

Investigating the metabolic reprogramming of cytokine-induced memory-like Natural Killer cells



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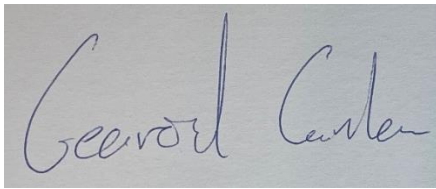
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Thesis Declaration

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Gearóid Conlon B.A. (Mod)

Declaration of collaborative input

For quantitative proteomics data presented in Chapters 3 and 4, sample processing was carried out by the staff, particularly Cara Henderson, of the Proteomics FingerPrints Facility in the University of Dundee, Scotland.

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Publications and awards

Publications

“Pharmacological Targets in Metabolic Diseases”

Chapter 21: “Polyphenols and the control of immunometabolism as a pharmacological target”

Gearóid Conlon, Nicole Campbell, Sinead O’Rourke¹ and Aisling Dunne
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Tumor-treating fields increase cytotoxic degranulation of natural killer cells against cancer cells

Eimear Mylod, **Gearóid Conlon**, Elise P.W. Jenkins, George G. Malliaras, Clair M. Gardiner
(Cell Reports Physical Science, 2024)

Awards

Best poster award, British Society for Immunology (BSI) Congress 2023
(600+ posters).

List of abbreviations

2-DG	2-deoxyglucose
5'NT	5 prime nucleotidase
ACLY	ATP-citrate lyase
ADCC	Antibody-dependent cellular cytotoxicity
ADK	Adenosine kinase
ADP	Adenosine diphosphate
ALDO	Aldolase
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
AML	Acute myeloid leukemia
APC	Antigen presenting cell
APRT	Adenine Phosphoribosyltransferase
ARE	Antioxidant response element
ATAC-seq	Assay for Transposase-Accessible Chromatin with sequencing
ATP	Adenosine triphosphate
BCR	B cell receptor
BCG	Bacillus Calmette-Guérin
BMDM	Bone marrow-derived macrophages
CAR	Chimeric antigen receptor
CAT	Catalase
CIML	Cytokine-induced memory-like
CMS	Citrate-malate shuttle
CMV	Cytomegalovirus
CNS	Conserved non-coding sequences
DAMP	Damage-associated molecular patterns
DRP1	Dynamin-related protein 1
effcNK	Effector conventional natural killer cell
eML	Enriched memory-like
ENO	Enolase
ER	Endoplasmic reticulum

ETC	Electron transport chain
FADH ₂	Flavin adenine dinucleotide
FAO	Fatty acid oxidation
FAS	Fatty acid synthesis
FASL	FAS ligand
FIS1	Fission, mitochondrial 1
FTH	Ferritin heavy chain
FTL	Ferritin light chain
G6P	Glucose-6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT	Glucose transporter
GMP	Guanosine monophosphate
GPI	Glucose-6-phosphate isomerase
GSS	Glutathione synthetase
GVHD	Graft-versus-host disease
H6PD	Hexose-6-phosphate dehydrogenase
HAT	Histone acetyltransferase
HCMV	Human cytomegalovirus
HDAC	Histone deacetylase
HIF1 α	Hypoxia-inducible factor 1 alpha
HLA	Human leukocyte antigens
HMOX1	Heme oxygenase 1
HK	Hexokinase
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase
IDO1	Indoleamine 2,3-dioxygenase 1
IFN γ	Interferon gamma
IL	Interleukin
ILT	Immunoglobulin-like transcript receptor
IMM	Inner mitochondrial membrane
IMP	Inosine monophosphate
IMPDH	Inosine monophosphate dehydrogenase
iPSC	Induced pluripotent stem cells

KDM	Lysine demethylase
KIR	Killer-cell immunoglobulin-like receptors
KMT	Lysine methyltransferase
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MIC	Major histocompatibility complex class I chain-related proteins
MFN	Mitofusin
MIP	Macrophage inflammatory protein
mPTP	Mitochondrial permeability transition pore
mTORC1	Mechanistic target of rapamycin complex 1
Myc	Myelocytoma
NADH	Nicotinamide adenine dinucleotide + hydrogen
NADK	Nicotinamide adenine dinucleotide kinase
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate + hydrogen
NCR	Natural cytotoxicity receptor
NFAT	Nuclear factor of activated T cells
NK	Natural Killer
NQO1	NAD(P)H:quinone oxidoreductase 1
NRF2	Nuclear factor erythroid 2-related factor 2
OAA	Oxaloacetate
OPA1	Optic atrophy type 1
OXPHOS	Oxidative phosphorylation
oxLDL	Oxidised low-density lipoprotein
PAMP	Pathogen-associated molecular pattern
PDH	Pyruvate dehydrogenase
PFK	Phosphofructokinase
PGAM	Phosphoglycerate mutase
PGC1 α	Peroxisome proliferator-activated receptor- γ coactivator 1 alpha
PGD	6-Phosphogluconate dehydrogenase
PGK	Phosphoglycerate kinase

