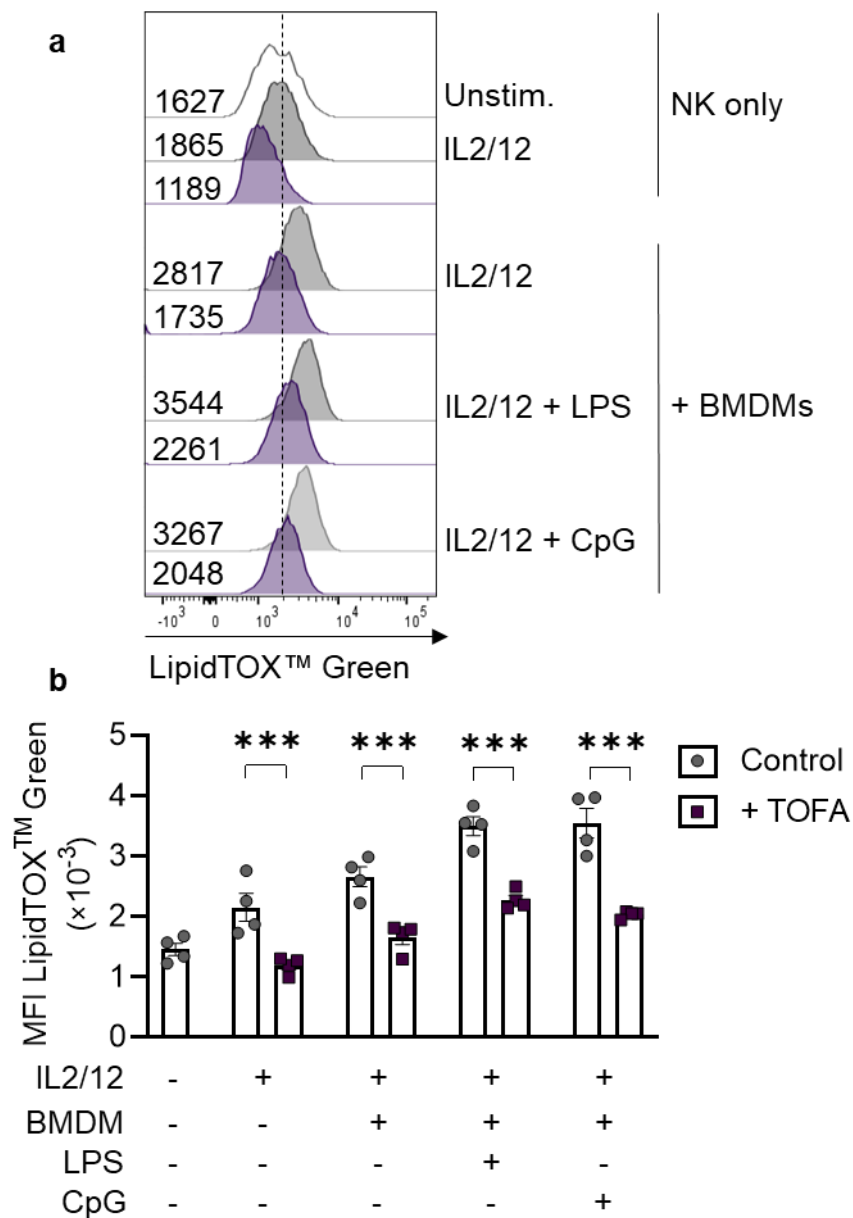


Figure 5.38 *De novo* fatty acid synthesis is required for neutral lipid accumulation in IL2-NK cells co-cultured with BMDMs.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 μ g/mL) for 6 h before co-culture with IL2-NK cells stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) in the presence or absence of TOFA (15 μ M) for 18 h. LipidTOX™ Green was used to determine intracellular neutral lipid content of NK cells by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 4 biological replicates (b). Statistical analysis was done using a two-way ANOVA with Šidák's post-test (** $p < 0.001$).

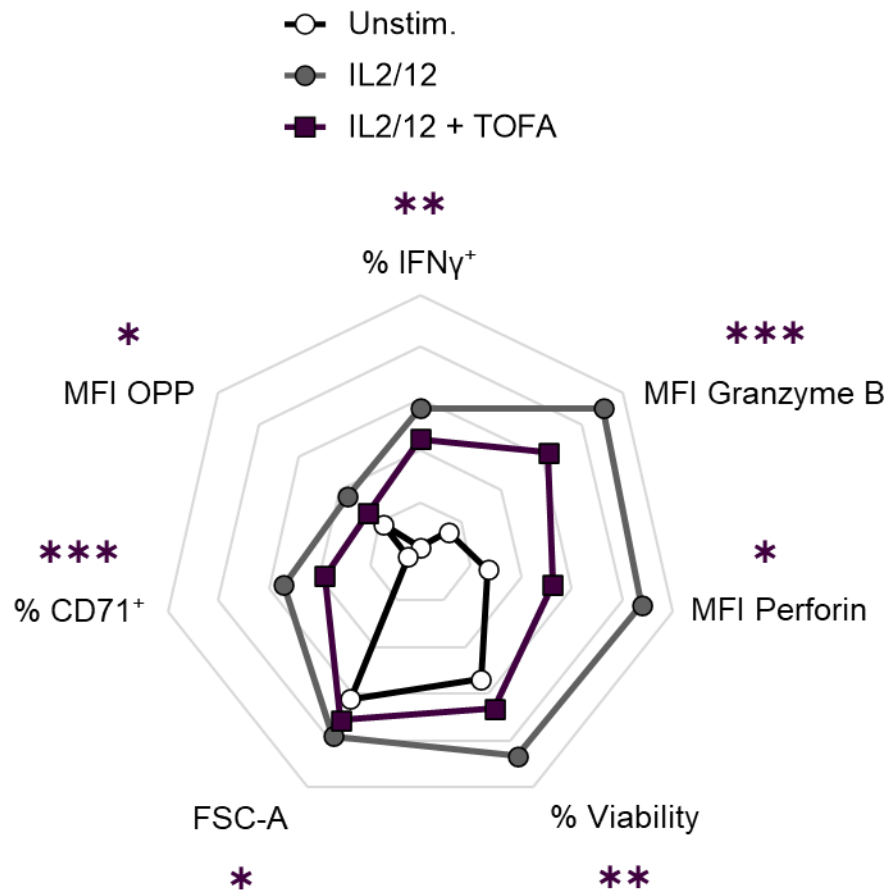


Figure 5.39 *De novo* fatty acid synthesis is required for activation of IL2-NK cells in response to IL2/12 stimulation.

IL2-NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) in the presence or absence of TOFA (15 μ M). IFN γ , granzyme B, perforin, NK cell viability, FSC-A, CD71 were measured by flow cytometry. OPP incorporation was measured by click chemistry and flow cytometry. Data is mean of 4 biological replicates. Statistical analysis was done using a one-way ANOVA with Tukey's post-test. All comparisons on the graph are made between IL2/12-stimulated NK cells with and without TOFA (* p <0.05, ** p <0.01, *** p <0.001).

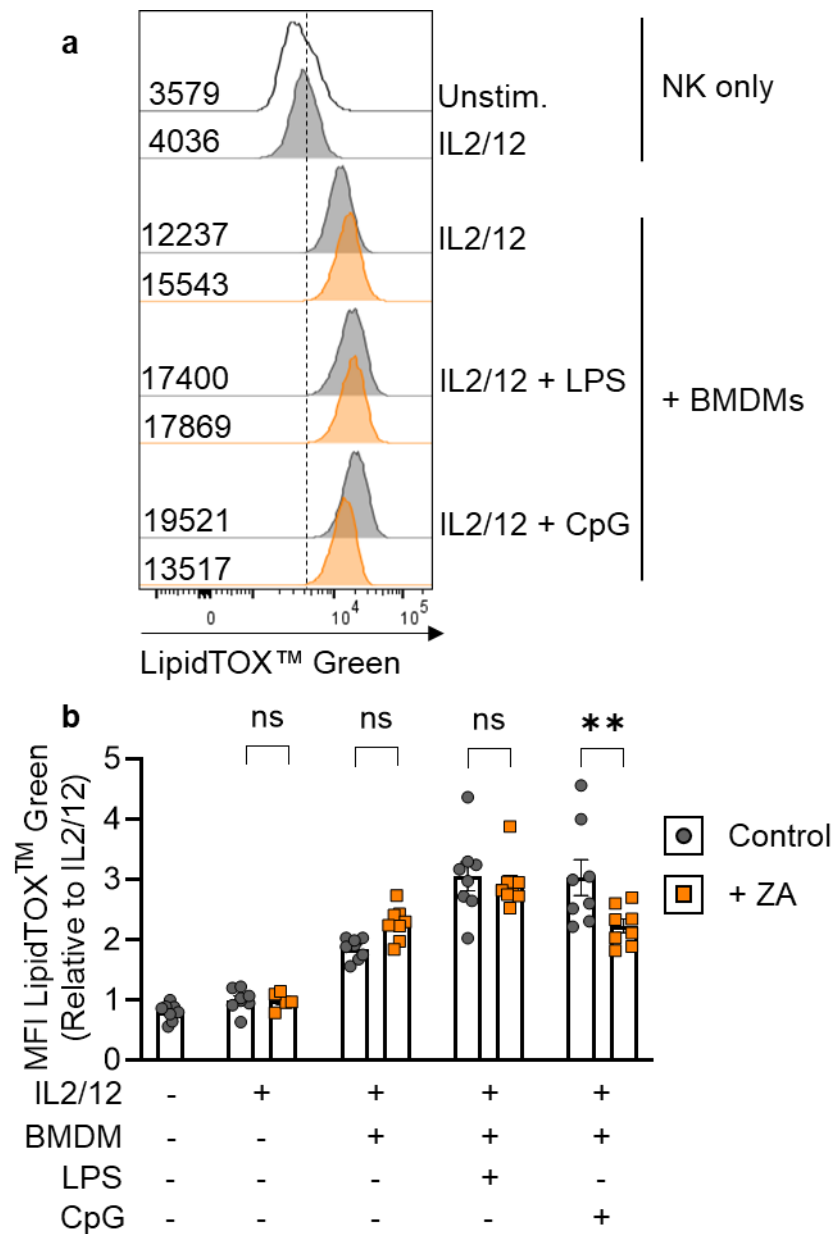


Figure 5.40 *De novo* cholesterol synthesis is required for neutral lipid accumulation in IL2-NK cells co-cultured with CpG-stimulated BMDMs.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 µg/mL) for 6 h before co-culture with IL2-NK cells stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) in the presence or absence of zaragozic acid (ZA) (10 µM) for 18 h. LipidTOX™ Green was used to determine intracellular neutral lipid content of NK cells by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 8 biological replicates across 3 independent experiments (b). Statistical analysis was done using a two-way ANOVA with Šidák's post-test (** $p < 0.01$, ns=non-significant).

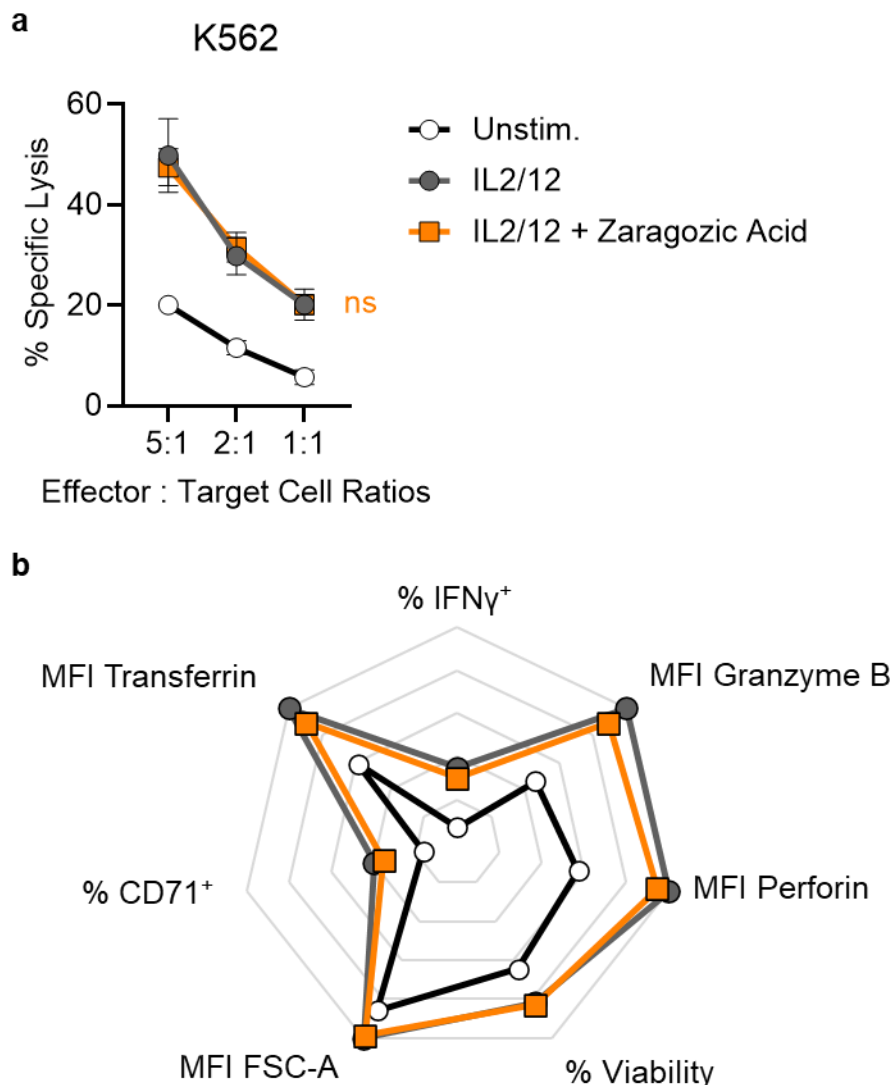


Figure 5.41 *De novo* cholesterol synthesis is not required for activation of IL2-NK cells in response to IL2/12 stimulation.

IL2-NK cells were left unstimulated or stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) in the presence or absence of zaragozic acid (10 μ M). NK cell cytotoxicity against K562 target cells was measured by fluorescence release assay (a). IFN γ , granzyme B, perforin, NK cell viability, FSC-A, CD71 and transferrin uptake were measured by flow cytometry (b). Data is mean of at least 3 biological replicates. Statistical analysis was done using a two-way ANOVA (a) or a one-way ANOVA with Tukey's post-test (b). The comparison is made versus IL2/12-stimulated NK cells only (a) (ns=non-significant).

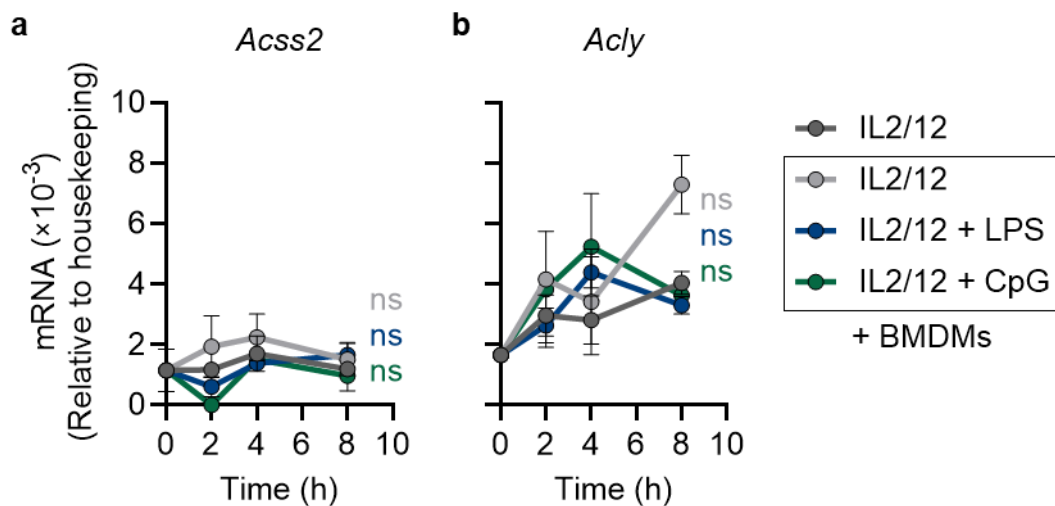


Figure 5.42 IL2-NK cells do not have altered expression of acetyl CoA synthesis genes when co-cultured with BMDMs.

IL2-NK cells were stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) in the presence or absence of BMDMs that were either unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL) for 2, 4 or 8 h. Cells were lysed and analysed by RT-qPCR for expression of *Acss2* (a) or *Acly* (b). Data is normalised to levels of control genes *Gapdh* and *Rplp0*. Data is mean $\pm$ SEM of 4 biological replicates. Statistical analysis was performed using a two-way ANOVA. Comparisons shown on the graph are made versus IL2/12-stimulated NK cells only (ns=non-significant).

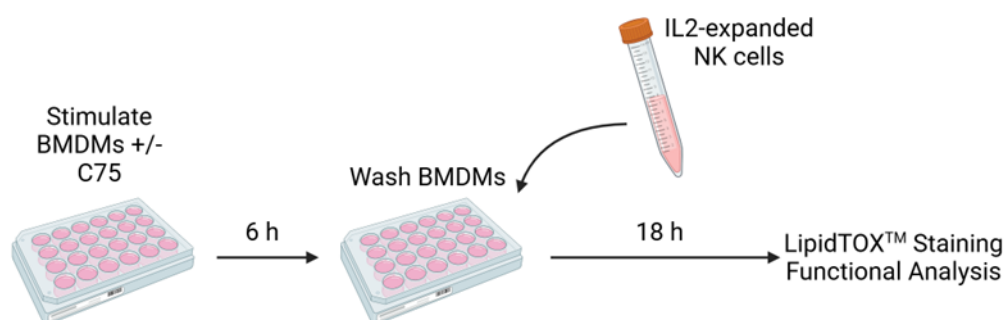


Figure 5.43 Experimental design for specific inhibition of FASN in macrophages co-cultured with NK cells.