PNP	Purine nucleoside phosphorylase
PPP	Pentose phosphate pathway
PRDX	Peroxiredoxin
PRPP	Phosphoribosyl pyrophosphate
PRPS	Phosphoribosyl pyrophosphate synthase
PRR	Pattern recognition receptor
R5P	Ribose-5-phosphate
RAS	Rat sarcoma
Redox	Oxidation and reduction
ROS	Reactive oxygen species
scFV	Single-chain variable fragment
SDH	Succinate dehydrogenase
SLC	Solute carrier
sMICA	soluble major histocompatibility complex class I chain-related protein A
SOD	Superoxide dismutase
SREBP	Sterol Regulatory Element-Binding Protein
TALDO1	Transaldolase 1
TB	Tuberculosis
TCA	Tricarboxylic acid
TCR	T cell receptor
TGFβ	Transforming growth factor beta
TKT	Transketolase
TME	Tumour microenvironment
TNFα	Tumour necrosis factor alpha
TPI	Triosephosphate isomerase
TRAIL	TNF-related apoptosis-inducing ligand
TXN	Thioredoxin
α-KG	Alpha-ketoglutarate

Abstract

Natural Killer (NK) cells play a crucial role in early immune defense by recognising and eliminating virally infected and malignant cells. Human NK cells are highly heterogeneous and, under cytokine stimulation, can acquire long-lasting functional adaptations known as cytokine-induced memory-like (CIML) properties. CIML NK cells fall under the bracket of “trained immunity”, meaning they can exhibit non-specific, memory-like properties, initiated and sustained by epigenetic modifications and metabolic reprogramming. CIML NK cells display enhanced cytotoxicity against cancer cells compared to conventional NK cells, yet the metabolic mechanisms that sustain this phenotype and underpin CIML NK cell enhanced function remain ill-defined.

This thesis presents one of the first integrated proteomic and metabolomic interrogations of CIML NK cell immunometabolism, using quantitative proteomics, targeted nutrient tracing, and flow cytometry to reveal insights into CIML NK cell metabolic reprogramming. The data presented herein demonstrates that CIML NK cells adopt a sustained proliferative and anabolic phenotype characterised by enhanced glycolysis, pentose phosphate pathway (PPP), purine metabolism, redox management, and accumulate mitochondrial mass poised for rapid mobilisation upon restimulation. This thesis also highlights the existence of functional and metabolic heterogeneity in recently identified CIML NK cell subsets.

Collectively, this work establishes a more comprehensive understanding of CIML NK cell metabolism by revealing a coordinated metabolic network that underpins their function. Not only does this advance our understanding of NK cell metabolism and its role in trained immunity, but it also highlights specific metabolic pathways that could be targeted to enhance NK cell-based immunotherapies to treat malignancy and/or viral infection.

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Chapter 1. Introduction.

1.1 The Immune System.

The human immune system is a complex network of tissues, cells, and molecules that has evolved over millions of years to protect us from infection and cellular damage. Environmental factors such as pathogens and pollutive agents, and more modern sources of disease, such as diet, can all result in disruptions to cellular metabolism and function that ultimately harm the host [1-3]. The primary role of the immune system is to detect these infected, transformed, or damaged cells and eliminate them without causing harm to healthy cells. Two main arms of the immune system exist: the innate and adaptive immune systems. The innate immune system is comprised of physical barriers like the skin and cells such as natural killer (NK) cells, macrophages, dendritic cells, and neutrophils that, without prior sensitisation, can recognise and rapidly respond to pathogens and cellular damage. The adaptive immune system is activated after this initial phase, and cells such as T and B lymphocytes elicit highly specific responses while also exhibiting immunological memory upon re-exposure to the agent [4].

The innate immune system consists of cells such as macrophages and dendritic cells that recognise molecules on the surface of, or derived from, pathogenic microbes called pathogen-associated molecular patterns (PAMPs). These molecules bind to and activate receptors known as pathogen recognition receptors (PRRs), which stimulate the cells to clear the pathogen. Cellular damage that arises from non-infectious agents, such as heat shock proteins from necrotic cells, is known as damage-associated molecular patterns (DAMPs), which activate innate cells similarly to PAMPs and result in the clearance of intrinsically damaged material [5, 6]. The material from these pathogens can be broken down by macrophages and dendritic cells, or other cells also known as antigen-presenting cells (APCs), and presented to naïve adaptive cells, which form immunological memory to the antigen in case of future re-exposure. Other innate cells, such as NK cells and neutrophils, exist primarily in the blood. Neutrophils are effective at clearing pathogens such as fungi and bacteria, while NK cells are effective at killing cancer and virally infected cells [7, 8].

In contrast to innate cells, T and B cells express highly specific antigen receptors known as the T-cell receptor (TCR) and B-cell receptor (BCR). These antigen-binding receptors display variability in specificity through rearrangement of their immunoglobulin variable, diversity, and joining segments, termed V(D)J recombination, which occurs early in cell development. This allows for the recognition of a broad range of antigens expressed on a pathogen [9]. T cells are comprised mainly of CD4⁺ helper T cells (of which there are several subsets) and CD8⁺ cytotoxic T cells that carry out the killing of target cells. B cells are potent producers of various antibodies, such as immunoglobulin (Ig) M, D, G, E, and A, which opsonise target cells, making them vulnerable to events such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity, and antibody-dependent phagocytosis [10].

1.2 Natural Killer cells.

Natural Killer cells are innate, granular effector lymphocytes that perform cell-mediated cytotoxicity and can release an array of soluble factors, including cytokines, perforin, and granzymes. They are distinct from T and B lymphocytes due to their ability to directly kill target cells without any prior sensitisation. Furthermore, in contrast to T and B cells, which undergo gene rearrangement to express T and B cell receptors, NK cells express germline-encoded receptors [8]. NK cell development takes place primarily in the bone marrow, where early hematopoietic growth factors flt-3 ligand and c-kit, in conjunction with interleukin (IL)-15, have been shown to be important [11]. NK cells have the capacity to differentiate between aberrant cells, such as tumour/infected cells and normal cells, primarily through expression of either activating or inhibitory receptors that bind ligands on the surface of target cells. Following the association of NK cell receptors with target cell surface molecules, the various signals produced are transduced and integrated by the NK cell, which dictates whether the NK cell will become activated or not. Distinct NK cell subsets have been identified in both mice and humans in relation to functional, anatomical, and phenotypic features [12]. Human NK cells are phenotypically categorised by the extent of their cell surface expression of the CD56 marker and absence of the CD3 receptor. NK cells expressing high or low levels of cell surface CD56 are divided into CD56^{bright}

and CD56^{dim} subsets, respectively and are further defined by the expression of FcγRIII (CD16). Differences in CD56^{bright} and CD56^{dim} NK cell metabolism can also distinguish between the subsets and account for their contrasting functions. CD56^{dim}CD16⁺ cells make up 90% of peripheral blood and spleen NK cells, produce perforin and are particularly cytotoxic, while CD56^{bright}CD16⁻ cells are found predominantly in tissues and are associated with increased cytokine production [8, 13].

1.2.1 Soluble factors produced by NK cells.

NK cells produce and respond to an array of cytokines and chemokines that enable them to carry out their effector functions [14]. They also constitutively express receptors that respond to activating cytokines such as IL-12, IL-15, and IL-18, which are released by monocytes and infected cells, and are also activated by IL-2 derived from T cells [15]. The efficiency of NK cell cytotoxicity relies on “priming” by stimulation with cytokines, such as those aforementioned, as certain activating receptors are not fully competent in triggering killing until after NK cell stimulation [16]. Cytokines can also serve to downregulate NK cell responses rather than promote them. Transforming growth factor (TGF)-β is a key immunosuppressive cytokine that prevents self-reactivity by dampening the response of immune cells such as NK cells [17]. Interferon γ (IFNγ) is the prototype effector cytokine produced by NK cells and is essential in anti-tumour responses, as it has pleiotropic effector actions on other immune cells as well as target cells [18]. The important role of IFNγ in tumour clearance has been shown in experiments in mice implanted with IFNγ-insensitive sarcoma cells, which displayed increased tumourigenicity in comparison with control cells [19]. Tumour necrosis Factor α (TNFα), another essential cytokine released by NK cells, is a multifunctional cytokine that plays a key role in apoptosis, inflammation and immunity. In experiments carried out on TNFα-deficient mice, NK cells displayed impaired cytotoxic functions, indicating that it is an essential factor in both controlling NK cell cytotoxicity and tumour rejection *in vivo* [20]. It has been shown that the release of IFNγ and TNFα synergistically enhances the cytolytic function of NK cells and are an integral part of NK effector machinery [21]. NK cells possess receptors for chemokines such as CXCR3 and CX3CR1 that allow

them to extravasate through vessels and migrate through tumour tissue [22]. NK cells also secrete chemokines such as macrophage inflammatory protein (MIP)-1 α or CCL3, MIP-1 β (CCL4), and RANTES that promote recruitment of both self and other immune cells to the target site [23].

1.2.2 NK cell-mediated immunosurveillance.

Immunosurveillance involves the surveying of host tissues for developing transformed or infected cells. NK cells mediate immunosurveillance by identifying the expression of the major histocompatibility complex (MHC) Class I molecules and stress markers on the surface of cells [24]. NK cells perform immunosurveillance via receptors that generate an inhibitory or activating signal. Notable human inhibitory receptors include killer cell immunoglobulin-like receptors (KIR), immunoglobulin-like transcripts (ILT), and NKG2A, whilst activating NK cell receptors include NKG2D, the natural cytotoxicity receptors (NCRs), and DNAM-1 [25]. NKG2D, a member of the NKG2 family, is one of the better characterised activating NK receptors and interacts with MHC class I chain-related proteins (MIC) A and B, which are induced upon stress, damage, or upon the transformation of cells [26]. Originally, it was believed that NK cells could differentiate between healthy and aberrant cells solely through the identification of MHC class I molecules, by virtue of inhibitory receptors that prevent the NK cell from attacking the target cell. Human leukocyte antigen (HLA) class I is the identified MHC class I in humans [27]. This “missing self” hypothesis, proposed in 1986 by Kärre and colleagues, is indeed partly true, as reduced MHC class I expression is a prominent characteristic of abnormal cells. However, it was shown that NK cells could still eliminate cells while their inhibitory receptors were associated with MHC class I molecules. A study performed in mice with induced tumour cells displaying a normal MHC class I expression, illustrated that NK cells could still reject the tumour cells while their inhibitory receptors were ligated [28]. Moreover, a study on *Drosophila* cells with cell surface ligands for NK cell activating receptors did not stimulate natural cytotoxicity unless there was collective engagement of a number of these activating receptors. This co-engagement of activating receptors produced a signal necessary to surpass the inhibitory signal from HLA-E association with its respective inhibitory receptor

[29]. Thus, it is the combination of integrated activating and inhibitory signals that determines whether the NK cell will exert its effector functions, with inhibitory signals serving to diminish, rather than completely abort, NK cell effector function [30].