Because C75 is an irreversible inhibitor of FASN, it can be used to specifically inhibit this enzyme in macrophages co-cultured with NK cells. BMDMs were pre-stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL) in the presence or absence of C75 (40 μ M) for 6 h. TLR agonists and excess C75 were then washed from the macrophages with PBS. IL2-NK cells were then added to the co-cultures and stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for a further 18 h before analysis. *This figure was made using Biorender.com.*

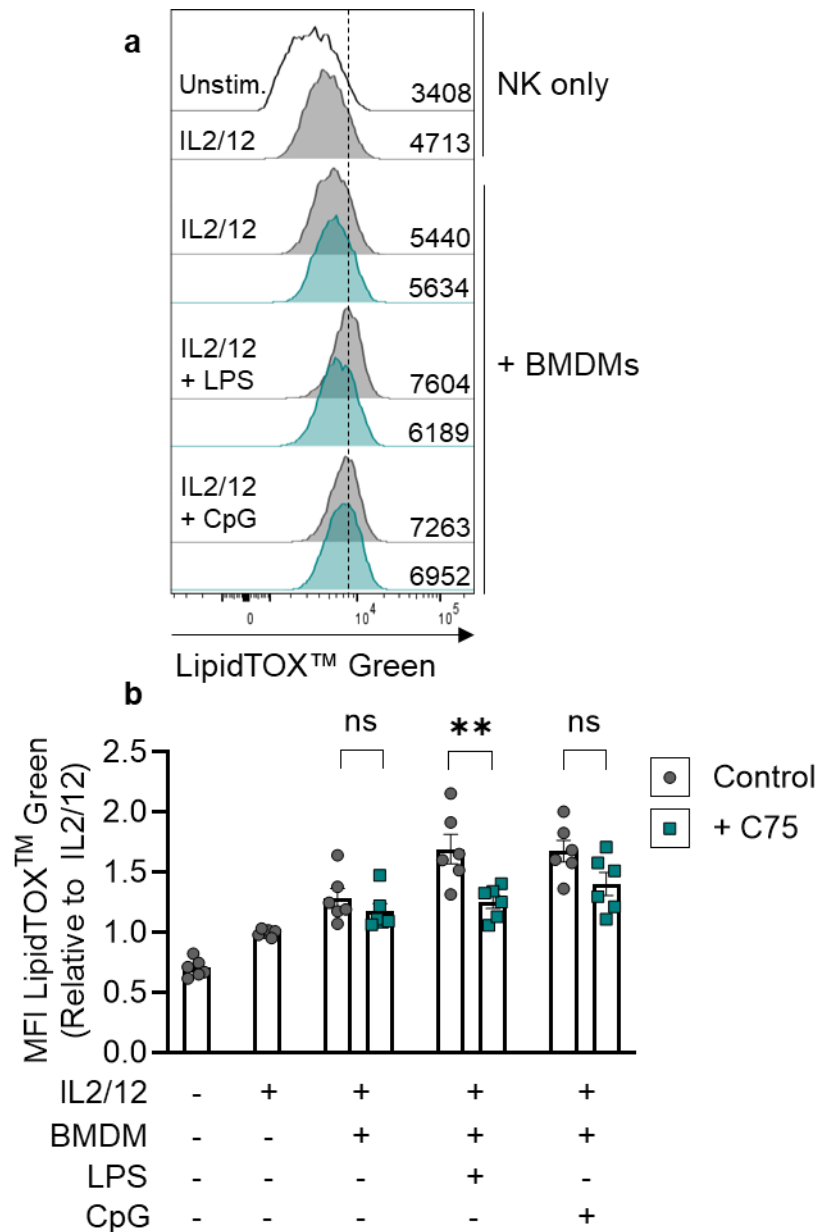


Figure 5.44 Specific inhibition of *de novo* fatty acid synthesis in LPS-stimulated BMDMs reduces neutral lipid accumulation in IL2-NK cells following co-culture.

BMDMs were left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 µg/mL), in the presence or absence of C75 (40 µM) for 6 h before co-culture with IL2-NK cells stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for a further 18 h. LipidTOX™ Green was used to determine the intracellular neutral lipid content of NK cells by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 6 biological replicates across 3 independent experiments (b). Statistical analysis was done using a two-way ANOVA with Šidák's post-test (** $p < 0.01$, ns=non-significant).

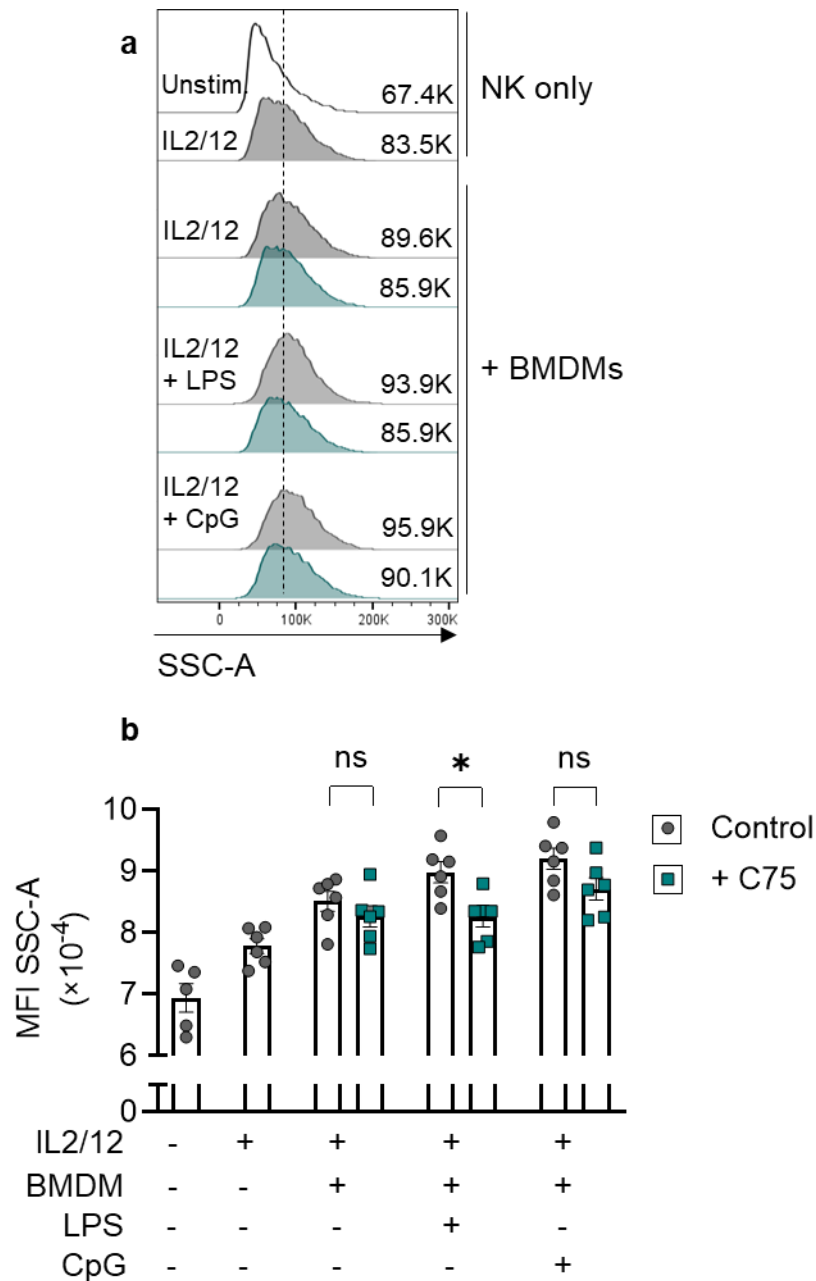


Figure 5.45 Specific inhibition of *de novo* fatty acid synthesis in LPS-stimulated BMDMs reduces internal complexity of IL2-NK cells following co-culture.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 μ g/mL), in the presence of absence of C75 (40 μ M) for 6 h before co-culture with IL2-NK cells stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for a further 18 h. SSC-A was used as a measure of internal complexity and was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 6 biological replicates across 3 independent experiments (b). Statistical analysis was done using a two-way ANOVA with Šidák's post-test (** $p < 0.01$, ns=nonsignificant).

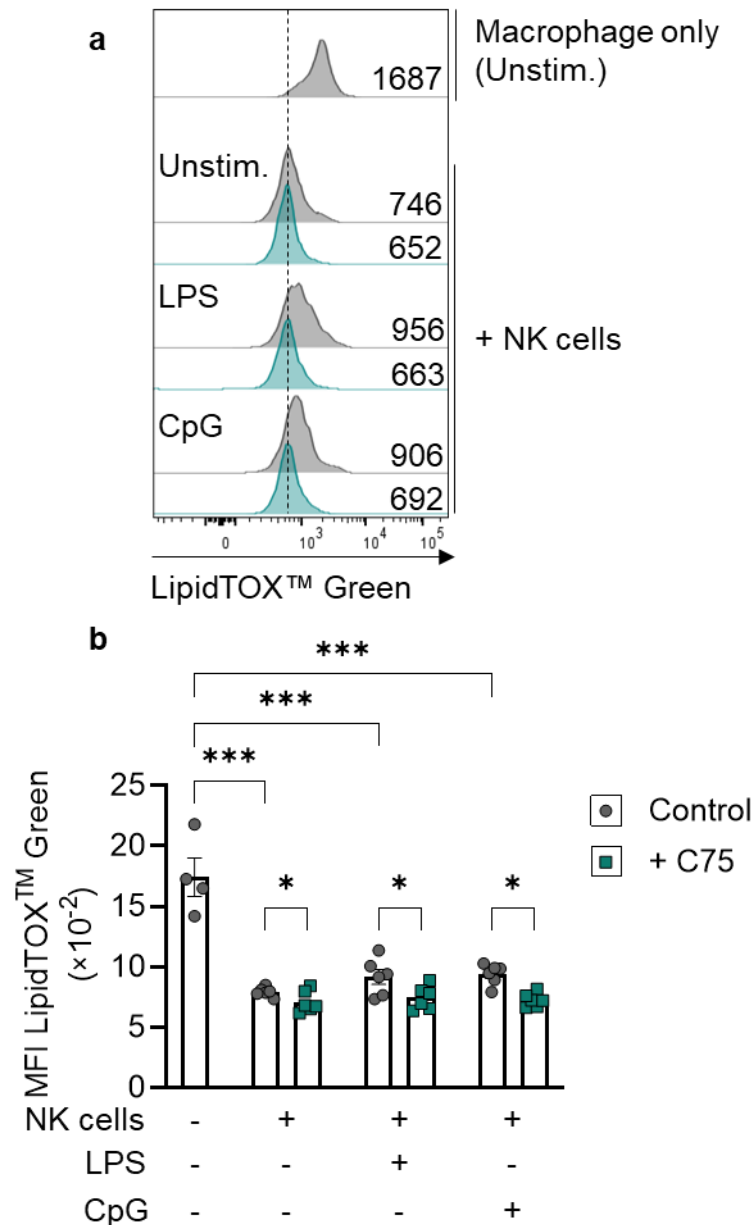


Figure 5.46 Inhibition of *de novo* fatty acid synthesis decreases intracellular neutral lipid content of peritoneal macrophages cultured with splenic NK cells.

Peritoneal macrophages were cultured in the presence of absence of C75 (40 μ M) for 2 h before being stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL), in the presence of absence of splenic NK cells for a further 18 h. All NK cells were cultured in low-dose murine IL15 (2 ng/mL). LipidTOX™ Green was used to determine intracellular neutral lipid content of macrophages by flow cytometry. Data is representative (a) or mean $\pm$ SEM of at least 4 biological replicates across 3 independent experiments (b). Statistical analysis was done using a two-way ANOVA with Tukey's post-test (* p <0.05, *** p <0.001 ns=nonsignificant).

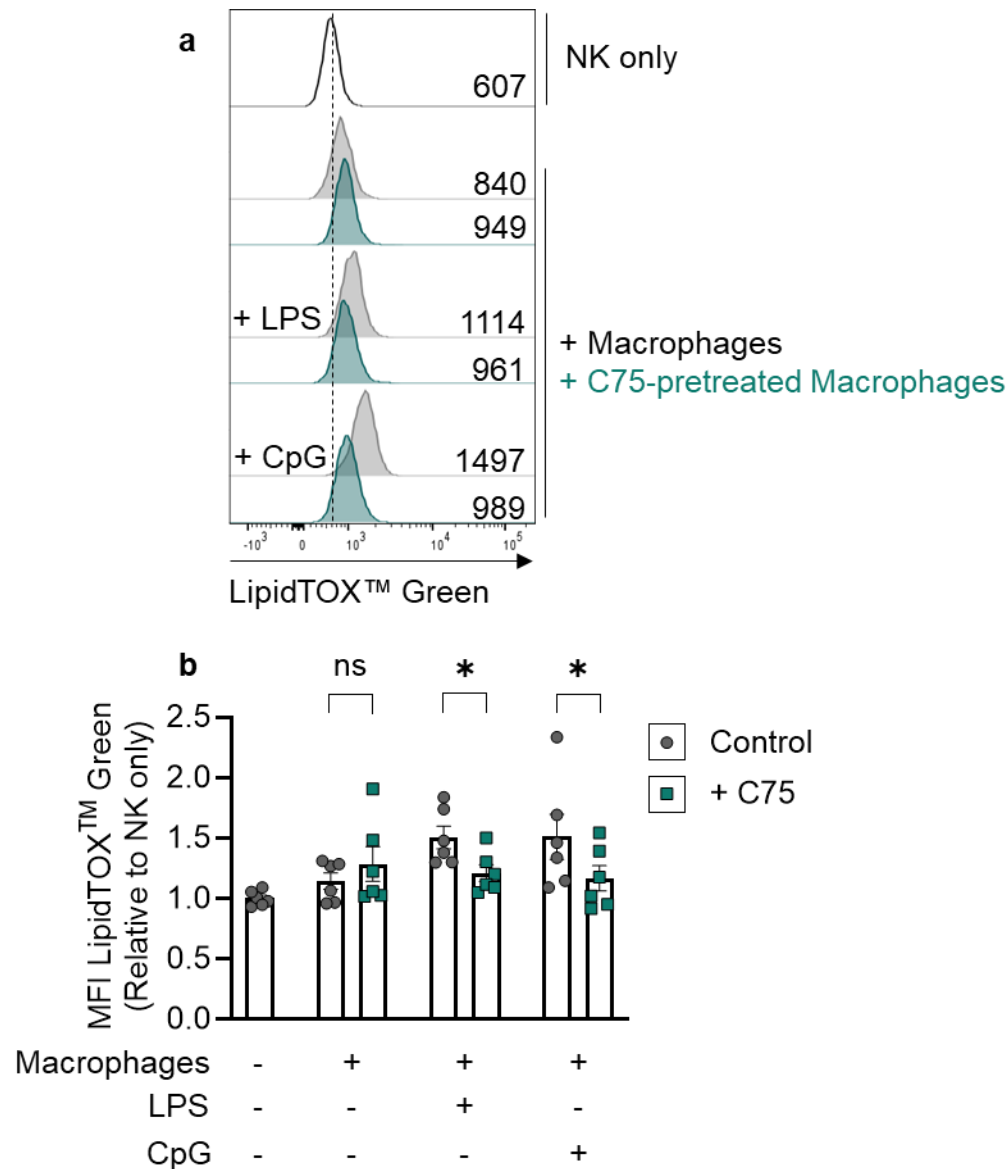


Figure 5.47 Specific inhibition of *de novo* fatty acid synthesis in peritoneal macrophages decreases intracellular neutral lipid accumulation by splenic NK cells following co-culture.

Peritoneal macrophages were cultured in the presence of absence of C75 (40 μ M) for 2 h before being stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL), in the presence of absence of splenic NK cells for a further 18 h. All NK cells were cultured in low-dose murine IL15 (2 ng/mL). LipidTOX™ Green was used to determine intracellular neutral lipid content of NK cells by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 6 biological replicates across 3 independent experiments (b). Statistical analysis was done using a two-way ANOVA with Šidák's post-test (* p <0.05, ns=nonsignificant).

5.5 Discussion

Lipids are emerging as a key regulator of lymphocyte functions. Initial studies investigating the effects of lipids on lymphocytes, focused on pathological lipid accumulation in conditions such as obesity or cancer. In these cases, increased lipid loading was associated with dysregulated lipid metabolism, leading to impaired effector functions (Tobin *et al.*, 2017; Viel *et al.*, 2017; Michelet *et al.*, 2018; Spielmann, Naujoks, *et al.*, 2020; Kedia-Mehta *et al.*, 2021; De Barra *et al.*, 2023). Because obesity is a metabolic disease, it is difficult to make a direct link between increased intracellular lipids and the altered functions of NK cells. Whether particular lipids elevated in obesity are responsible for inducing NK cell dysfunction is unknown. For example, fatty acids have been associated with impaired mTORC1-signalling (Michelet *et al.*, 2018). Increased cholesterol could also be responsible for NK cell dysfunction through inhibition of SREBP-mediated metabolic reprogramming (Brown and Goldstein, 2009; Assmann *et al.*, 2017). However, the data presented in this chapter suggests a controlled accumulation of lipids in NK cells, which may have important implications for NK cell function. In fact, it has been shown that loss of homeostatic levels of neutral lipids from healthy human NK cells impaired cytotoxicity and this was restored following supplementation with exogenous oleic acid (Li *et al.*, 2024). The role of lipids in control of NK cell functions is clearly more nuanced than previously believed.

Studies of NK cell metabolism using cultured systems have never shown an accumulation of lipids, nor have they shown any dependence on lipids to support energy metabolism. More recently studies using NK cells *ex vivo* have shown that lipids may have important roles for NK cells in viral infections (Schimmer *et al.*, 2023; Sheppard *et al.*, 2024). The increase in CD36 expression in both splenic and peritoneal NK cells following LPS-immunisation, suggests that lipid uptake may have an important role in supporting NK cell's functions in bacterial infections. CD36 is a lipid scavenging receptor that has a number of defined ligands, including oxidised low-density lipoproteins (oxLDL), oxidised phospholipids, thrombospondin-1 and long chain fatty acids (Endemann *et al.*, 1993; Dawson *et al.*, 1997; Podrez *et al.*, 2002). It is known that CD36 has fatty acid binding capacity (Abumrad *et al.*, 1993), but is not a translocase, as previously believed (Xu *et al.*, 2013). In fact, the mechanism of fatty acid transport

across the plasma membrane by CD36 is not understood. CD36 has also been described to be involved in clearance of apoptotic cells (Fadok *et al.*, 1998). The wide variety of roles that this protein carries out is not surprising considering its wide expression and its orthologues identified in many species including nematodes and sponges (Müller *et al.*, 2004). Through adding macrophages to cultured NK cells, the increase in CD36 expression observed *in vivo* could be recapitulated. This highlights that signals from cells in *in vivo* niches are important for modulating other cell's functions. In this case direct contact with macrophages was required for CD36 expression on NK cells.

Even more interesting is the mechanism that the NK cells are using to rapidly increase CD36 expression. This rapid transfer is not due to an increase in expression of the protein by the NK cell, but because of transfer of CD36 protein from macrophages to NK cells. The process of cells sharing proteins is called trogocytosis, which comes from the Greek word meaning “to gnaw”. In these co-cultures, NK cells are essentially extracting pieces of macrophage membrane and re-expressing this gained protein, giving them a new cellular function. The mechanism of trogocytosis has not been fully unravelled though it is known to involve actin polymerisation and is similar to RhoG GTPase-dependent phagocytosis (Hudrisier *et al.*, 2007; Martínez-Martín *et al.*, 2011). This process is particularly associated with lymphocytes and has been described in CD8⁺ T, CD4⁺ T, γδ T, B and NK cells (Espinosa *et al.*, 2002; Tabiasco *et al.*, 2002; Puaux *et al.*, 2006; Hudrisier *et al.*, 2007). Some myeloid cells have also been described to carry out trogocytosis with dendritic cells using it to scavenge antigens and MHC molecules (Wakim and Bevan, 2011; Hughes *et al.*, 2020). In the case of NK cells trogocytosis has been described to be an inhibitory process in cancer and is an obstacle to overcome for NK cell-based immunotherapies. One study from CLL patients treated with CAR-NK cells showed that CAR-NK cells were using trogocytosis to obtain CD19, the cognate antigen, which reduced recognition of tumour cells and also led to fratricide of other CAR-NK cells (Li *et al.*, 2022). Another study using a murine RMA/S model of leukaemia showed that NK cells were using trogocytosis to obtain PD-1 *in vivo* which attenuated NK cell anti-tumour responses (Hasim *et al.*, 2022). It is hypothesised that trogocytosis evolved in primitive cells as a means to rapidly increase expression of proteins

(Joly and Hudrisier, 2003). These studies show that cancers may take advantage of this process in an effort to evade anti-tumour immune responses.