1.2.3 NK cell-mediated cytotoxicity.

The effector functions of NK cells are enabled after the cell becomes stimulated by an adequate activation signal. NK cells contain cytotoxic granules that package factors, namely perforin and granzymes, which are released during a process called degranulation and induce a range of cell-death pathways that kill target cells. Granzymes are serine proteases that cause cell death by triggering the activation of cellular cysteine proteases called caspases that orchestrate apoptosis. There are 5 granzymes identified in humans, including granzyme A, H, K, and M, with the most commonly studied, in relation to cytotoxicity, being Granzyme B. Perforin enables granzyme B-mediated apoptosis by disrupting the target cell membrane, allowing granzyme entry [31]. Other effector molecules released from granules include granulysin, which triggers endonuclease activity [32, 33]. Furthermore, ADCC is an effective mechanism used by NK cells to eliminate target cells. The expression of the Fc receptor, CD16, on NK cells links the humoral and cellular branches of the immune system [34]. Antibodies such as IgG opsonise target cells, and their Fc portions are free to engage with CD16, thus mediating the interaction between NK cells and their targets. The signal transduced by NK cells upon CD16 ligation triggers cytokine release, degranulation, and the lysis of the target cell [31, 35]. For NK cells to degranulate efficiently and direct cytolytic contents towards a target cell, NK cells must first form an immunological synapse with the target cell. This is mediated by receptor-ligand ligation, followed by firmer adhesion by receptor-ligand interactions of higher affinity, and eventually interaction with more potent activating receptors, such as those belonging to the NCR family. When the inhibitory and activating signals are integrated in the NK cell and the inhibitory signals are overridden, the synapse can reorganise, forming a cleft into which cytolytic molecules are secreted [36]. NK cells also express ligands such as tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and the Fas ligand (FASL), which bind

death receptors 4 and 5 and the Fas receptor, respectively. This leads to direct apoptosis of target cells [37, 38] (Figure 1.1).

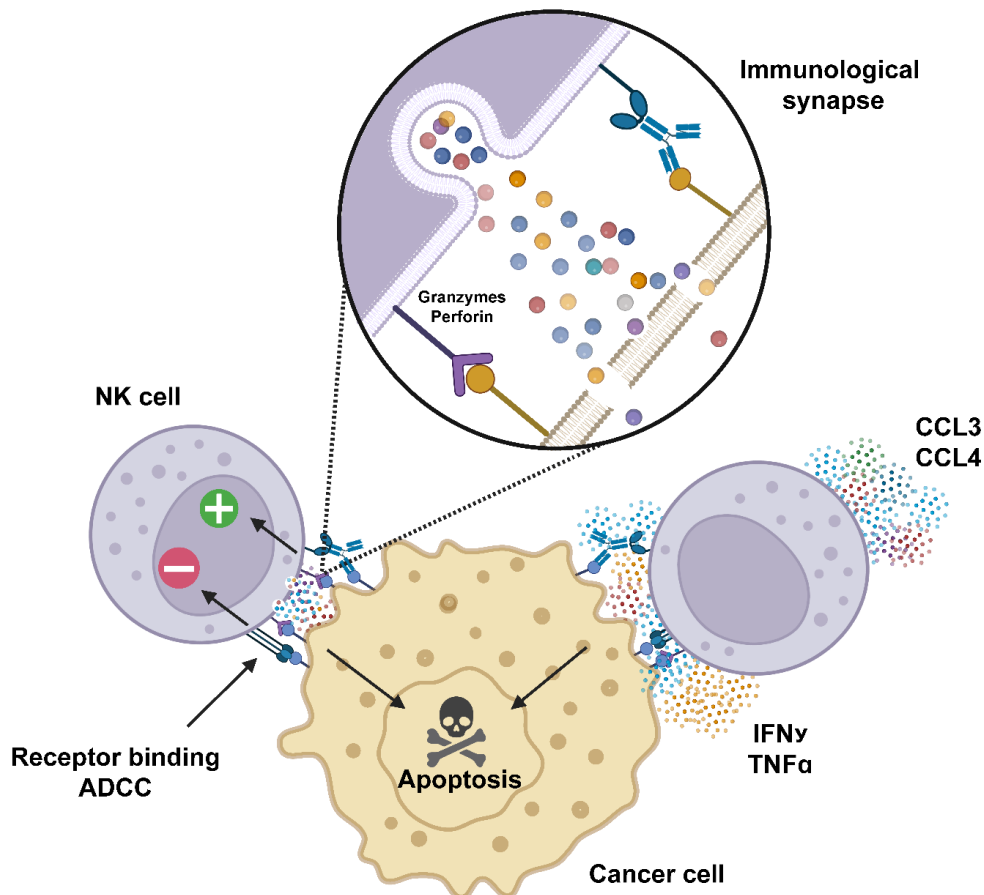


Figure 1.1. NK cell-mediated cytotoxicity.

Illustration of NK cell mechanisms of cytotoxicity. NK cells recognise infected or transformed cells via immunosurveillance using activating receptors or CD16 (ADCC) that induce an activating signal, which is balanced against inhibitory signals. NK cells directly induce apoptosis in target cells via death ligands or form an immunological synapse with the tumour cells, where they localise cytolytic granules laden with granzymes and perforin. Upon degranulation, NK cells release their granule contents into the immunological synapse, which contact, enter and induce apoptosis in the cancer cell. NK cells also produce the cytokines TNF α and IFN γ to promote inflammation and tumour killing, and chemokines such as CCL3 and CCL4 to recruit other cells to the site. Illustration made using Biorender.

1.3 Immunometabolism.

Immunometabolism refers to the metabolic pathways and events that sustain immune function and support the mechanisms by which the immune system protects the host from disease [39]. Immune cells with different effector functions use numerous metabolic pathways to produce adequate energy stores that support cell survival and generate intermediates allowing for growth and proliferation. These different metabolic pathways are linked closely due to shared fuel inputs and by the products of one pathway feeding into another, acting as biosynthetic precursors. Specific augmentations in these metabolic pathways couple to immune cell function and allow for dynamic adaptations in immune cell activity [39].

1.3.1 Glycolysis.

Glycolysis is the glucose-utilising metabolic pathway that takes place in the cytosol and involves 10 enzymatic reactions. The first reaction breaks down exogenously derived glucose via hexokinase (HK) to form glucose-6-phosphate (G6P), and subsequent reactions involving phosphoglucose isomerase (GPI), phosphofructokinase (PFK), aldolase (ALDO), and triose phosphate isomerase (TPI) result in the formation of 2 glyceraldehyde 3-phosphate molecules [40, 41]. This portion of glycolysis requires energy in the form of adenosine triphosphate (ATP), the energetic currency of the cell, resulting in energy investment to form glyceraldehyde 3-phosphate (2) from glucose [42]. Subsequently, 2 molecules of 1,3-bisphosphoglycerate are formed via glyceraldehyde 3-phosphate dehydrogenase (GAPDH), producing 2 reduced nicotinamide adenine dinucleotide (NADH) molecules, which can be used for energy production under aerobic conditions. The reactions catalysed by phosphoglycerate kinase (PGK1) and pyruvate kinase (PK) produce 4 ATP molecules, with intermediary reactions carried out by phosphoglycerate mutase (PGAM) and enolase (ENO), neither spending nor earning ATP [41, 43, 44]. Ultimately, glycolysis nets 2 ATP, biosynthetic precursors, NADH, and pyruvate. Under aerobic conditions, pyruvate is transported into the mitochondria, where it is oxidatively decarboxylated by the pyruvate dehydrogenase (PDH) complex (PDC) to form acetyl-coenzyme A (Acetyl-CoA). Alternatively, under anaerobic or hypoxic

conditions, pyruvate remains in the cytosol and is reduced to lactate by lactate dehydrogenase (LDH), a reaction that regenerates NAD^+ to sustain glycolytic flux [45].

Glycolysis is the most studied metabolic pathway in immune cells, having been investigated across multiple immune cell types [46-50]. The glycolysis pathway starts with the uptake of glucose into the cell through glucose transporters such as the solute carrier (SLC) 2A family, or the GLUT family of glucose transporters, the most studied of which is SLC2A1-4 (GLUT1-4) [51]. Once inside the cell, glucose can be broken down under several conditions, mainly through aerobic or anaerobic glycolysis, which occur in the presence or absence of oxygen, respectively [52]. Aerobic glycolysis is used by lymphocytes such as T cells and NK cells, which demand precursors needed for growth and development, with glycolytic intermediates acting as valuable sources of carbon that feed a range of other pathways, resulting in nucleotide, lipid, and amino acid synthesis [53]. Glycolysis only nets 2 ATP per glucose molecule; therefore, its primary purpose is to provide components that facilitate proliferation and the generation of effector molecules [54, 55]. Immune cells have evolved to become highly sensitive to environmental glucose availability, with elements such as the kinase mammalian target of rapamycin complex 1 (mTORC1) and the transcription factor cytosolic myelocytomatosis oncogene (Myc) acting in synergy to induce glycolytic gene expression upon immune activation [56-58]. Alternatively, in low glucose environments unsuitable for immune cell anabolism or functional activity, mTORC1 and downstream events such as c-Myc activation are inhibited. This is likely to be mediated by adenosine monophosphate (AMP) kinase (AMPK), an energy sensor that regulates mTORC1 through detection of increases in AMP:ATP ratios that result from energy depletion under glucose shortages [59]. Myc is also an essential regulator of human NK cell anabolism, murine NK cell immunosurveillance, and amino acid-dependent functional responses [60-62]. The transcription factor hypoxia inducible factor 1- α (HIF1 α) also has pro-glycolytic roles in immune cells, promoting glycolytic reprogramming in lymphocytes such as T cells and NK cells, and is sensitive to immune-activating stimuli such as IL-2, which is produced during the response to infection. HIF1 α is also an essential element of metabolic adaptivity in NK cells during viral

infections, with its knockout resulting in increased morbidity in mice [63-65]. Glycolysis also results in the formation of G6P and pyruvate, which feed the pentose phosphate pathway (PPP) and mitochondrial metabolism, respectively [39].