This chapter suggests that the re-expression of CD36 by NK cells is rapid, controlled and deliberate, as there was increased CD36 expression by the NK cells as soon as 1 h following co-culture. Furthermore, NK cells did not obtain F4/80 from macrophages following co-culture, suggesting this protein was either not taken from the macrophages or was degraded by the NK cells. The fact that F4/80, a highly expressed, macrophage-specific protein was not expressed by NK cells, suggests that mechanisms are in place to control the re-expression of macrophage-derived CD36. It is thought that trogocytosis has evolved to quickly confer a new biological function to recipient cells. The data presented in this chapter agrees with this as CD36⁺ splenic NK cells co-cultured with peritoneal macrophages had higher accumulation of intracellular lipids than CD36⁻ NK cells. This suggests that in specific tissue niches where NK cells and macrophages are in close proximity, trogocytosis may act as a mechanism to change NK cells functions rapidly, without having to change the pattern of protein expression which can take hours.

Experiments have shown that the likely mechanism of CD36 transfer from macrophages to NK cells is trogocytosis, enabled by direct interactions between the cells and subsequent actin reorganisation required for endocytosis and re-expression of CD36 by NK cells. However, it must also be considered whether the expression of CD36 is mediated by soluble transfer following an initial direct interaction between the cells. Macrophages have been shown to shed CD36 both *in vitro* and *in vivo* through the activity of ADAM17 (Driscoll *et al.*, 2013). This study detected increased levels of soluble CD36 in cell culture medium, which agrees with other studies that have shown that circulating soluble CD36 can act as a biomarker for viral infections (Himoto *et al.*, 2013; Cai *et al.*, 2022). Although the source of soluble CD36 is not known to be macrophages, in atherosclerosis the increase in soluble CD36 was shown to be in macrophage-rich regions of the plaque (Handberg *et al.*, 2008). It is unlikely that this mechanism of CD36 transfer is occurring in these experiments as NK cells are not described to scavenge proteins independently of exocytic vesicles (EVs), nor is soluble CD36 described

to be endocytosed by any other cells. Overall, there is mounting evidence in this chapter that the macrophage-derived CD36 on NK cells is due to trogocytosis.

One result that conflicts with the hypothesis that trogocytosis is occurring is the increase in *Cd36* mRNA observed in NK cells co-cultured with BMDMs. However, the kinetics of CD36 protein expression versus *Cd36* mRNA expression is discordant with endogenous expression, as protein appears very quickly, before any increase in *Cd36* mRNA. There is no evidence in literature that cells store *Cd36* mRNA in P-bodies to facilitate the rapid protein expression that occurs in these co-cultures, suggesting that the NK cells are obtaining CD36 from exogenous sources. Furthermore, the increase in *Cd36* mRNA expression could be occurring because of an increase in intracellular fatty acids which can activate PPAR signalling. *Cd36* has a peroxisome proliferator-responsive element (PPRE) in its promoter region meaning that it is a target gene of PPAR transcription factors (Nagy *et al.*, 1998). PPAR α/δ signalling in particular has been described to induce *Cd36* mRNA expression in NK cells (Michelet *et al.*, 2018).

The co-cultures used in this chapter revealed that in the presence of macrophages, NK cells increase their intracellular neutral lipid content, and that this is further increased when the macrophages receive stimulation from TLR agonists. This result was particularly interesting because it suggested a macrophage-lipid-NK cell axis that could be essential for intercellular communication between the cells and modulate their function. The idea that metabolites can act as paracrine signalling molecules is emerging in relation to immune modulation (Zeng *et al.*, 2023). Data already discussed in this thesis has investigated whether macrophage-derived 25HC could be an important player in the regulation of NK cell responses. Although, there was no correlation between levels of 25HC-production by macrophages and NK cell dysfunction, the fact that there is a significant increase in intracellular lipids reestablished the idea that metabolites could be involved in intercellular communication. However, the presence of the lipids in the NK cells does not mean that they are being released in soluble form from macrophages and entering NK cells. In fact, through use of direct co-cultures, trans-well assays and culture of NK cells in BMDM-conditioned medium we can see that the lipids are not transferred through soluble

mechanisms. This was further supported by metabolomics of co-culture medium, where there were very modest changes in concentration of lipids. Because trogocytosis of CD36 did not appear to be essential for lipid accumulation in NK cells other mechanisms of lipid accumulation were investigated. Although CD36⁺ NK cells had higher lipid accumulation following interactions with macrophages, other CD36⁻ NK cells were also shown to increase their lipid content, suggesting another CD36-independent mechanism of lipid accumulation was occurring.

The enhanced IFN γ expression in IL2-NK cells co-cultured with LPS-stimulated, CD36^{KO} BMDMs suggests that the presence of CD36 may be responsible for enhanced uptake of particular lipid species that may impact IFN γ expression. It was shown that CD36⁺ NK cells had enhanced lipids, though the exact lipid species present in NK cells are not known. Data in this chapter has shown that CD36 is not required for oleic acid uptake, though uptake of other lipid species may be impacted. It should also be considered whether CD36^{KO} BMDMs have altered function in comparison to CD36^{WT} BMDMs. In fact, it has been shown that CD36^{KO} BMDMs had enhanced IL1 β , TNF α and IL12 production when stimulated with IFN γ *in vitro* (Wang *et al.*, 2020). This study revealed that co-culture of these CD36^{KO} BMDMs with CD8⁺ T cells enhanced expression of IFN γ by T cells. Given the importance for IL12 for NK cell activation in certain contexts, it is also possible that enhanced IL12 production by BMDMs could be supporting NK cell IFN γ production. This is unlikely as the addition of IL12 to these co-cultures ensures that this cytokine is in excess, though it does not exclude other macrophage-derived factors having synergistic effects.

Among the changes observed in the co-culture medium lipidome was an accumulation of phospholipids. Although modest, this could have impacts for NK cell functions. A study investigating NK cells in the ascites of ovarian cancer patients has shown that phospholipid uptake, particularly phosphatidylcholine (PC) 36:1, led to dysregulated lipid metabolism and impairments in killing of target cells (Slattery *et al.*, 2025). Concentrations of PC 36:1 in the ascites were in the micromolar range, whereas in the NK cell-BMDM co-culture medium the mean concentration was 70 nM, meaning it is an unlikely candidate for NK cell dysfunction. It is also possible that the phospholipids present in the co-culture medium are present in EVs. Lipidomics of EVs isolated from serum of healthy

human donors showed that the predominant component lipid species are triglycerides, phosphatidylcholines and sphingomyelins (Peterka *et al.*, 2020). The most abundant phosphatidylcholine identified in human serum EVs was PC 34:2, which agrees with the metabolomics of the co-culture medium. Furthermore, the most abundant sphingomyelin (SM) in human serum EVs was SM 34:1, also mirroring the co-culture medium. Other studies have also suggested that phosphatidylcholines and sphingomyelins are abundant lipid species in EVs (Wubbolts *et al.*, 2003; Laulagnier *et al.*, 2004; Llorente *et al.*, 2013; Chapuy-Regaud *et al.*, 2017), suggesting that in the co-culture medium the lipids detected are primarily in these vesicles. It is possible that EVs present in BMDM-NK cell co-culture medium are from both BMDMs and NK cells as both have been described to release them (Zhang *et al.*, 2021; Aarsund *et al.*, 2022).

The presence of these lipids in NK cell-BMDM co-culture medium led to the hypothesis that the lipids could potentially be transferred in exocytic vesicles. In fact exosomes have been described to be responsible for the transfer of bioactive lipids including fatty acids between cell types *in vitro* (Subra *et al.*, 2010). The data shown in this chapter does not suggest that the lipid accumulation in NK cells is due to macrophage-derived EVs as the co-culture of these cells across a trans-well insert did not induce lipid accumulation in the NK cells. EVs from BMDMs have been measured and are typically 40-60 nm in diameter meaning they could cross the 0.4 μm pores in the trans-well supports (Zhang *et al.*, 2021). The requirement for direct contact between the NK cells and macrophages could lead to lipid accumulation through a number of mechanisms. There are several described ligand-receptor interactions between NK cells and macrophages that have been shown to alter NK cell functions. For example, following stimulation with an immunomodulatory glycan, peritoneal macrophages were shown to increase IFN γ expression in splenic NK cells following CD40-CD40L interactions (Atochina and Harn, 2005). CD40-CD40L was also shown to induce CD69 expression on splenic NK cells in co-culture with LPS-stimulated peritoneal macrophages (Scott *et al.*, 2004). Whether this interaction leads to a signal, from macrophages to NK cells inducing lipid uptake or *de novo* lipogenesis is not known.

Experiments using pharmacological inhibitors of both *de novo* fatty acid synthesis and *de novo* cholesterol synthesis determined that in the case of LPS-stimulation, macrophages are transferring fatty acids to NK cells. Interestingly, in the case of CpG-stimulated macrophages, there seems to be a contribution of cholesterol or CEs to the lipids transferred to NK cells. This highlights that different TLR agonists can induce distinct lipidomes in macrophages. This has already been shown in BMDMs, where TLR4-stimulation was shown to result in TAG accumulation, whereas TLR9-stimulation did not (Hsieh *et al.*, 2020). Although this study did not show significant accumulation of CEs when BMDMs were stimulated with TLR9, the presence of NK cells in co-cultures may provide further signals to alter the BMDM lipidome. Furthermore, the treatment of co-cultures with zaragozic acid, to inhibit squalene synthase can occur in co-cultures affects both IL2-NK cells and BMDMs. This means that the results should be interpreted with caution, as the reduction in lipid accumulation could be due to reduced CE synthesis by IL2-NK cells. Given the effects of LPS-stimulated macrophages on IFN γ production and the likelihood that these cells were feeding fatty acids to NK cells, fatty acid transfer was more thoroughly investigated in this chapter.

One described method that macrophages could be using to pass fatty acids to NK cells is through the passive process of 'flip-flop' across the plasma membrane. The charged carboxyl group of a fatty acid means that it can insert easily into plasma membranes but would be slow to pass through the membrane because of ionic interactions with phospholipids. However, it has been suggested that in aqueous solutions the pK_a of fatty acids is about 4.5 and then this shifts to 7.6 following insertion into the membrane (Kamp and Hamilton, 1992). This effect was observed for a number of long chain fatty acids, meaning that in the plasma membrane they become protonated and lose their charge. This reduces the ionic interactions between the fatty acid and phospholipids in the membrane and allows the unionised fatty acid to easily 'flip' into another adjacent membrane leaflet. Once the fatty acid transfers into the recipient cell membrane it can lose the proton and become available for desorption into the cytosol. Although this was initially proposed in vesicles made of phospholipid bilayers, it has since been shown to occur in adipocytes (Kamp *et al.*, 2003). Because the macrophages and NK cells in these co-cultures are forming direct interactions, this could facilitate

the really rapid 'flip flop' of fatty acids across the membrane. Moreover, because this process occurs so quickly the macrophages and NK cells would not need to be in close proximity for long. It is suggested that unionised fatty acid 'flip flop' can occur in milliseconds (Kamp *et al.*, 1995), whereas for ionised fatty acids this can take minutes (Kamp and Hamilton, 1992). This may explain why the entire NK cell population's lipid content increases despite images showing that not every NK cell forms long-lasting synapses with macrophages. The mechanism described here does not require any changes in protein expression and suggests that there is no barrier to fatty acid transport across membranes. However, other mechanisms have been described to control the 'flip flop' of fatty acids more tightly across the membrane. For example, uncoupling protein (UCP) 2 has been described to use fatty acids in order to translocate protons across the mitochondrial inner membrane through protonation and subsequent flipping of fatty acids from the inner to outer leaflet of the membrane (Berardi and Chou, 2014). Transport across the mitochondrial inner membrane is tightly controlled in order to maintain the proton electrochemical gradient. As a result, UCP2 is specifically expressed in the mitochondria with no plasma membrane-associated variant being described to exhibit free fatty acid flippase activity. The only protein described to carry out any flippase activity at the plasma membrane is major facilitator superfamily domain containing (MFSD) 2A, which acts on lysophosphatidylcholine-bound fatty acids (Chua *et al.*, 2023). Enzyme-controlled fatty acid 'flip flop' has not yet been described in immune cells, but it is possible that the NK cells could be controlling fatty acid transfer at the level of intracellular solubilisation.

In accordance with the increase in intracellular lipids, NK cells were shown to increase expression of a number of genes involved in lipid handling. Since inhibition of *de novo* fatty acid synthesis was shown to be required for lipid accumulation in NK cells co-cultured with macrophages, it is likely that the lipids stored in the NK cells are in esterified DAG and TAG forms, which are derived from fatty acids. It is known that there is a pool of free fatty acids (FFAs) in many cell types which have important roles in supporting mitochondrial metabolism and intracellular signalling (Hara *et al.*, 2013; Xiang *et al.*, 2025), however, this pool needs to be tightly regulated to prevent aberrant fatty acid accumulation leading

to lipotoxicity. The increase in expression of *Fabp5* mRNA in NK cells co-cultured with BMDMs suggests that NK cells are in fact accumulating fatty acids, but also that they are altering their gene expression to do this in a controlled way. In fact in many cell types expression of FABPs is essential for lipid uptake as they are required to solubilise intracellular fatty acids (Storch and Thumser, 2000). FABPs have also been linked with immune cell functions. For example, a subset of TAMs from hepatocellular carcinoma patients were shown to have elevated *Fabp5* expression leading to lipid accumulation. These lipids were then linked with PPAR γ activation leading to increased PDL1 expression and subsequent inhibition of CD8⁺ T cell responses (Yang *et al.*, 2025). However, increased lipid uptake and solubility is not always associated with immune cell dysfunction. Expression of FABP5 by murine CD8⁺ T cells was shown to be linked with enhanced metabolic fitness as it made fatty acids available for β -oxidation, increasing mitochondrial respiration (Hwang *et al.*, 2024). Cultured NK cells have not been described to use β -oxidation to support energy metabolism and *Cpt1a* gene expression was not detected in this thesis. Without the ability to burn FFAs, the resulting oxidative stress has been shown to induce apoptosis in many cell types, through a process called lipotoxicity (Koves *et al.*, 2008; Akoumi *et al.*, 2017).

There is no evidence in this thesis that the lipid accumulation in NK cells is inducing lipotoxicity, however, all analysis was carried out following 24 h co-culture of NK cells with macrophages. Data from cultures of IL2-NK cells and TLR-stimulated BMDMs showed that even after 24 h co-culture, NK cells will continually accumulate lipids, leading to a further doubling of lipid content at the 48 h time point. It is possible that this further increase in intracellular lipid accumulation in NK cells crosses a threshold where the lipids could have negative impacts on NK cell survival. The exact mechanisms of lipotoxicity are not known but it is thought to involve disruptions to mitochondrial structure resulting in elevated ROS (Szeto *et al.*, 2016), pro-longed activation of the ER stress response leading to CHOP activation (Haywood and Yammani, 2016) or ferroptosis (Wan Seok Yang *et al.*, 2016). In fact, NK cells co-cultured with macrophages showed impaired holotransferrin uptake, suggesting that the NK cells may be reducing iron uptake in order to escape ferroptosis. Lipid

accumulation has not been explicitly linked with apoptosis in NK cells, however, in mice and individuals with obesity there were reduced numbers of circulating NK cells (Michelet *et al.*, 2018). This could potentially be linked with the pro-apoptotic effects of free fatty acids in the context of uncontrolled lipid uptake in obesity. Although the lipid species stored within the NK cells were not measured in this study, they are likely fatty acid-derived due to the inhibition of lipid accumulation in NK cells if *de novo* fatty acid synthesis is impaired. Prolonged lipid accumulation in NK cells co-cultured with macrophages did not have any effects on their viability in these experiments, potentially due to the NK cells changing gene expression to correctly solubilise and store the fatty acids in a manner that is not harmful to the cell. Perilipin (PLIN) 2 is a lipid-handling protein, with elevated mRNA expression in NK cells co-cultured with BMDMs. It is an essential protein for maintaining integrity of lipid droplets composed of DAGs and TAGs, through blocking the activity of lipases (Itabe *et al.*, 2017). This suggests that the lipid accumulation in NK cells co-cultured with macrophages leads to their storage as DAGs and TAGs in lipid droplets, which was confirmed by confocal microscopy. By sequestering FFAs as DAGs and TAGs in lipid droplets, these NK cells are likely escaping the effects of FFA-induced lipotoxicity.

It was also considered whether the increase in intracellular lipids following interactions with macrophages was the reason for impaired iron uptake, as shown in chapter 4. An increase in lipids combined with an increase in intracellular iron can lead to ferroptosis (Wan Seok Yang *et al.*, 2016). Therefore, the loss of CD71 expression and reduced holotransferrin uptake could be a protective mechanism to prevent NK cell death in co-cultures with macrophages. Given the reduced expression of CD71 in NK cells cultured in BMDM-conditioned medium, it is unlikely that an increase in intracellular lipids is a cause for the loss of CD71 in direct co-cultures. Another link between iron and lipid accumulation comes from the requirement of iron to support the activity of mitochondrial aconitase. This enzyme functions in the TCA cycle to convert citrate to isocitrate. It contains 4 Fe-S clusters, meaning that in the absence of iron, it cannot function properly, leading to an accumulation of citrate which then enters the *de novo* fatty acid synthesis pathway (Crooks *et al.*, 2018; Berteau, 2018). It is unlikely that the decrease in holotransferrin uptake is responsible for the increase in lipid droplets

observed in NK cells interacting with macrophages, as this chapter has shown that the macrophages are indeed the source of the fatty acids accumulating in NK cells.

The transfer of macrophage-derived lipids to NK cells was shown to be dependent on *de novo* fatty acid synthesis, which is strongly associated with pro-inflammatory macrophage functions. *In vitro* studies using M1 and M2 phenotype classifications have provided useful information to try and understand the metabolic configurations of pro-inflammatory versus anti-inflammatory macrophages. While M1 macrophages are more associated with *de novo* fatty acid synthesis, M2 macrophages are more associated with uptake of exogenous lipids and subsequent increases in β -oxidation (Vats *et al.*, 2006; Huang *et al.*, 2014). In this study all co-cultures of NK cells and macrophages were carried out in the presence of TLR agonists, meaning that whether this lipid accumulation occurs in the presence of anti-inflammatory macrophages is not known. The increase in FASN activity by pro-inflammatory macrophages is not surprising considering the cells' increased demand for fatty acids as biosynthetic precursors for membrane synthesis. However, there are also several links described between FASN activity and pro-inflammatory macrophage effector functions. Inhibition of *de novo* fatty acid synthesis has been shown to prevent M1 macrophage polarisation in response to TLR agonists or cytokines, decreasing NLRP3 inflammasome activation and cytokine production (Carroll *et al.*, 2018; Xiong *et al.*, 2021; Leishman *et al.*, 2024). It has also been shown that the increase in *de novo* fatty acid synthesis is required for sustained TLR4 signalling. When FASN was inhibited macrophages were unable to form the lipid rafts that are required for MyD88-dependent TLR4 signalling which resulted in impairments in macrophage cytokine production (Carroll *et al.*, 2018). However, this study revealed that FASN inhibition also had the off-target effect of impairing cholesterol synthesis which was the essential component of the lipid rafts required for signalling. Aside from supporting cholesterol biosynthesis, newly synthesised fatty acids were also an important precursor for palmitoylation of Akt. Inhibition of FASN activity not only reduced Akt expression, but also prevented its association with the plasma membrane and subsequent phosphorylation and activation by mTORc2 and PDK1 (Xiong *et al.*, 2021). Furthermore, NLRP3 itself

can be palmitoylated which is essential for its localisation to the dispersed trans-Golgi network where it is aggregates to form ASC specks (Leishman *et al.*, 2024).