1.3.2 The pentose phosphate pathway.

The PPP is an auxiliary pathway that uses the glycolytic product, G6P, to produce factors used for oxidation-reduction (redox) reactions and nucleotide synthesis [66]. The PPP consists of 2 stages, an oxidative stage and a non-oxidative stage. The oxidative stage occurs first and is initiated by the conversion of G6P to 6-phosphogluconolactone via glucose 6-phosphate dehydrogenase (G6PD). This reaction uses nicotinamide adenine dinucleotide phosphate (NADP^+) and results in the formation of NADPH, the reduced form of NADP^+ . Subsequent enzymatic reactions by hexose 6-phosphate dehydrogenase (H6PD) and 6-phosphogluconate dehydrogenase (PGD) result in the formation of ribulose-5-phosphate and another NADPH molecule [67]. The PPP is a major source of NADPH, which is a powerful reductive agent that is used for redox reactions as well as fatty acid synthesis [67]. Ribulose-5-phosphate is then converted to ribose-5-phosphate (R5P), which feeds nucleotide synthesis or further non-oxidative PPP metabolism by transketolase (TKT) and transaldolase 1 (TALDO1), which can feed intermediates back into glycolysis [68] (Figure 1.2). The PPP has been well described in terms of cancer metabolism, where oncogenic regulation of the PPP supports rapid cell proliferation through nucleic acid, fatty acid, and NADPH synthesis. Oncogenes such as rat sarcoma (RAS) transcription factor drive non-oxidative PPP in cancer cells for nucleotide biosynthesis, as well as upstream hexokinase 2 (HK2) expression in order to maintain G6P flux into the PPP [69, 70]. Nuclear factor erythroid 2-related factor 2 (NRF2) is a redox-sensitive transcription factor that activates oxidative and non-oxidative PPP, and its activation has also been observed in glioblastoma and lung cancer [71, 72]. Oxidative PPP can also be activated by mTORC1 through a set of metabolic-regulating transcription factors, including HIF1 α and sterol-regulatory-element binding proteins (SREBP) [73]. Thus, the PPP serves mostly as a supporting pathway of cell anabolism, proliferation, and redox control. The

PPP remains ill-defined regarding immune cells compared to other metabolic pathways, but some progress has been made recently in T cell studies. Non-oxidative PPP has been shown to act as a link between metabolism and epigenetic control in regulatory T cells, and in CD8⁺ memory T cells, glycogen drives the PPP through glycogen-derived glucose-1-phosphate interaction with G6PD [74, 75]. Furthermore, in macrophages stimulated with lipopolysaccharide (LPS), PPP production of NADPH was essential for generating oxidative burst via NADPH oxidase, which is crucial for driving macrophage responses [76].

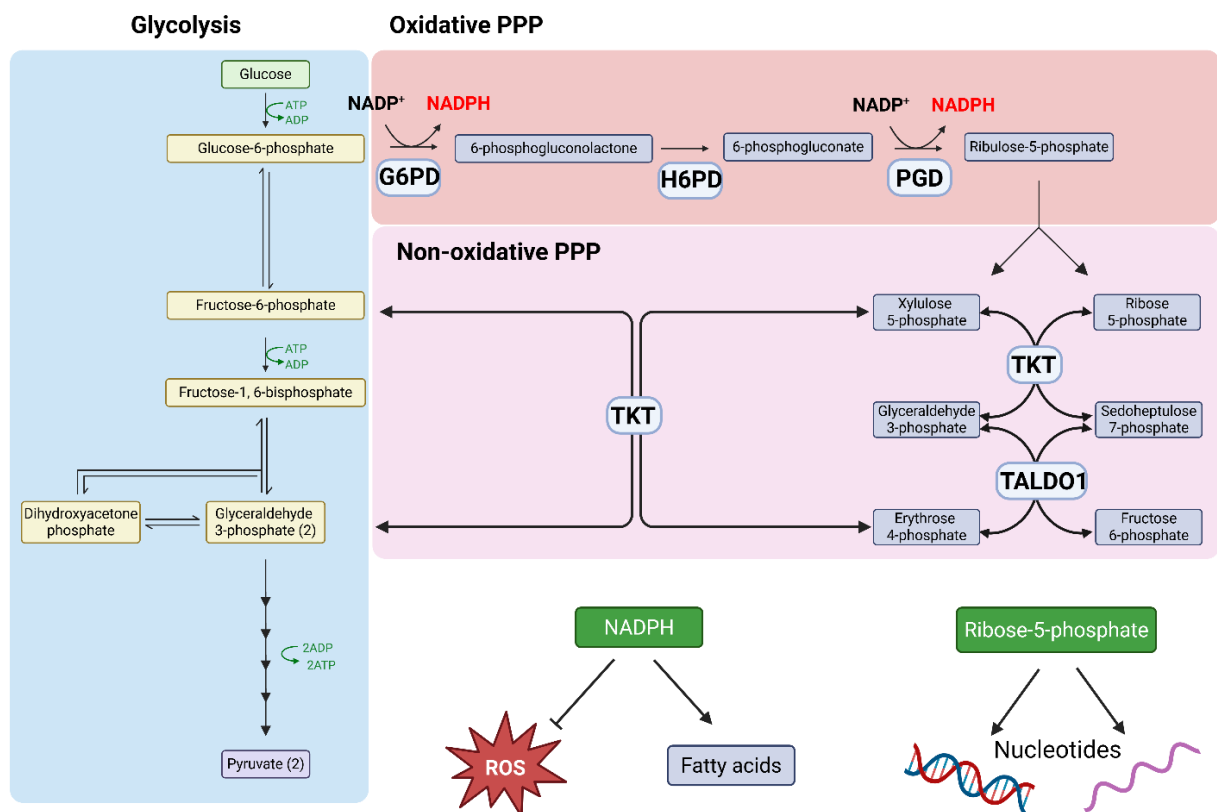


Figure 1.2. Glycolysis and the pentose phosphate pathway.

Glycolysis involves the breakdown of glucose, producing biosynthetic precursors and net 2 ATP molecules. The PPP siphons glucose-6-phosphate from glycolysis and consists of an oxidative and non-oxidative stage. Oxidative PPP results in NADPH formation, which can be used for redox reactions and fatty acid synthesis. Ribose-5-phosphate (R5P) feeds the non-oxidative stage of the PPP, which recycles intermediates back to glycolysis and/or supports nucleotide synthesis. Illustration made using Biorender.