Whether fatty acid transfer from macrophages to NK cells occurs *in vivo* is not known. Because macrophages are such long-lived cells, existing in every tissue in the body, it is plausible that they are often in proximity to NK cells. In this chapter, peritoneal NK cells were shown to increase lipid content following LPS-immunisation. Although NK cells are found in relatively low abundance in the peritoneal cavity following LPS immunisation or infection, it is possible that they are interacting with macrophages which make up the largest proportion of immune cells (Kubicka *et al.*, 1996). Interestingly, immune cell movement in the peritoneal cavity has not been attributed to chemokine gradients but rather the constant mechanical movement of interstitial fluid due to expansion and contraction of the chest cavity due to breathing. *In situ* imaging has revealed that peritoneal cells are constantly being moved around the cavity due to the contraction of the diaphragm, allowing cells in the interstitial fluid to continually contact one another (Zhang *et al.*, 2019). If the lipid transfer between macrophages and NK cells is occurring in milliseconds due to fatty acid ‘flip flop’, these interactions could be sufficient to allow for transfer of biomolecules between the cells.

This chapter highlights that the metabolism of immune cells cannot be properly evaluated in isolation. Macrophage FASN activity has been shown to be essential for many intracellular processes required to support effector functions, but the concept of fatty acids being directly transferred from one cell to another is completely novel. Lipid accumulation in NK cells has primarily been associated with cellular dysfunction, but recent studies have shown that lipid metabolism can be beneficial for supporting NK cell effector functions in viral infections. However, the role of macrophage-derived fatty acids in altering NK cell functions is not understood and will be the subject of the next chapter in this thesis.

Chapter 6

Macrophage-derived lipids dampen IFN γ production by NK cells

6.1 Introduction

The role of fatty acids in regulating lymphocyte functions varies depending on the physiological context. For example, in obesity, the increased levels of fatty acids in the environment cause increased lipid accumulation which is associated with decreased expression of IFN γ as well as diminished capacity to kill tumour target cells *in vitro* (Perdu *et al.*, 2016; Tobin *et al.*, 2017; Viel *et al.*, 2017; Michelet *et al.*, 2018; Kedia-Mehta *et al.*, 2021; De Barra *et al.*, 2023). In obese individuals this was specifically associated with an increase in fatty acid uptake, as opposed to glucose uptake (Michelet *et al.*, 2018). Studies of NK cells in obesity investigate the effects of lipid accumulation in a systemic metabolic disease and may not necessarily illustrate the effects fatty acids have on NK cell effector functions in all contexts. For example, it has also been shown that NK cells in viral infections have elevated lipid uptake to support energy metabolism, which improves viral clearance (Schimmer *et al.*, 2023; Sheppard *et al.*, 2024). In this thesis it has been shown that in LPS-induced peritonitis, a model of bacterial infection, NK cells also increase their lipid content, suggesting they may be using these lipids to support OxPhos. However, *in vitro* analysis demonstrated that macrophages are directly feeding the lipids into NK cells which suggests that lipid accumulation may also be an immunoregulatory strategy. Subsequent experiments investigated whether the accumulation of fatty acids in NK cells co-cultured with macrophages was acting to support NK cell metabolism or to dampen effector function.

6.2 Macrophage-derived lipids do not fuel NK cell oxidative phosphorylation.

In order to determine whether the lipids being accumulated by NK cells in co-culture with macrophages were being used to fuel β -oxidation, an inhibitor of the carnitine shuttle system required to introduce fatty acids to the mitochondria

named etomoxir was used. Due to off-target effects associated with the use of high doses of etomoxir for long periods (O'Connor *et al.*, 2018; Yao *et al.*, 2018; Raud *et al.*, 2018), cells were only exposed to etomoxir (5 μ M) for 40 mins before OPP incorporation into translated proteins was assessed. This dose of etomoxir is widely considered to be specific for the inhibition of CPT1A. There was no significant change in the rate of protein translation when IL2-NK cells were co-cultured with BMDMs in the presence of etomoxir compared to controls (Figure 6.1). Interestingly, IL2-NK cells cultured alone, in the presence of etomoxir showed significantly impaired rates of protein translation (Figure 6.1). Peritoneal NK cells from LPS immunised mice also displayed increased neutral lipid accumulation which may be important to support their elevated metabolism in their *in vivo* niche. Assessment of rates of protein translation in the presence of etomoxir directly *ex vivo* revealed that in peritoneal NK cells from LPS-immunised mice β -oxidation of fatty acids contributes to energy production (Figure 6.2). In contrast there was no effect of etomoxir on energy production by splenic NK cells. In order to assess whether the ATP produced by β -oxidation of fatty acids in peritoneal NK cells was important to support certain functional responses, cells from LPS-immunised mice were exposed to etomoxir for 4 h *in vitro* before IFN γ , perforin and granzyme B were measured. These experiments showed no dependence on β -oxidation to support expression of effector molecules by peritoneal NK cells following LPS-immunisation (Figure 6.3).

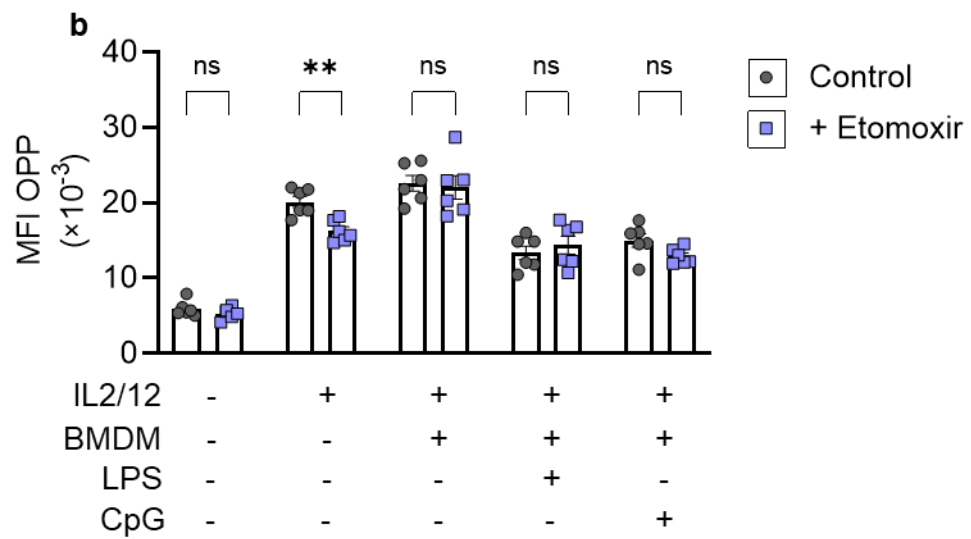
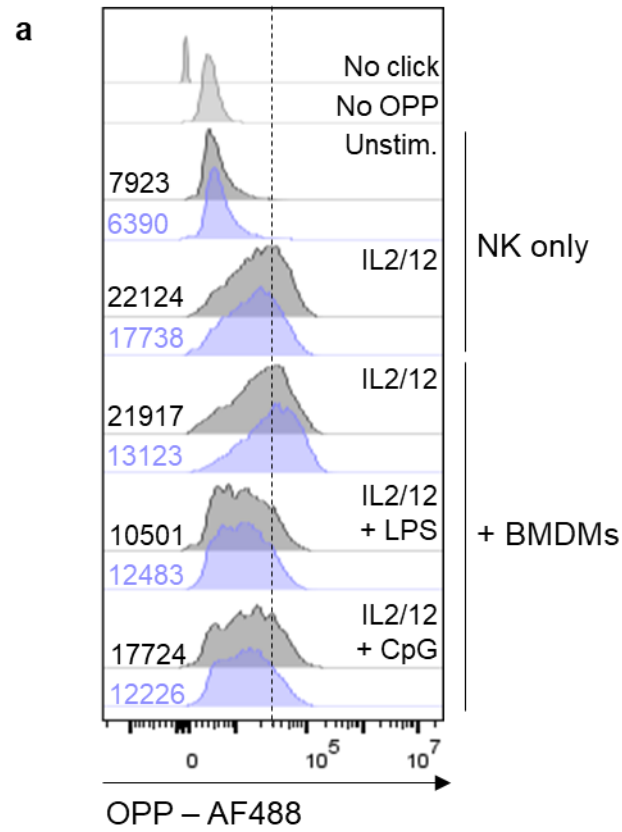


Figure 6.1 IL2-NK cells co-cultured with BMDMs do not use β -oxidation of fatty acids to support rates of protein translation.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 μ g/mL) for 6 h before co-culture with IL2-NK cells stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for a further 18 h. Cells were left untreated or treated with etomoxir (5 μ M) for 40 mins. O-propargyl puromycin (OPP) was administered for the final 30 mins of the incubation. OPP incorporation into translated proteins of NK cells was measured using click chemistry and flow cytometry. Data is representative (a) or mean $\pm$ SEM of 6 biological replicates across 2 independent experiments (b). Statistical analysis was done using a two-way ANOVA with Šidák's post-test (** p <0.01, ns=nonsignificant).

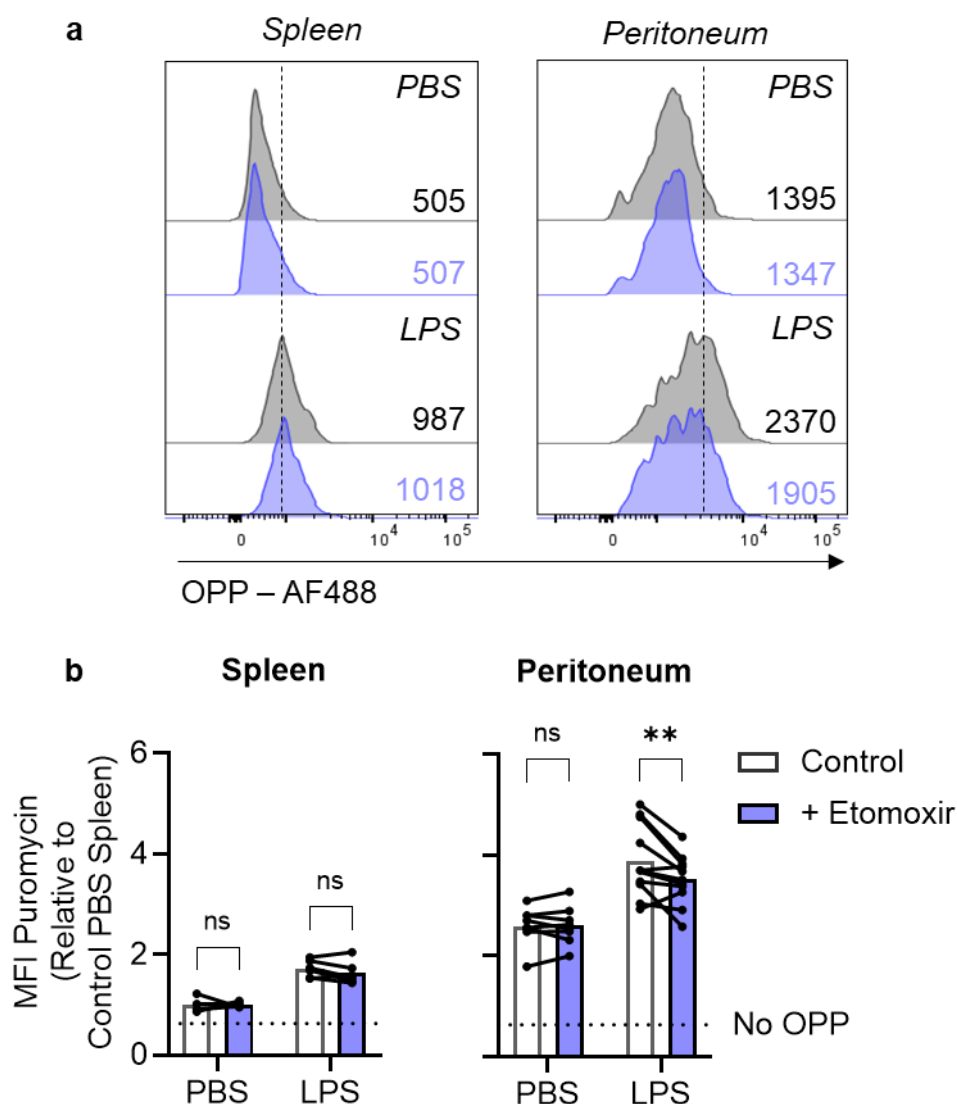


Figure 6.2 Peritoneal NK cells use β -oxidation of fatty acids to support protein translation following LPS-immunisation.

Mice were administered PBS or LPS (2.4 mg/kg) by intraperitoneal injection. Spleens and peritoneal cells were harvested 24 h post-injection. Cells were left untreated or treated with etomoxir (5 μ M) for 40 mins. O-propargyl puromycin (OPP) was administered for the final 30 mins of the incubation. OPP incorporation into translated proteins of NK cells (CD3⁻, CD19⁻, CD49b⁺, NK1.1⁺) was measured using click chemistry and flow cytometry. A minimum of 4 mice were used per group across two independent experiments. Data is representative (a) or pooled (b). Statistical analysis was done using a two-way repeated measures ANOVA with Šidák's post-test (**p<0.01, ns=nonsignificant).

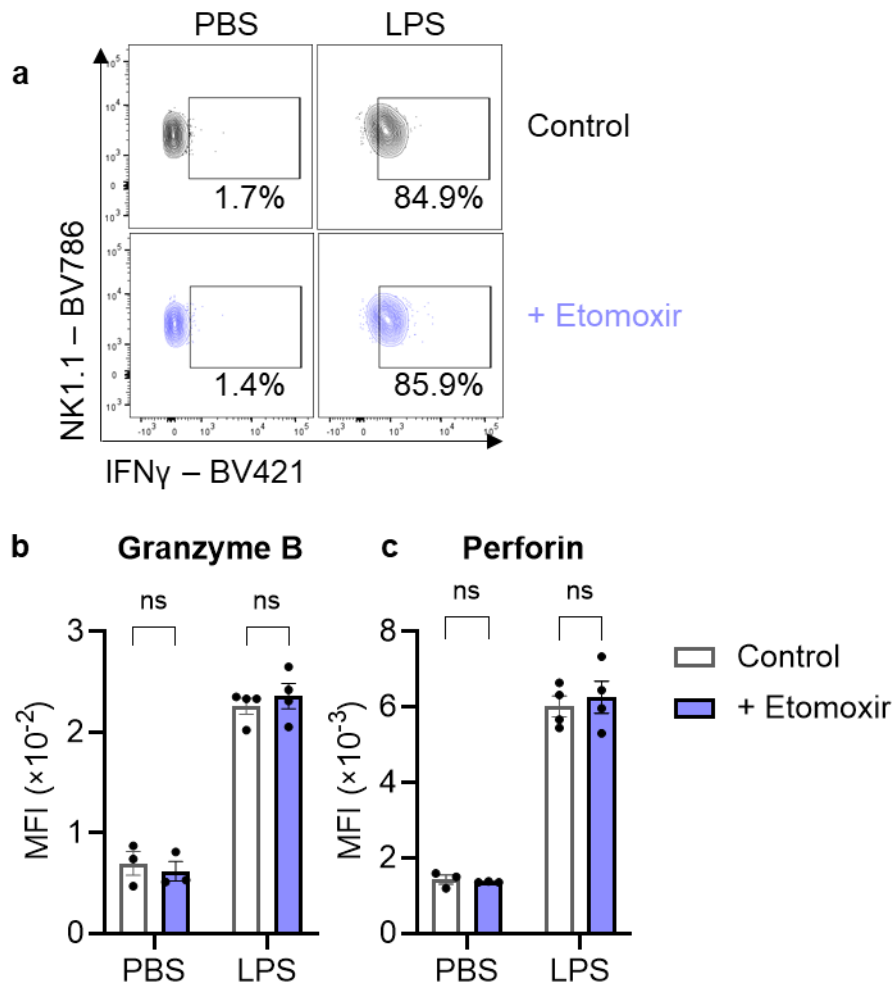


Figure 6.3 Inhibition of β -oxidation of fatty acids does not impair effector molecule expression by peritoneal NK cells following LPS-immunisation.

Mice were administered PBS or LPS (2.4 mg/kg) by intraperitoneal injection. Spleens and peritoneal cells were harvested 24 h post-injection. Cells were left untreated or treated with etomoxir (5 μ M) for 4 h. IFN γ (a), granzyme B (b) and perforin (c) expression by NK cells (CD3⁻, CD19⁻, CD49b⁺, NK1.1⁺) were measured by flow cytometry. A minimum of 3 mice were used per group across 2 independent experiments. Data is representative (a) or pooled $\pm$ SEM (b,c). Statistical analysis was done using a two-way ANOVA with Šidák's post-test (ns=nonsignificant).

6.3 Inhibition of *de novo* fatty acid synthesis in macrophages rescues NK cell IFN γ production *in vitro*.

Previously in this thesis it was shown that inhibition of *de novo* fatty acid synthesis in LPS-stimulated BMDMs and peritoneal macrophages with the irreversible inhibitor: C75, resulted in a decrease in neutral lipid accumulation by NK cells following co-culture (Figure 5.44, Figure 5.47). The following experiments use C75 in a similar context (experimental design outlined in figure 5.43) to assess whether inhibiting transfer of fatty acids from macrophages to NK cells could rescue NK cell metabolic and functional parameters which were previously shown to be impaired in direct co-cultures. In order to assess whether these changes were occurring in more physiological cell types, these co-cultures were repeated with peritoneal macrophages and splenic NK cells. Peritoneal macrophages were adhered to culture dishes for 4 h before treatment with C75 for 2 h, which reduced fatty acid transfer from macrophages to splenic NK cells following co-culture (Figure 5.47).

Before investigating the impact of C75-pre-treated macrophages on NK cell metabolism and effector function, it was important to assess the impact of C75 treatment on macrophage effector functions. These experiments sought to measure the production of soluble factors and surface proteins produced by macrophages that could have an impact on NK cell activation, following pre-treatment with C75. Cell culture supernatants were taken and analysed for chemokine and cytokine production using a cytokine bead array. Importantly, these supernatants were taken from cultures of peritoneal macrophages alone, to ensure the soluble factors were derived from macrophages. Firstly, the production of pro-inflammatory cytokines canonically associated with M1 polarisation were measured in the cell culture medium. These parameters were measured to confirm that peritoneal macrophages were activated *in vitro* and to determine whether *de novo* fatty acid synthesis was required for this process. The increased production of IL6 and TNF α when peritoneal macrophages were stimulated with LPS and CpG was significantly reduced when *de novo* fatty acid synthesis was inhibited (Figure 6.4). Next, expression of cytokines that are known to alter the activation of NK cells were measured in the cell culture supernatants. Among the NK cell-activating cytokines produced by peritoneal macrophages,

IL12p70 was the most abundant following stimulation with LPS (Figure 6.5a). This increased production of IL12p70 by peritoneal macrophages was significantly impaired when *de novo* fatty acid synthesis was inhibited (Figure 6.5a). There was little to no detection of IL15, IL18 or IL2 in the culture supernatant of peritoneal macrophages in the presence or absence of C75 (Figure 6.5b,c,d). CpG-stimulation did not significantly increase production of any of these cytokines. IL10 is another cytokine which has been described to directly activate human NK cells (Wang *et al.*, 2021). There was a slight increase in IL10 production by LPS-stimulated peritoneal macrophages which was impaired when *de novo* fatty acid synthesis was inhibited (Figure 6.5e). TGF β 1 was also measured as it has been shown to impair NK cell activation (Viel *et al.*, 2016; Zaiatz-Bittencourt *et al.*, 2018; Slattery *et al.*, 2021). There was no change in TGF β 1 production in cell culture supernatants regardless of TLR stimulation or the presence or absence of C75 (Figure 6.5f).

It has been shown that direct interactions with macrophages are required for lipid transfer to NK cells. Taking this into account, chemokine production by macrophages was measured in the cell culture supernatants. It is possible that chemokine production by macrophages could create a gradient that would increase interactions with NK cells required for lipid transfer. In this chapter CCL2 and CCL5 were measured as NK cells have been described to respond to these chemokines. CXCL10 was also measured though NK cells are less responsive to this chemokine (Yao and Matosevic, 2021). CCR5 and CXCR3 were detected in a proteomics dataset of splenic NK cells in the Finlay lab at relatively low copy numbers, indicating that they may be able to migrate in response to CCL5 and CXCL10. Following stimulation with LPS, peritoneal macrophages increased expression of CCL2, CCL5 and CXCL10 which was significantly reduced when *de novo* fatty acid synthesis is inhibited (Figure 6.6). There was no increase in expression of these chemokines when peritoneal macrophages were stimulated with CpG (Figure 6.6). One of the receptor-ligand interactions described to occur between macrophages and NK cells following direct interactions involves binding of CD40 on macrophages to CD40L on NK cells, leading to enhanced CD69 expression (Scott *et al.*, 2004; Atochina and Harn, 2005). Taking this into account, expression of CD40 by peritoneal macrophages was measured. There

was a modest, but significant, increase in expression of CD40 on peritoneal macrophages stimulated with LPS, with no increase seen if they were stimulated with CpG (Figure 6.7). Inhibition of *de novo* fatty acid synthesis in peritoneal macrophages with C75 significantly decreased CD40 expression (Figure 6.7). A summary of the impact of C75 treatment on LPS-stimulated peritoneal macrophage effector functions is shown in figure 6.8.

Having characterised the impact of C75 treatment on peritoneal macrophage cytokine and chemokine production, C75-pre-treated peritoneal macrophages were co-cultured with splenic NK cells to block fatty acid transfer. Expression of CD71 by splenic NK cells in these co-cultures was significantly rescued when macrophages were pre-treated with C75, in comparison with untreated controls (Figure 6.9). Expression of perforin and granzyme B by splenic NK cells co-cultured with peritoneal macrophages was measured to determine whether C75 pre-treatment could rescue expression of cytotoxic machinery. In these experiments, cytokine stimulation did not seem to significantly increase expression of these proteins by NK cells (Figure 6.10), meaning the effect of blocking fatty acid transfer from macrophages to NK cells could not be measured. Nevertheless, the NK cells from these experiments had significantly increased expression of IFN γ (Figure 6.11). Blocking fatty acid transfer from peritoneal macrophages to splenic NK cells through pre-treatment with C75 significantly rescued the defect in IFN γ production seen in control co-cultures (Figure 6.11).

These experiments were then repeated using co-cultures of IL2-NK cells and BMDMs. Pre-treatment of LPS-stimulated BMDMs with C75 before co-culture with IL2-NK cells significantly rescued expression of CD71 (Figure 6.12). This was mirrored in a rescue of holotransferrin uptake by IL-2 NK cells co-cultured with LPS-stimulated BMDMs following inhibition of fatty acid transfer between the cells (Figure 6.13). There was also a rescue in holotransferrin uptake seen in IL2-NK cells co-cultured with CpG-stimulated BMDMs, independent of any changes in CD71 expression (Figure 6.13). Cytotoxicity of IL2-NK cells against RMA/S target cells showed no change in target cell killing when NK cells were co-cultured with C75-pretreated BMDMs, compared to untreated controls (Figure 6.14). In agreement with this, IL2-NK cells co-cultured with C75-pretreated BMDMs also showed no change in expression of perforin or granzyme B (Figure

6.15). However, direct contacts with LPS-stimulated BMDMs were consistently shown to impair IFN γ production by IL2-NK cells throughout this thesis. Through pre-treating LPS-stimulated BMDMs with C75 and blocking lipid transfer from BMDMs to IL2-NK cells there was a significant rescue in IFN γ production in these co-cultures (Figure 6.16).

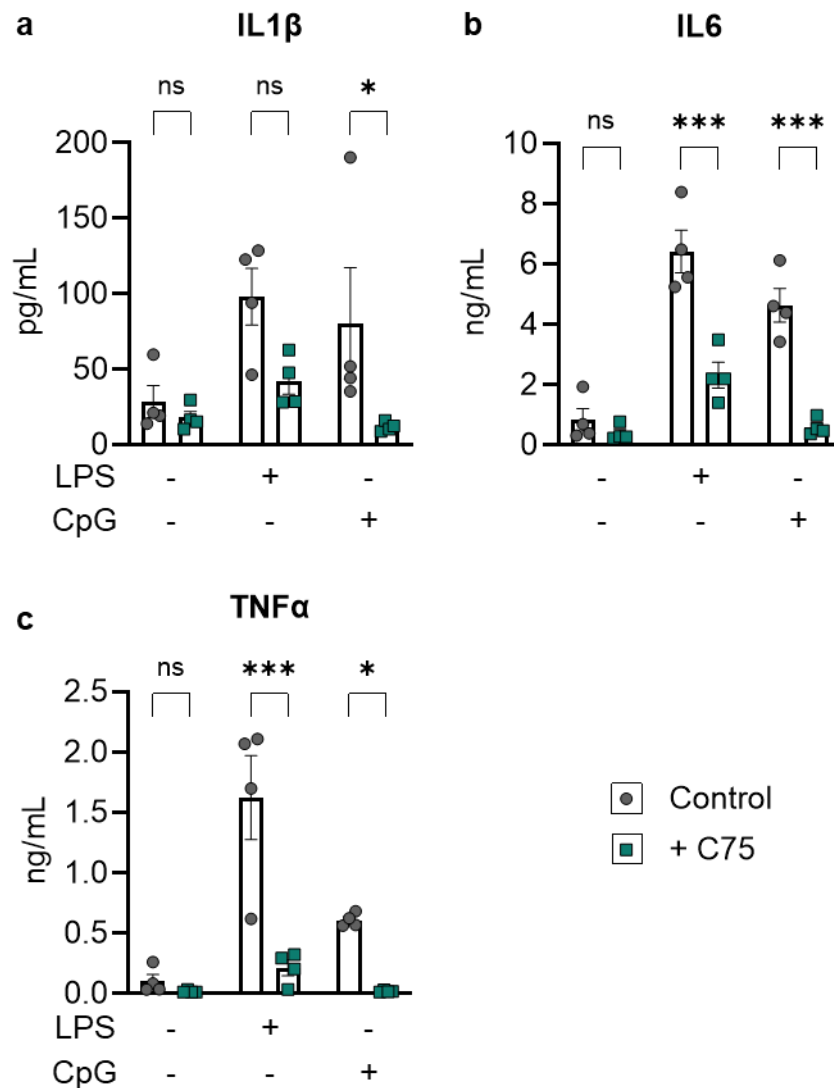


Figure 6.4 Inhibition of *de novo* fatty acid synthesis impairs pro-inflammatory cytokine production by LPS-stimulated peritoneal macrophages *in vitro*.

Peritoneal macrophages were cultured in the presence of absence of C75 (40 μ M) for 2 h before being left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL) for a further 18 h. IL1 β , IL6 and TNF α were measured in the culture medium using a cytokine bead array by flow cytometry. Data is mean $\pm$ SEM of 4 biological replicates. Statistical analysis was performed using a two-way ANOVA with Šidák's post-test (* p <0.05, *** p <0.001, ns=non-significant). Cytokine bead array was performed with Carrie Corkish, Finlay Lab, Trinity College Dublin.

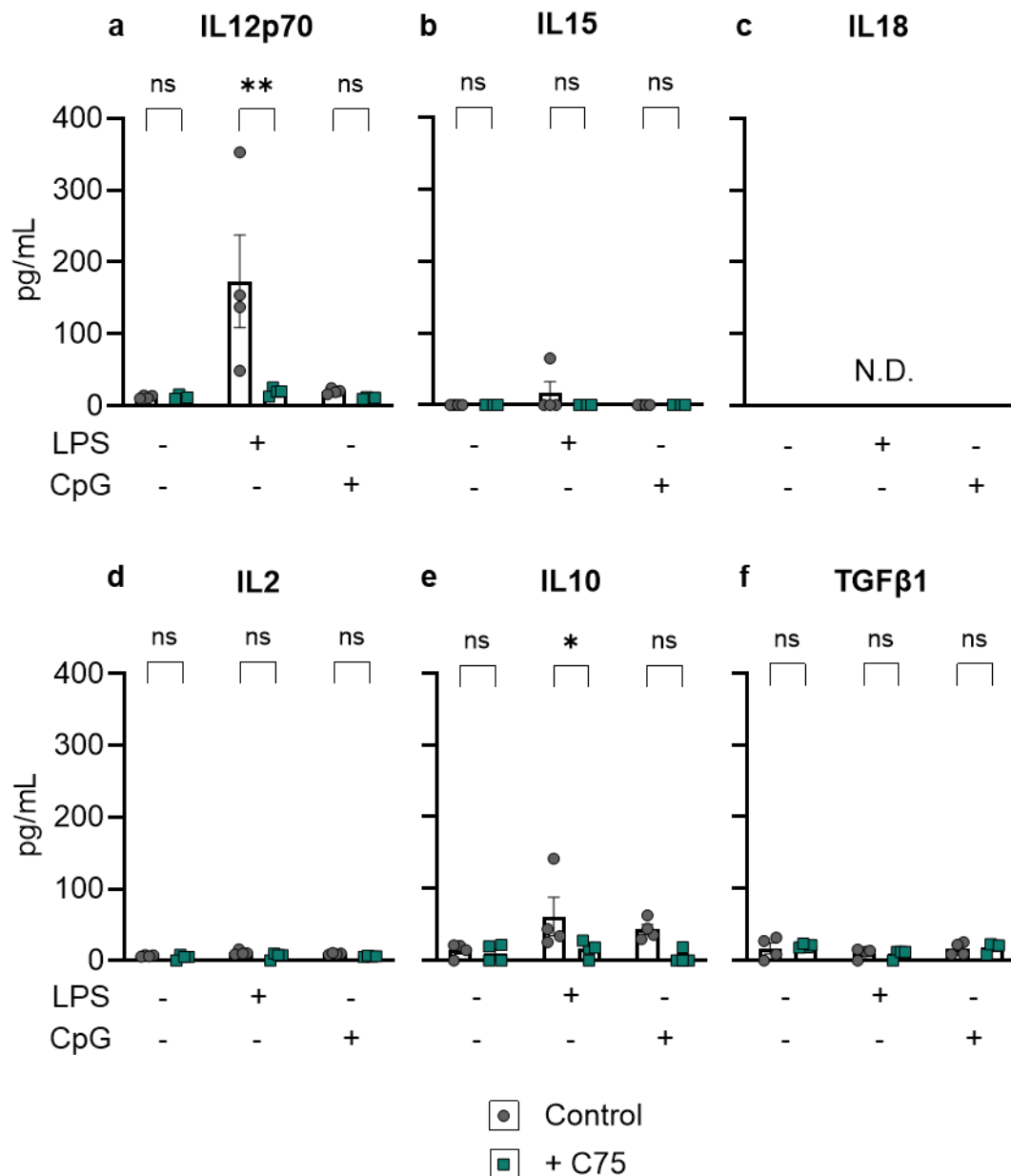


Figure 6.5 Inhibition of *de novo* fatty acid synthesis impairs IL12p70 production by LPS-stimulated peritoneal macrophages *in vitro*.

Peritoneal macrophages were cultured in the presence of absence of C75 (40 μ M) for 2 h before being left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL) for a further 18 h. IL12p70, IL15, IL18, IL2, TGF β 1 and IL10 were measured in the culture medium using a cytokine bead array by flow cytometry. Data is mean $\pm$ SEM of 4 biological replicates. Statistical analysis was performed using a two-way ANOVA with Šidák's post-test (* p <0.05, ** p <0.01, ns=non-significant). (ND=not detected) *Cytokine bead array was performed with Carrie Corkish, Finlay Lab, Trinity College Dublin.*

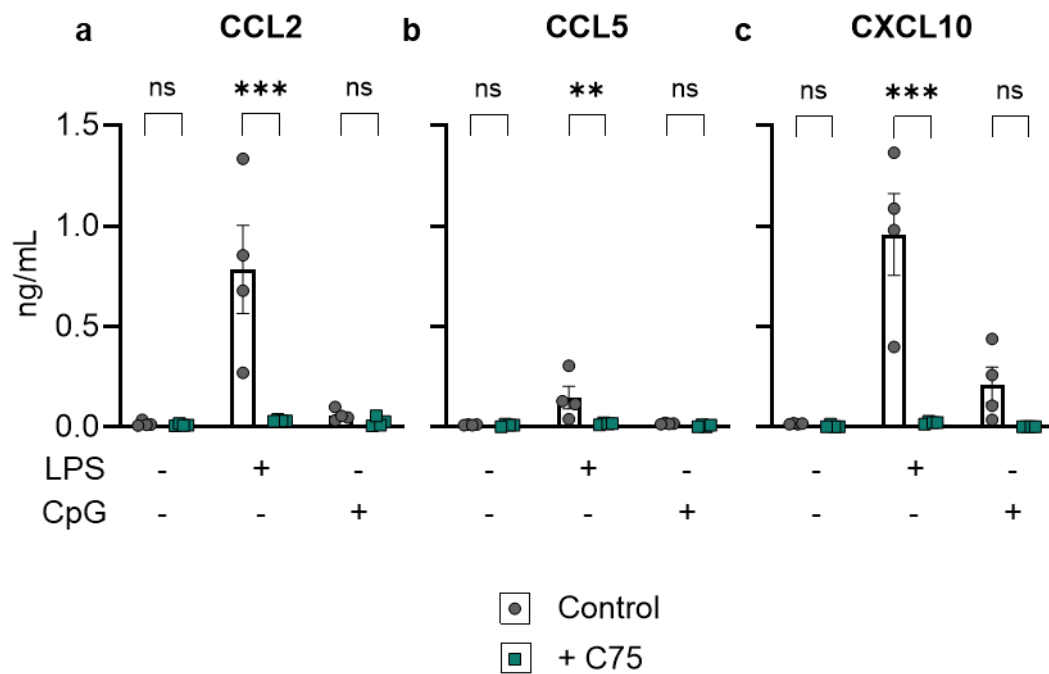


Figure 6.6 Inhibition of *de novo* fatty acid synthesis impairs production of NK cell-recruiting chemokines in LPS-stimulated peritoneal macrophages *in vitro*.

Peritoneal macrophages were cultured in the presence of absence of C75 (40 μ M) for 2 h before being left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL) for a further 18 h. CCL2, CCL5 and CXCL10 were measured in the culture medium using a cytokine bead array by flow cytometry. Data is mean $\pm$ SEM of 4 biological replicates. Statistical analysis was performed using a two-way ANOVA with Šidák's post-test (** $p < 0.01$, *** $p < 0.001$, ns=non-significant). Cytokine bead array was performed with Carrie Corkish, Finlay Lab, Trinity College Dublin.

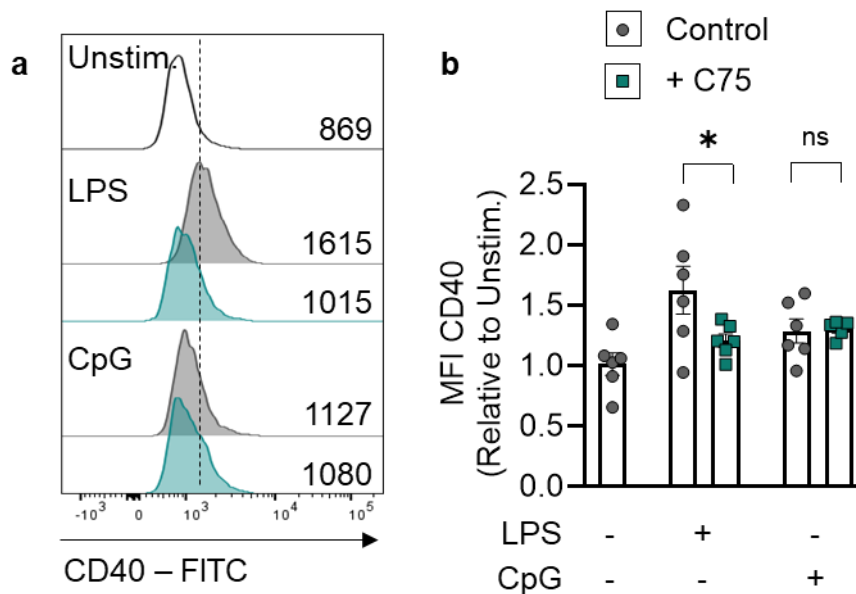


Figure 6.7 Inhibition of *de novo* fatty acid synthesis impairs CD40 expression by LPS-stimulated peritoneal macrophages *in vitro*.

Peritoneal macrophages were cultured in the presence of absence of C75 (40 μ M) for 2 h before being left unstimulated or stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL) for a further 18 h. CD40 was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 6 biological replicates across 2 independent experiments (b). Statistical analysis was performed using a two-way ANOVA with Šidák's post-test (ns=non-significant).

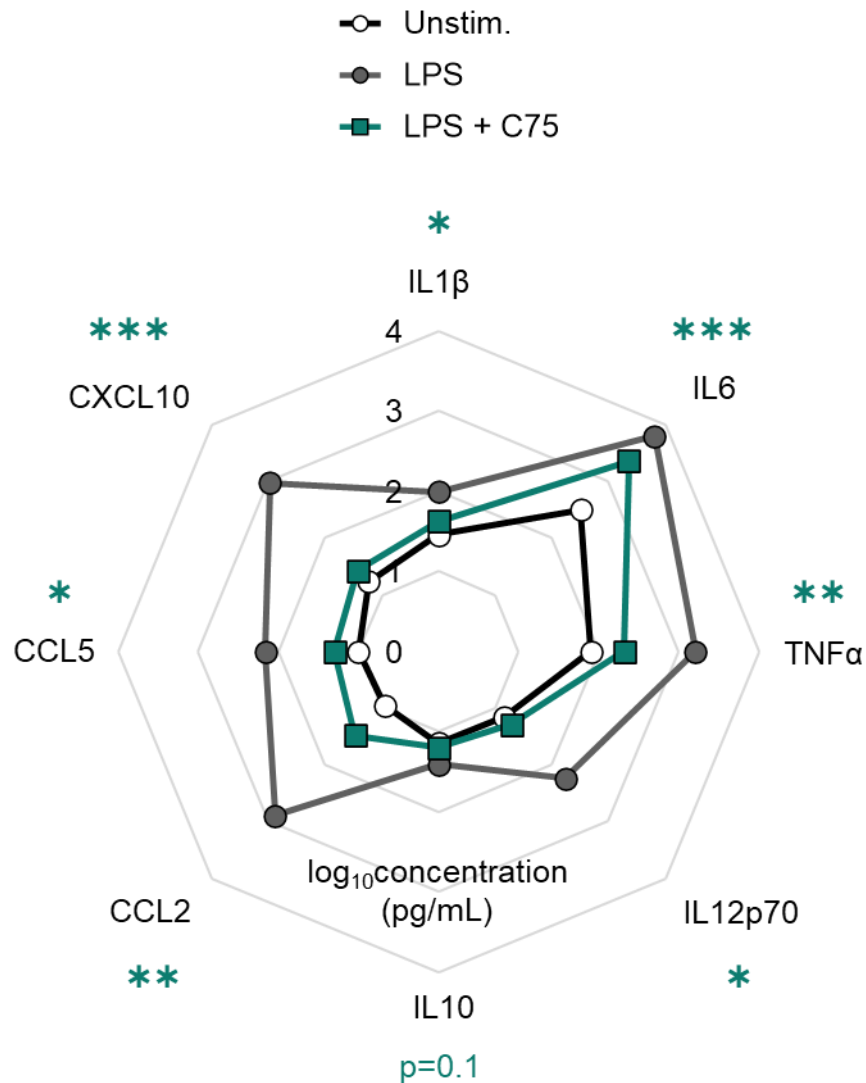


Figure 6.8 Summary of the inhibition of LPS-stimulated peritoneal macrophage effector function by C75 *in vitro*.