R5P made from the PPP is used for the synthesis of 5-phosphoribosyl-1-pyrophosphate (PRPP), which is catalysed by PRPP synthase (PRPS) [77]. Further synthesis of 5-phosphoribosylamine from both PRPP and glutamine is the committed step for purine synthesis, and further reactions culminate in inosine monophosphate (IMP) generation. IMP can then be used for the synthesis of guanosine monophosphate (GMP) through IMP dehydrogenase (IMPDH) and GMP synthase or for the synthesis of AMP via adenylosuccinate synthase and lyase. AMP can be reversibly metabolised back to IMP using AMP deaminase. 5'nucleotidases (5'NT) then carry out the reversible conversion of these monophosphate nucleosides to their respective nucleosides, adenosine, inosine, and guanosine [78-80]. Purine nucleoside phosphorylase (PNP) subsequently catalyses the conversion of inosine and guanosine to their respective bases, hypoxanthine and guanine, liberating the sugar, ribose-1-phosphate, which can be recycled for upstream reactions [81]. A purine salvage pathway also exists, mediated by enzymes such as hypoxanthine phosphoribosyltransferase 1 (HPRT1), adenine phosphoribosyltransferase (APRT1) and adenosine kinase (ADK), and allows for more efficient nucleotide synthesis, using less ATP than de novo synthesis [82, 83] (Figure 1.3). These nucleotide-associated pathways have been recently discovered to have important roles in immune cell function, such as in activated macrophage metabolic reprogramming and in acting as an alternative carbon source for T cells under glucose deprivation [84, 85].

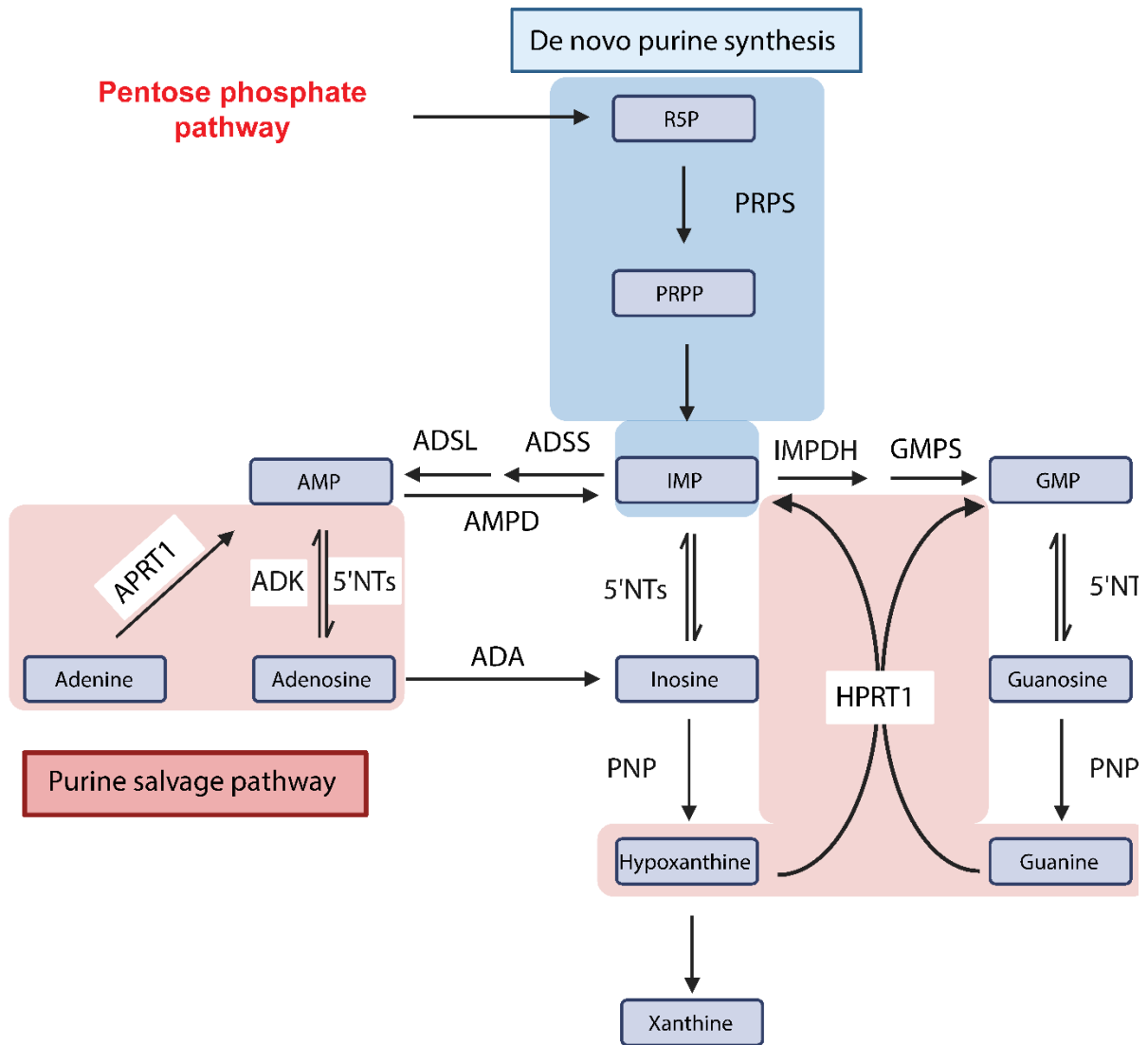


Figure 1.3. Purine biosynthesis and salvage.