Peritoneal macrophages were cultured in the presence or absence of C75 (40 μ M) for 2 h before being left unstimulated or stimulated with LPS (10 ng/mL) for 18 h. IL1 β , IL6, TNF α , IL12p70, IL10, CCL2, CCL5 and CXCL10 were measured in the culture medium using a cytokine bead array by flow cytometry. Data is mean of 4 biological replicates. For all analytes, data is presented as log₁₀concentration (pg/mL). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test. Comparisons shown on the graph are between LPS-stimulated macrophages in the presence or absence of C75 (* p <0.05, ** p <0.01, *** p <0.001). *Cytokine bead array was performed with Carrie Corkish, Finlay Lab, Trinity College Dublin.*

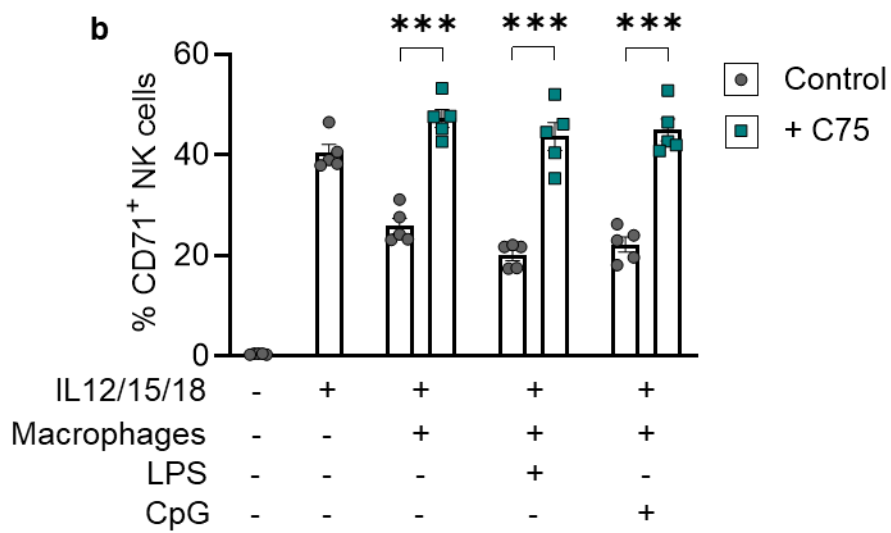
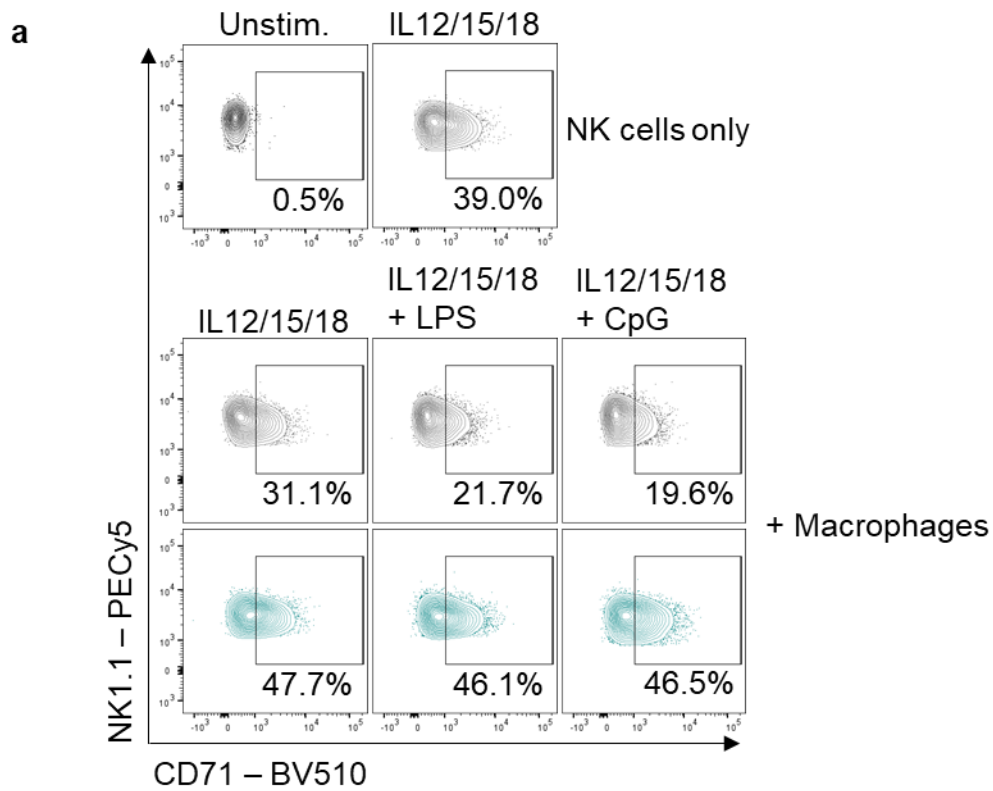


Figure 6.9 Specific inhibition of *de novo* fatty acid synthesis in peritoneal macrophages rescues CD71 expression by splenic NK cells.

Peritoneal macrophages were cultured in the presence or absence of C75 (40 μ M) for 2 h before being stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL), in the presence or absence of splenic NK cells for a further 18 h. NK cells were left unstimulated or stimulated with IL12 (20 ng/mL), IL15 (10 ng/mL) and IL18 (50 ng/mL). CD71 was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 5 biological replicates across 2 independent experiments (b). Statistical analysis was done using a two-way ANOVA with Šidák's post-test (** p <0.001).

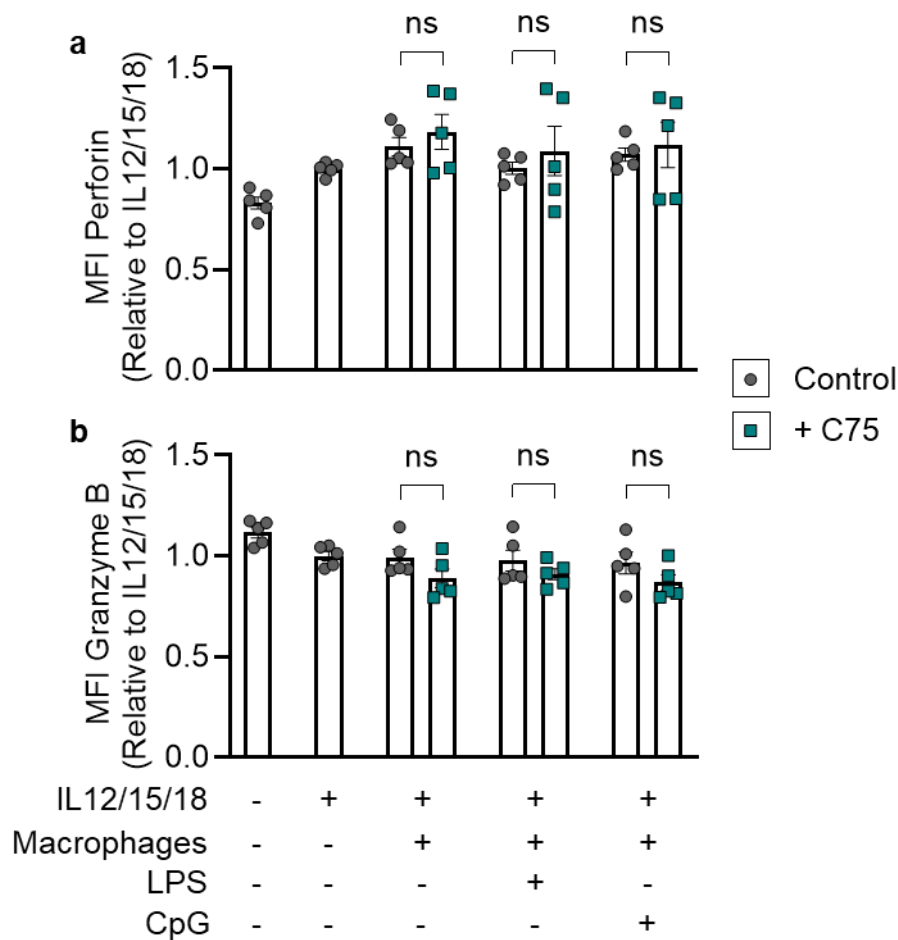


Figure 6.10 Specific inhibition of *de novo* fatty acid synthesis in peritoneal macrophage does not alter perforin or granzyme B expression by splenic NK cells.

Peritoneal macrophages were cultured in the presence of absence of C75 (40 μ M) for 2 h before being stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL), in the presence of absence of splenic NK cells for a further 18 h. NK cells were left unstimulated or stimulated with IL12 (20 ng/mL), IL15 (10 ng/mL) and IL18 (50 ng/mL). Perforin (a) and granzyme B (b) were measured by flow cytometry. Data is mean $\pm$ SEM of 5 biological replicates across 2 independent experiments. Statistical analysis was done using a two-way ANOVA with Šidák's post-test (ns=non-significant).

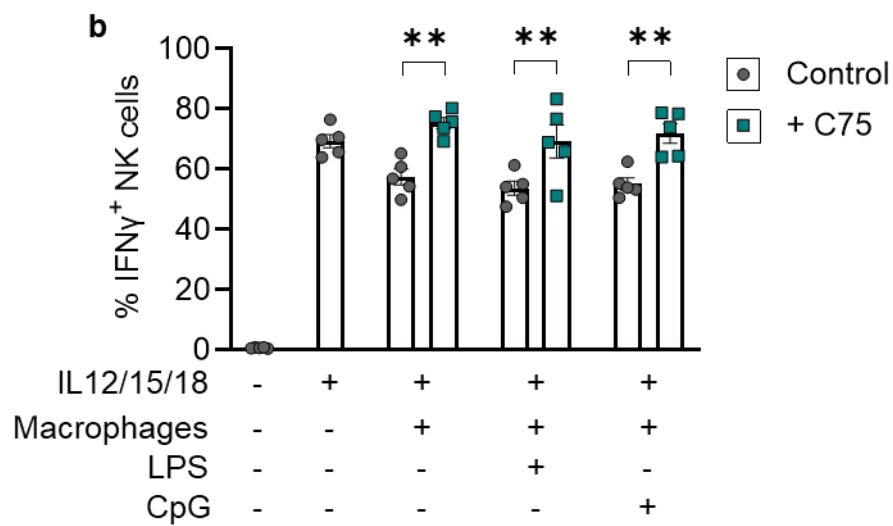
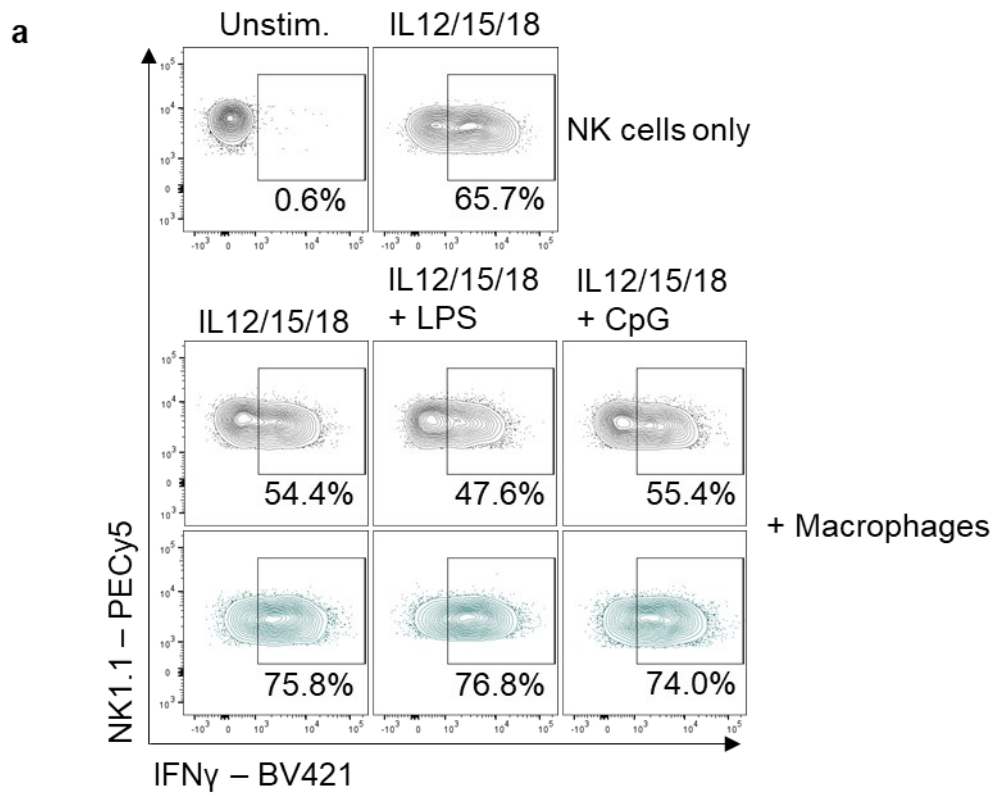


Figure 6.11 Specific inhibition of *de novo* fatty acid synthesis in peritoneal macrophages rescues IFN γ expression by splenic NK cells.

Peritoneal macrophages were cultured in the presence or absence of C75 (40 μ M) for 2 h before being stimulated with LPS (10 ng/mL) or CpG (4 μ g/mL), in the presence or absence of splenic NK cells for a further 18 h. NK cells were left unstimulated or stimulated with IL12 (20 ng/mL), IL15 (10 ng/mL) and IL18 (50 ng/mL). IFN γ was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 5 biological replicates across 2 independent experiments (b). Statistical analysis was done using a two-way ANOVA with Šidák's post-test (** $p < 0.01$).

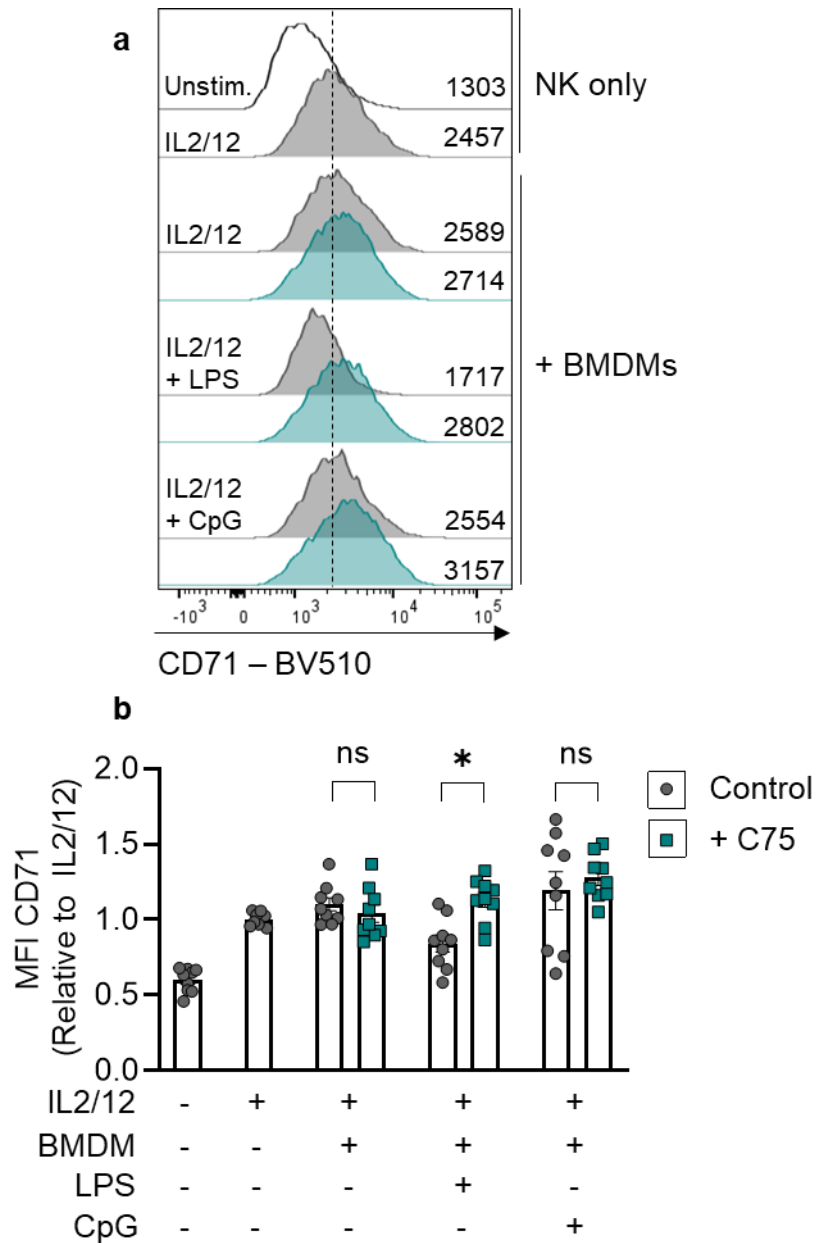


Figure 6.12 Specific inhibition of *de novo* fatty acid synthesis in BMDMs rescues CD71 expression in IL2-NK cells following co-culture.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 μ g/mL), in the presence or absence of C75 (40 μ M) for 6 h before co-culture with IL2-NK cells stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for a further 18 h. CD71 was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 9 biological replicates across 3 independent experiments (b). Statistical analysis was done using a two-way ANOVA with Šidák's post-test (ns=nonsignificant).

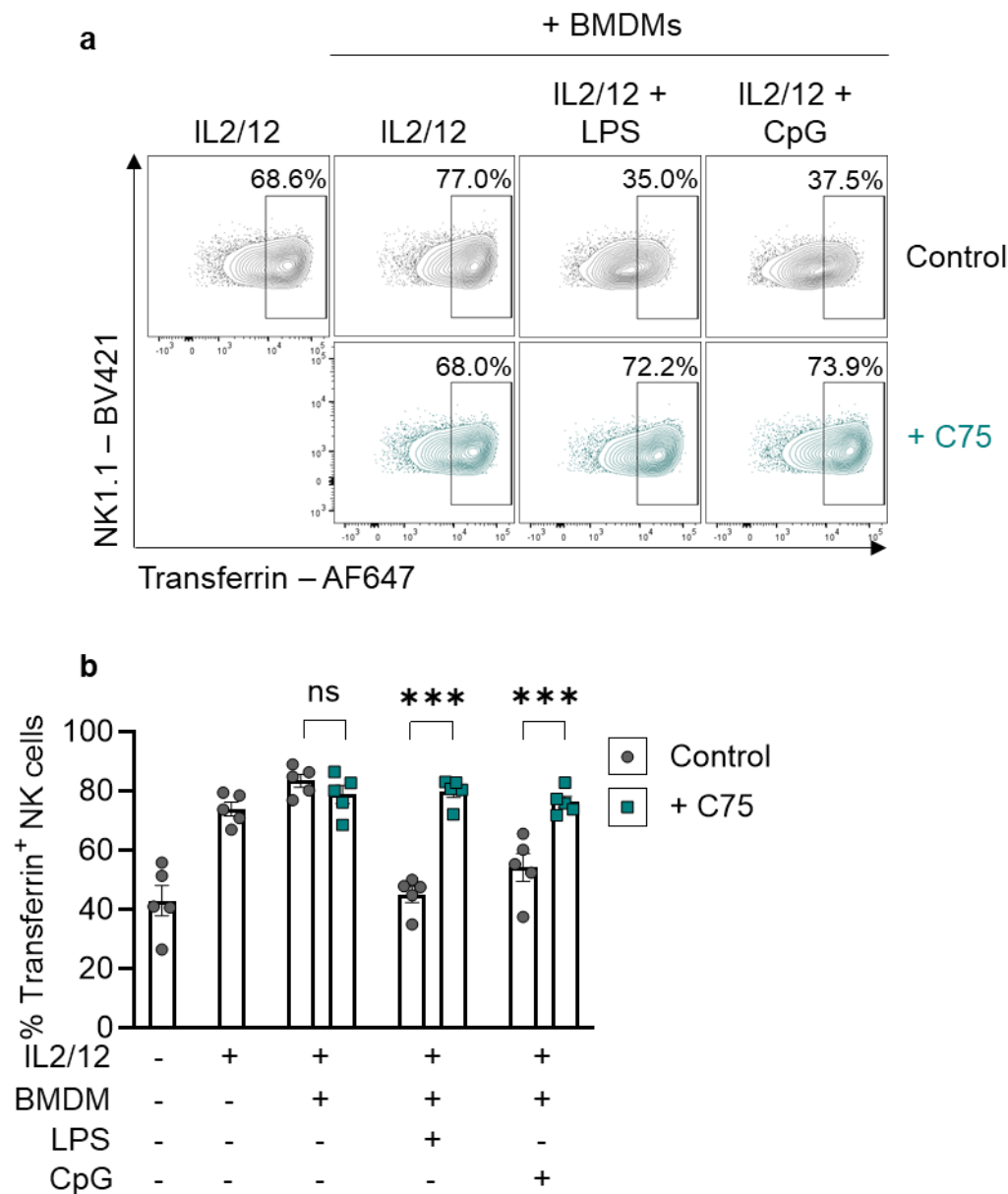


Figure 6.13 Specific inhibition of *de novo* fatty acid synthesis in BMDMs rescues holotransferrin uptake by IL2-NK cells following co-culture.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 µg/mL), in the presence or absence of C75 (40 µM) for 6 h before co-culture with IL2-NK cells stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for a further 18 h. Holotransferrin uptake was measured using fluorescently labelled transferrin by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 5 biological replicates across 2 independent experiments (b). Statistical analysis was done using a two-way ANOVA with Šidák's post-test (** $p < 0.001$, ns=nonsignificant).

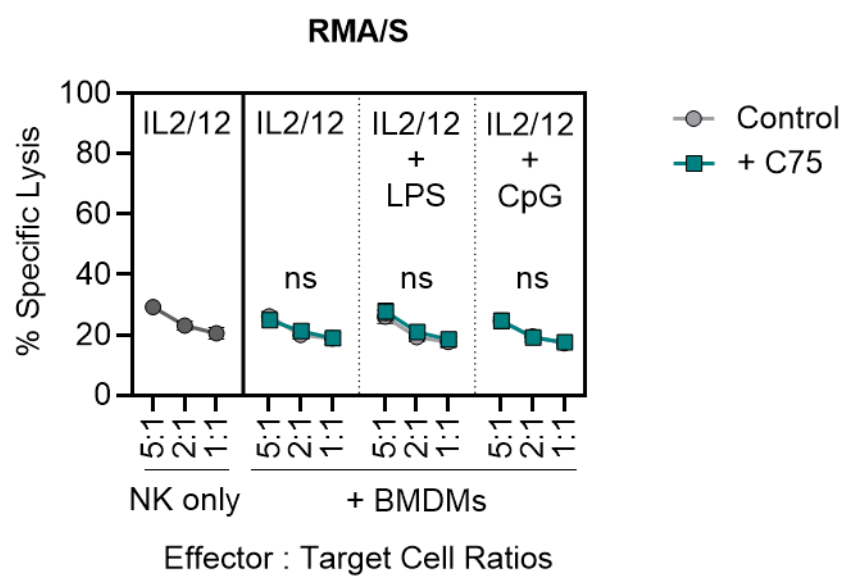


Figure 6.14 Specific inhibition of *de novo* fatty acid synthesis in BMDMs does not alter IL2-NK cell cytotoxicity following co-culture.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 µg/mL), in the presence or absence of C75 (40 µM) for 6 h before co-culture with IL2-NK cells stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for a further 18 h. NK cell cytotoxicity against RMA/S target cells was determined by fluorescence release assay. Data is mean $\pm$ SEM of 4 biological replicates across 2 independent experiments. Statistical analysis was done using a two-way ANOVA (ns=nonsignificant).

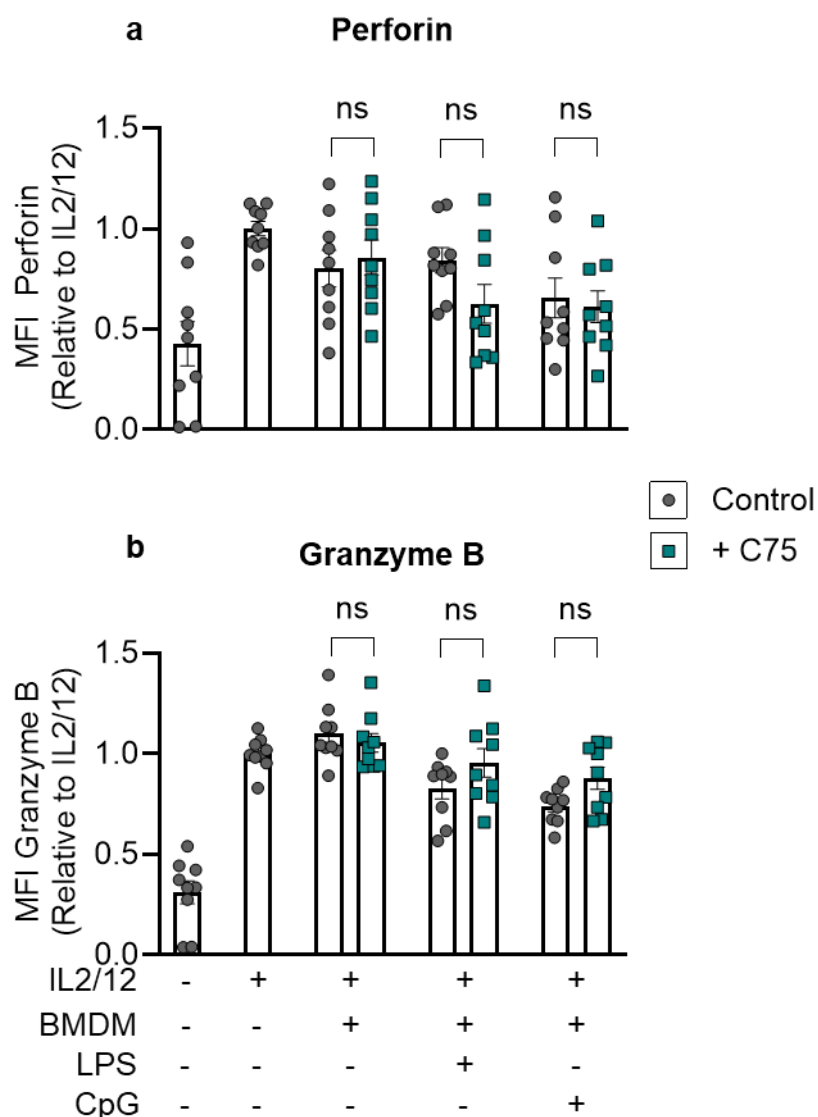


Figure 6.15 Specific inhibition of *de novo* fatty acid synthesis in BMDMs does not alter perforin or granzyme B expression by IL2-NK cells following co-culture.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 μ g/mL), in the presence of absence of C75 (40 μ M) for 6 h before co-culture with IL2-NK cells stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for a further 18 h. Granzyme B (a) and perforin (b) were measured by flow cytometry. Data is mean $\pm$ SEM of 9 biological replicates across 3 independent experiments. Statistical analysis was done using a two-way ANOVA with Šidák's post-test (ns=nonsignificant).

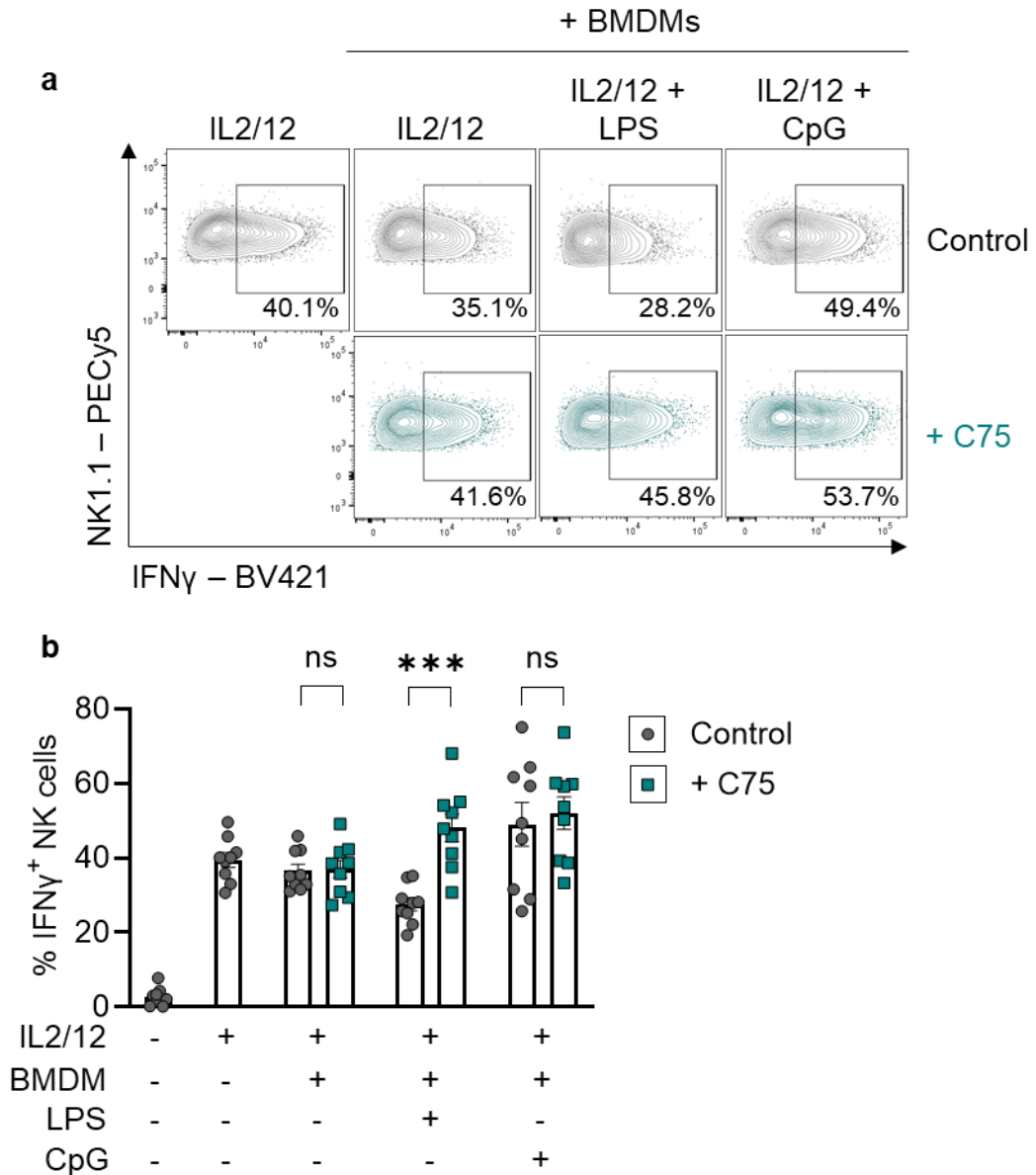


Figure 6.16 Specific inhibition of *de novo* fatty acid synthesis in LPS-stimulated BMDMs rescues IFN γ expression by IL2-NK cells following co-culture.

BMDMs were left unstimulated or pre-treated with LPS (10 ng/mL) or CpG (4 μ g/mL), in the presence of absence of C75 (40 μ M) for 6 h before co-culture with IL2-NK cells stimulated with IL2 (20 ng/mL) and IL12 (10 ng/mL) for a further 18 h. IFN γ expression was measured by flow cytometry. Data is representative (a) or mean $\pm$ SEM of 9 biological replicates across 3 independent experiments (b). Statistical analysis was done using a two-way ANOVA with Šidák's post-test (***p<0.001, ns=nonsignificant).

6.4 Discussion

Lipid synthesis and uptake are essential processes for supporting cellular processes such as energy metabolism, membrane structure and signalling. Advances in techniques for assessing immune cell metabolism has also revealed that lipids play an important role in shaping the immune response in different contexts. Lipid synthesis from acetyl CoA encompasses a wide variety of metabolic pathways producing a diverse number of lipid species. This diversity is also mirrored in the wide variety of functions that lipids can have in immune cells. For example, it has been shown that treatment of macrophages *in vitro* with unsaturated fatty acids such as oleic acid and linoleic acid can selectively promote production of IL1 α , whereas saturated fatty acids such as stearic acid and palmitic acid had no effect on IL1 α production (Freigang *et al.*, 2013). In another study it was shown that fatty acids are essential to support activation of CD4⁺ T cells, though these cells specifically required saturated fatty acids. In fatty acid free medium, supplementation with the saturated fatty acids, decanoic acid and myristic acid supported CD4⁺ T cell proliferation, whereas supplementation with the unsaturated fatty acids palmitoleic acid and linoleic acid had no effect (Angela *et al.*, 2016). These studies highlight that very discrete changes in the structure of lipid species can have opposing effects on cellular processes. In this thesis, the precise fatty acid species being transferred from macrophages to NK cells are not known though the data here suggests that they are immunosuppressive and may represent part of an immunoregulatory axis between these cells.

Initially it was assessed whether NK cells were using the fatty acids acquired from macrophages to support OxPhos through β -oxidation. Assessment of fatty acid metabolism *in vitro* has shown that β -oxidation of fatty acids is primarily associated with anti-inflammatory immune cell types such as M2 macrophages and T_{reg} cells. Stimulation of BMDMs with IL4 has been shown to induce increased rates of β -oxidation, which were essential for their polarisation (Vats *et al.*, 2006; Huang *et al.*, 2014). Some studies have shown that lipid uptake is essential to support this metabolic configuration, though other studies have suggested that *de novo* fatty acid synthesis from glucose-derived carbons are essential for this process (Huang *et al.*, 2016). This dependence on β -oxidation

has also been described in TAMs from both murine and human tumours (Su *et al.*, 2020). In NK cells, the use of β -oxidation has opposing effects depending on the context the NK cells are in. For example, it has been shown that in NK cells treated with palmitate, there was an increase in fatty transport into the mitochondria, and that inhibition of this process rescued rates of glycolysis which were more important to support NK cell cytotoxicity (Michelet *et al.*, 2018). However, this is likely a context dependent process as in viral infections NK cells had increased uptake of fatty acids which was associated with elevated rates of β -oxidation. Although this was important to support cytotoxicity, it was not required for cytokine production (Schimmer *et al.*, 2023; Sheppard *et al.*, 2024). This demonstrates another situation where immune cells seem to control which metabolic processes can provide ATP to support specific effector functions. These studies also show that engagement of β -oxidation is not a definitive parameter to differentiate between a regulatory or an effector cell, as has previously been described. In this chapter, peritoneal NK cells from LPS-immunised mice had a small dependence on β -oxidation to support rates of protein synthesis, though it is possible that this small contribution to the overall ATP levels in the cell is important for a discrete process that hasn't been measured in this chapter. Alternatively, this minor use of β -oxidation to support ATP production likely allows the NK cells a degree of metabolic flexibility through using a range of different fuels. Treatment of NK cells with etomoxir for 4 h did not induce any changes to expression of IFN γ or cytotoxic molecules, in agreement with data from chapter 4, which showed that OxPhos was not essential to support expression of IFN γ or perforin.

There are a number of studies that have suggested that altering expression of CPT1A in a cell can dramatically alter their phenotype. For example, in pro-inflammatory macrophages, expression of constitutively active CPT1A significantly reduced production of pro-inflammatory cytokines (Malandrino *et al.*, 2015). In NK cells there is also evidence that the expression of CPT1A not only acts as a metabolic switch but also has significant functional outcomes. Human NK cells treated with PD-1 increased expression of CPT1A which facilitated increased rates of β -oxidation. In this setting the increased rates of β -oxidation were associated with enhanced infiltration of NK cells into tumours, where they

had sustained effector functions (Srpan *et al.*, 2025). This study suggests that if NK cells in co-culture with macrophages were to express CPT1A they may be able to eliminate intracellular lipids and potentially rescue expression of IFN γ . In mice fed a high fat diet it has been shown that increased fatty acid uptake induces a metabolic switch to β -oxidation due to CPT1A expression. This was induced by fatty acids signalling through PPAR α/δ (Michelet *et al.*, 2018). In this thesis it was shown that IL2-NK cells do not express *Cpt1a* mRNA, even in the presence of BMDMs. However, assessment of rates of protein translation in the presence of etomoxir showed that ATP production by IL2-NK cells alone was dependent on β -oxidation of fatty acids, suggesting there is some expression of CPT1A at protein level. Whether this is a true result is unclear. Previous data in this thesis has shown that inhibition of OxPhos with oligomycin did not affect rates of protein translation. Furthermore, etomoxir has a number of off-target effects, meaning experiments involving use of this inhibitor should be interpreted with caution (O'Connor *et al.*, 2018; Yao *et al.*, 2018; Raud *et al.*, 2018). The concentration of etomoxir used in the experiments in this chapter is widely considered to inhibit β -oxidation independently of any described off-target effects, though there may be increased sensitivity to these off-target effects depending on the cell type being investigated.

As discussed, it has been shown that activation of PPAR α/δ signalling with fatty acids can facilitate a switch to β -oxidation (Michelet *et al.*, 2018). However, it is possible that the increased abundance of fatty acids in NK cells in these co-cultures could alter other signalling pathways. For example, palmitic acid has been described to activate Akt in numerous cell types leading to enhanced mTORc1 activation (Pu *et al.*, 2011; Calvo-Ochoa *et al.*, 2017). However, it is unlikely that enhanced mTORc1 activation would be inhibitory to NK cells in this context. It has also been shown that fatty acyl CoA molecules can bind to AMPK to induce a switch to β -oxidation, independently of any change to the ATP:AMP ratio (Clark *et al.*, 2004). Again, it is unlikely that this is occurring in this co-culture system as there is no evidence that NK cells are switching to β -oxidation to support energy metabolism *in vitro*. Increased levels of fatty acids have also been shown to alter the structure of the plasma membrane by increasing plasma membrane disorder (Muranushi *et al.*, 1981; Langner and Hui, 2000). This has

potential implications for cytokine signalling in immune cells. However, data shown in chapter 3 showed that both LPS- and CpG-stimulated BMDMs had no impact on NK cell plasma membrane order. Furthermore, there were no changes detected in expression of CD25 or SREBP target genes, indicating that IL2 signalling in these co-cultures was not interrupted. There is also evidence that some medium chain fatty acids can activate LXR signalling *in vitro* (Bedi *et al.*, 2017), whereas long chain acids have been shown to antagonise LXR *in vitro* (Ou *et al.*, 2001). Although the precise fatty acid species present in the NK cells in these co-cultures is not known it is unlikely that activation or inhibition of LXR signalling would alter NK cell functions. In fact, data not shown in this thesis showed that performing NK cell-macrophage co-cultures in the presence of LXR agonists did not change the level of lipid accumulation or expression of effector molecules by NK cells. In order to fully understand whether there are changes to signalling pathways occurring in NK cells in the presence of macrophages, the fatty acid species accumulating in the NK cells would need to be identified.

Another consideration for the role of these fatty acids in altering NK cell signalling is how they are stored. As shown in chapter 5 the lipids accumulating in NK cells are stored in lipid droplets where they are sequestered from the rest of the cell through the activity of PLINs (Itabe *et al.*, 2017). In order for these NK cells to mobilise free fatty acids to alter signalling would require the activity of lipases. The only lipase detected in a proteomics dataset of splenic NK cells in the Finlay lab was lysosomal lipase. Although this enzyme is primarily associated with the lysosomes, it has also been shown to mobilise fatty acids from cytosolic lipid droplets (Tuohetahuntala *et al.*, 2017). However, its role in NK cells has not been investigated. There is evidence that lipid droplets themselves can also alter the function of other organelles within the cell. For example, lipid droplets have been shown to interact with many different organelles within the cell such as the ER and mitochondria, facilitating transfer of lipids into or out of, these organelles (Hong Wang *et al.*, 2011; Du *et al.*, 2020). Tethering of lipid droplets to the mitochondria has been shown to facilitate the rapid uptake of fatty acids into the mitochondria as fatty acyl CoA molecules to support β -oxidation (Miner *et al.*, 2023). Whether lipid droplet tethering to other organelles occurs in NK cells has not been investigated.

As already mentioned, *de novo* fatty acid synthesis is commonly associated with pro-inflammatory cell types, including macrophages (Posokhova *et al.*, 2008; Feingold *et al.*, 2012; Castoldi *et al.*, 2020). In fact, many studies have shown that *de novo* fatty acid synthesis is also required for macrophages to respond to pro-inflammatory stimuli. Activation of FASN was shown to be required for correct formation of lipid rafts at the plasma membrane to which are required for TLR and TNF α signalling (Triantafilou *et al.*, 2002; Legler *et al.*, 2003; Nakahira *et al.*, 2006). Interestingly, it has been shown that inhibiting *de novo* fatty acid synthesis in BMDMs can also result in changes to cholesterol levels, which is the reason for the alterations to the plasma membrane (Carroll *et al.*, 2018). This suggests that the use of C75 in this thesis may be changing the transfer of cholesterol or CEs to NK cells. However, this was addressed in chapter 5 using a specific inhibitor of *de novo* cholesterol synthesis, showing that cholesterol is not transferred to IL2-NK cells by LPS-stimulated BMDMs. Considering macrophages need FASN activity to support TLR4 signalling, it was also considered whether C75 could induce a change in another communication axis that exists between macrophages and NK cells, independently of changes in fatty acids. For example, if C75-treated macrophages were to enhance production of NK cell-activating cytokines, this could account for the rescue in IFN γ expression. On the contrary, if C75-treated macrophages were to lose production of cytokines that are inhibitory to NK cells, this could also account for the rescue of IFN γ expression. To investigate this, levels of pro-inflammatory cytokines were measured showing a significant impairment in their expression when peritoneal macrophages were treated with C75, mirroring data already showing this effect in BMDMs (Carroll *et al.*, 2018). It is unlikely that a loss of IL1 β , IL6, TNF α or IL12 production is responsible for a rescue in NK cell functions in co-cultures with C75-pre-treated macrophages as all of these cytokines have been described to activate NK cells *in vitro* (Rabinowich *et al.*, 1993; Nguyen *et al.*, 2000; Cooper *et al.*, 2001; Khan *et al.*, 2023; Duggan *et al.*, 2018). Furthermore, there was no detected loss of TGF β 1, a potent inhibitor of NK cell IFN γ production (Viel *et al.*, 2016; Zaiatz-Bittencourt *et al.*, 2018; Slattery *et al.*, 2021). IL10 signalling has been associated with enhanced rates of metabolism in human NK cells, supporting increased IFN γ production (Wang *et al.*, 2021). After caecal ligation and puncture, IL10 was associated with impaired NK cell activation, though this

effect is likely not due to a direct effect on NK cells, but rather inhibiting production of IL12 by macrophages and DCs required for sustained activation (Scott *et al.*, 2006). Regardless of the direct effects IL10 may have on NK cell activation, a proteomics dataset from the Finlay lab did not detect the IL10 receptor on splenic NK cells. Therefore, it is unlikely that a loss of IL10 could be responsible for a rescue in NK cell IFN γ production. The decrease in chemokine production by peritoneal macrophages pre-treated with C75 may result in decreased contacts being formed between macrophages and NK cells. However, imaging of co-cultures of peritoneal macrophages and splenic NK cells did not suggest any chemokine gradients were promoting interactions as NK cells continually moved around the dish and did not cluster around macrophages, with the exception of the few long-lasting contacts that were shown in chapter 5. Of all the parameters altered by C75 in peritoneal macrophages in this chapter, none are likely candidates for enhancing NK cell activation. Furthermore, when NK cell effector functions are investigated in this *in vitro* co-culture exogenous cytokines are in excess, meaning that changes in the cytokine expression by the macrophages would be inconsequential. This strengthens the argument that transfer of fatty acids from macrophages to NK cells in these co-cultures is the cause of dampened NK cell IFN γ production.

Although the focus of this chapter has been on NK cell IFN γ production, it has consistently been shown that inhibition of lipid accumulation in NK cells also rescues CD71 expression by NK cells, which was mirrored in a rescue of holotransferrin uptake. It has previously been shown that activation of PPAR α/δ signalling in NK cells is associated with a loss of cMyc (Michelet *et al.*, 2018; Jiao *et al.*, 2023), the transcription factor for CD71. Data in chapter 4 showed a significant decrease in expression of *Tfrc* mRNA, which encodes for CD71, supporting a loss of cMyc activity. Although, it is not clear whether this mechanism is occurring in these co-cultures, it has been shown that increased fatty acid accumulation leads to decreased cMyc expression in NK cells, which was associated with impaired IFN γ production and cytotoxicity (Jiao *et al.*, 2023). This study showed that oleic acid accumulation in NK cells inhibited p300, a histone acetyltransferase. This was linked with reduced acetylation of cMyc, which reduced its half-life. The decrease in active cMyc led to reduced expression of

cMyc target genes, revealing a possible fatty-acid dependent mechanism that macrophages could be using to alter NK cell effector functions. Studies have previously shown that cMyc is essential to support NK cell effector functions following cytokine stimulation (Loftus *et al.*, 2018).

There are numerous studies which demonstrate the importance of certain epigenetic marks for controlling NK cell effector functions (Shi *et al.*, 2016; Cribbs *et al.*, 2018). The study by Jiao *et al.* (2023) also revealed that inhibition of p300 by oleic acid led to decreased acetylation of H3K27, reduced chromatin accessibility to the promoters of genes encoding NK cell effector proteins. Although it is not known if macrophages are feeding oleic acid specifically into NK cells in co-cultures, it is possible that increased abundance of this fatty acid could suppress IFN γ production and warrants further investigation. Oleic acid in particular has previously been associated with impaired NK cell function when present at supraphysiological concentrations (Michelet *et al.*, 2018).

This thesis hypothesises that macrophages are using fatty acids to dampen IFN γ production by NK cells in order to prevent hyperinflammation due to sustained macrophage activation. Whether rescue of IFN γ production through blocking lipid transfer, results in enhanced macrophage activation is unclear. The experiments in this chapter blocked lipid transfer with C75 which was shown to significantly impair macrophage activation *in vitro*, meaning the impact of the increased IFN γ could not be assessed. In order to properly assess this an *in vivo* strategy to specifically block lipid transfer from macrophages to NK cells would be required. However, this comes with significant difficulties as inhibition of fatty acid synthesis in macrophages *in vivo* would suppress their activation, leading to decreased NK cell activation in many contexts. Based on the data shown in this chapter, the transfer of fatty acids from macrophages to NK cells likely represents a metabolic signalling axis between the two cell types to control the levels of IFN γ production by NK cells in an effort to resolve elevated levels of inflammation.

Chapter 7

General Discussion

This thesis primarily sought to understand how NK cells and macrophages communicate with one another. Macrophages are ubiquitous in the body, meaning that regardless of the tissue niche an NK cell is carrying out its effector functions, it will likely be in close proximity to a macrophage. The mechanisms of macrophage-NK cell interactions have primarily been described in the context of providing cytokines to enhance immune responses. However, the resolution of inflammation is crucial for restoring tissue homeostasis after injury or infection, meaning that negative feedback mechanisms between the two cell types are essential to prevent hyperinflammatory conditions. Perforin deficient mice have provided information about the importance of cytotoxicity by NK cells and CD8⁺ T cells to resolve inflammation and have shown an important regulatory link between lymphocytes and pro-inflammatory myeloid cells. Following infection of perforin deficient mice with LCMV there was evidence of severe inflammation characterised by elevated IL6, IL10, IL18, IFN γ and TNF α (Binder *et al.*, 1998; Matloubian *et al.*, 1999; Jordan *et al.*, 2004). Ultimately the mice were unable to resolve the activation of macrophages, leading to a fatal inflammatory response. These effects were further exacerbated when mice were vaccinated with an LCMV antigen, leading to higher rates of death following LCMV challenge in perforin deficient mice (Badovinac *et al.*, 2003). These studies highlight the importance of the immune editing features of lymphocytes to control the numbers of pro-inflammatory cells in infection. However, these studies also revealed a feed forward effect whereby the increased cytokine production by macrophages in turn lead to enhanced cytokine production by NK cells and CD8⁺ T cells. Interestingly, neutralisation of IFN γ reduced the rate of death in perforin deficient mice challenged with LCMV (Jordan *et al.*, 2004).

Similar effects can be seen in patients with mutations in the machinery used by NK cells to release cytotoxic granules. This leads to a severe autoimmune disease in humans called familial hemophagocytic lymphohistiocytosis (FHL). There are a number of inherited forms of this disease caused by biallelic mutations in genes involved in the secretory lysosome-dependent exocytosis

pathway (Sepulveda and de Saint Basile, 2017; Canna and Marsh, 2020). Among these are *Prf1*, which encodes for perforin (Stepp *et al.*, 1999), mimicking the effects seen in perforin-deficient mice. Other forms of the disease involve mutations in *Lyst* and *Unc13d* which are essential components of the machinery required for the exocytosis of cytotoxic granules (Barbosa *et al.*, 1996; Côte *et al.*, 2009). This generally manifests early in life leading to increased susceptibility to viral infections but also presents with hyperinflammation due to excessive activation of lymphocytes and macrophages, that would otherwise be directly killed by NK cells to resolve inflammation (Sepulveda and de Saint Basile, 2017; Canna and Marsh, 2020).

NK cell-derived IFN γ has been shown to be important to enhance the antimicrobial activity of myeloid cells, though sustained IFN γ production is thought to be damaging to the host, as shown in the studies investigating LCMV. There are a number of studies which demonstrate that NK cell activation is a significant risk factor for progression to sepsis. In sepsis models caused by caecal ligation and puncture or infection with live bacteria, depletion of NK cells reduced the levels of systemic inflammation, prevented tissue damage and prolonged survival (Badgwell *et al.*, 2002; Sherwood *et al.*, 2004; Goldmann *et al.*, 2005; Enoh *et al.*, 2006; Christaki *et al.*, 2015). Clinical studies have also shown that in sepsis patients, heightened levels of NK cells are associated with early mortality following septic shock (Andaluz-Ojeda *et al.*, 2011; de Pablo *et al.*, 2012). Furthermore, neutralisation of IFN γ has been shown to prevent septic shock in mice immunised with LPS (Ito *et al.*, 2006). Soluble IL15 complexed with IL15R α , which enhances proliferation and activation of NK cells, has been shown to cause significant immunotoxicity in mice, though this effect is not observed in IFN γ -null mice (Guo *et al.*, 2015). These studies highlight the importance of immunoregulatory mechanisms to prevent excessive IFN γ production during infections.

NK cells have been shown to use certain endogenous mechanisms to control their production of IFN γ , though they may not always be sufficient in hyperinflammatory conditions. Thioredoxin-interacting protein (TXNIP) is a multifunctional protein involved in resolving oxidative stress and glucose metabolism, however, it has also been shown to be important for regulation of

IFN γ production by NK cells during models of bacterial infection. NK cells from TXNIP-null mice showed enhanced IFN γ production following stimulation *in vitro*. Following adoptive transfer into wild-type mice, TXNIP-null NK cells could elicit increased activation of splenic macrophages *in vivo* (Kim et al., 2020). Other mechanisms that have been shown to regulate IFN γ production include IL10 production by NK cells, which has been shown to limit IL12 production by DCs in mice infected with *Yersinia pestis* or *Toxoplasma gondii*. This IL10 production limited IL12 production by DCs, leading to reduced IFN γ production by NK cells (Perona-Wright et al., 2009). In fact IL10 production by NK cells has also been shown to be protective following caecal ligation and puncture, through preventing systemic inflammation in the host (Jensen et al., 2021). Whether these negative feedback mechanisms can act to curtail NK cell responses in severe infections is not fully understood due to conflicting reports of NK cells' importance in sepsis. It is thought NK cell activation following initial infection or septic shock can be lethal, whereas their production of anti-inflammatory mediators later in infection can improve survival in the host.

This thesis has investigated fatty acid delivery from macrophages to NK cells as a paracrine immunoregulatory mechanism that macrophages may use to attenuate NK cell responses and resolve inflammation. The roles of fatty acids in controlling NK cell effector functions are complex and dependent on the species of fatty acids and the metabolic configuration adopted by the NK cells (Michelet et al., 2018; Schimmer et al., 2023; Sheppard et al., 2024). In the context of macrophage to NK cell transfer the exact species of fatty acids have not been identified, though evidence in this thesis shows that they can impair iron uptake and IFN γ production by NK cells. Although this may mirror the decreased NK cell effector functions seen following fatty acid accumulation in conditions like cancer and obesity (Michelet et al., 2018; Niavarani et al., 2019; Kobayashi et al., 2020), the interactions between macrophages and NK cells investigated in this thesis seem to be more controlled and deliberate, resulting in a decrease in IFN γ production, while preserving NK cell cytotoxicity. Given the significant risk posed by sustained IFN γ production in infections, it is possible that this fatty acid transfer may have evolved as a homeostatic mechanism to dampen IFN γ and prevent hyperinflammation. The fact that it is dependent on direct cell-cell contacts

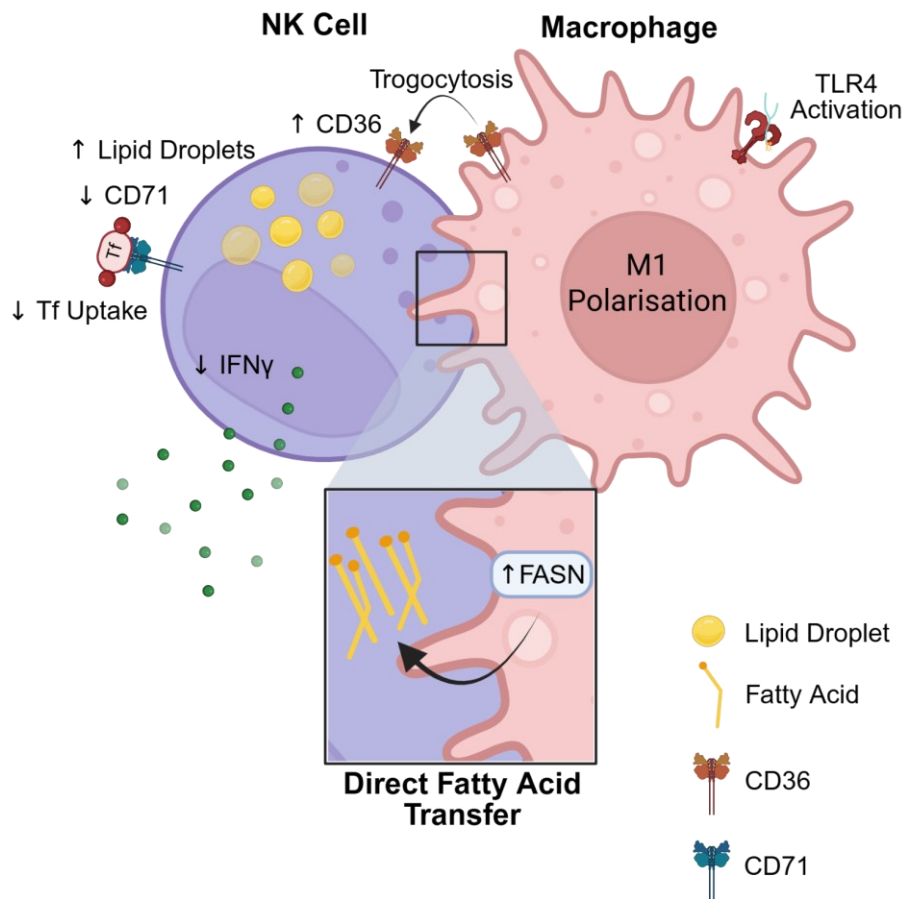


Figure 7.1 Macrophages directly feed fatty acids to NK cells to dampen IFN γ production.

Following the formation of direct contacts, LPS-stimulated macrophages directly feed fatty acids into NK cells, which corresponds with increased lipid droplet formation in NK cells. These direct contacts were also required for trogocytosis of CD36 from macrophages by NK cells. The increased lipid accumulation was associated with decreased expression of CD71 and impaired holotransferrin (Tf) uptake by NK cells. Furthermore, the enhanced lipid accumulation was associated with dampened IFN γ production by NK cells. *This figure was created using Biorender.com.*

suggests that it dampens NK cell IFN γ production locally, while allowing NK cells to carry out other important functions such as cytotoxicity against bacteria or infected cells.

Many questions still remain regarding the transfer of fatty acids from macrophages to NK cells. Firstly, the mechanism of transfer was not determined

in this thesis. Although it requires direct cell-cell contact it is not known whether this is a passive process or requires the expression of machinery to facilitate the transfer. Although the trogocytosis of CD36 was not shown to be essential for lipid accumulation by NK cells in co-culture with macrophages, it is possible that compensatory expression of other transporters may be occurring in these experiments. In fact, the role of CD36 trogocytosis is still not known but reveals a very interesting mechanism that cells can use to quickly gain proteins, independent of changes in endogenous expression. Finally, following the transfer of fatty acids to the NK cells and their storage in lipid droplets, it is not known what signalling pathways the fatty acids are using to impair IFN γ production. These could potentially be targeted therapeutically in conditions such as cancer to prevent TAM-mediated inhibition of NK cell IFN γ production.

Overall, this study highlights the importance of considering the metabolism of immune cells in more complex environments where they are integrating extrinsic signals received from other cells intrinsic regulators of their own metabolic configuration. In this study, the addition of one other cell type, a pro-inflammatory macrophage, was sufficient to induce CD36 expression and lipid storage by NK cells, which has not before been shown in culture. The direct feeding of fatty acids from macrophages to NK cells represents a novel regulatory mechanism using metabolic mediators to control NK cell IFN γ production. This study will inform future investigations of NK cell immunometabolism and effector function in complex *in vivo* environments.

Chapter 8

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