Illustration of de novo purine synthesis (shaded blue) and the purine salvage pathway (shaded red). The PPP forms R5P, which enters the de novo purine synthesis pathway. De novo synthesis involves the synthesis of IMP from PRPP and the subsequent formation of AMP and GMP monophosphate nucleosides. Hypoxanthine and guanine purine bases are then synthesised from further 5'NT and PNP activity. Purine salvage is mediated by HPRT1, ADK, and APRT1 and recycles purine bases back to nucleoside monophosphates, which can be reused in a more ATP-efficient manner. Illustration made using Biorender.

1.3.3 Mitochondria and cell metabolism.

Mitochondria are double-membraned cell organelles specialised for ATP production through a process called oxidative phosphorylation (OXPHOS). Peter Mitchell first defined chemiosmotic theory, which was a form of energy storage involving a proton gradient generated across the mitochondrial membrane that is harnessed in the form of proton motive force, which drives mitochondrial machinery in the inner mitochondrial membrane (IMM) for ATP production [86]. This proton gradient is generated by oxidative reactions carried out by the electron transport chain (ETC), which is a group of IMM-located enzymes that use NADH and flavin adenine dinucleotide (FADH_2) as electron donors [87, 88]. The tricarboxylic acid (TCA) cycle is a mitochondrial engine that is defined by a series of reactions in a closed loop that is fed by multiple metabolic pathways. Acetyl-CoA from the metabolism of pyruvate or fatty acids forms citrate with oxaloacetate (OAA) in the initial step, followed by isocitrate formation and subsequent α -ketoglutarate (α -KG) synthesis. These initial steps, followed by conversion of α -KG into succinyl-CoA, result in NADH (2) generation and subsequent succinate and fumarate formation, which produce guanosine triphosphate and FADH_2 . Succinate-coenzyme Q reductase, also known as succinate dehydrogenase (SDH), which carries out succinate to fumarate conversion, is also found within the ETC. Fumarate is then converted to malate and eventually back to OAA, which produces NADH and restarts the cycle [89-91]. NADH and FADH_2 are oxidised by NADH dehydrogenase (Complex I) and SDH (Complex II), respectively, and electrons are transferred to cytochrome c reductase (complex III) by the electron carrier coenzyme Q. Electrons are then shuttled to cytochrome c oxidase (complex IV) by cytochrome c, resulting in electron transport back into the mitochondrial matrix where they are used to reduce oxygen to water. This electron transfer results in proton pumping from the mitochondrial matrix through complexes I, III, and IV, generating the proton gradient in the intermembrane space, which is harnessed by the fifth and final complex (ATP synthase), which generates ATP from adenosine diphosphate (ADP) in the matrix. The proton gradient is defined by a difference in proton concentration across mitochondrial membranes, which also generates a difference in electrical charge, known as mitochondrial membrane potential. Together, this gives rise to proton motive force [86, 88, 92, 93] (Figure 1.4). It is

important to note, murine NK cells demonstrated the ability to use a TCA cycle utilising pathway called the citrate-malate shuttle (CMS). Citrate is exported to the cytosol from the mitochondria, where it is broken down into Acetyl-CoA and OAA by ATP citrate lyase (ACLY). OAA is converted back to malate, which is then imported back into the mitochondria, where it enters the TCA cycle [94].

NK cells rely on fully functional or “fit” mitochondria for survival and efficient effector responses [95]. Glycolysis and OXPHOS were shown to be essential for NKG2D and ADCC-mediated cell-cell cytotoxicity, with OXPHOS inhibition decreasing cytokine production [96]. Furthermore, TGF β produced by metastatic breast cancer cells drives NK cell exhaustion through the generation of dysfunctional mitochondria, resulting in abrogated effector functions [97]. Immunosenescence, or the weakening of the immune system due to ageing in humans, may also be driven by mitochondrial dysfunction in NK cells, with impaired NK cells from elderly people showing an inability to undergo mitochondrial biogenesis and generate membrane potential after IL-2 stimulation [98]. NK cell interaction with leukemia cells resulted in mitochondrial localisation at the immunological synapse, and ATP synthase inhibition decreased cytotoxicity, suggesting that NK cell-mediated killing is reliant on mitochondrial reorganisation and ATP synthesis [99]. Increased levels of mitochondrial reactive oxygen species (ROS) are associated with mitochondrial dysfunction [100]. NK cells rely on the peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC1 α), which manages mitochondrial ROS, for enhanced mitochondrial mass biogenesis and membrane potential generation. Inhibition of PGC1 α results in downregulated IFN γ expression following stimulation of NK cells with IL-2 [98, 101].

Mitochondria are also dynamic organelles, meaning they can undergo various changes in their shape and distribution, known as mitochondrial dynamics. Mitochondrial cristae are bioenergetic compartments whose structure and shape have a direct impact on metabolism, with condensed cristae being associated with increased ATP production [102, 103]. Mitochondrial fission and fusion frequently occur and drive changes in the shape and function of mitochondria. Mitochondrial fission is mediated by proteins such as dynamin-related/-like protein 1 (DRP1) and fission, mitochondrial 1 (FIS1), while fusion is commonly

mediated by mitofusins (MFN) and optic atrophy 1 (OPA1) proteins [104-106]. Mitochondrial fusion is characterised by a long, tubular mitochondrial phenotype and is associated with healthy NK cells, which have increased mitochondrial mass, enhanced OXPHOS, long-term persistence, and enhanced cytokine production [95, 97, 107]. Thus, it is clear that mitochondrial function, organisation and fitness is essential for NK cell persistence and their associated effector functions.

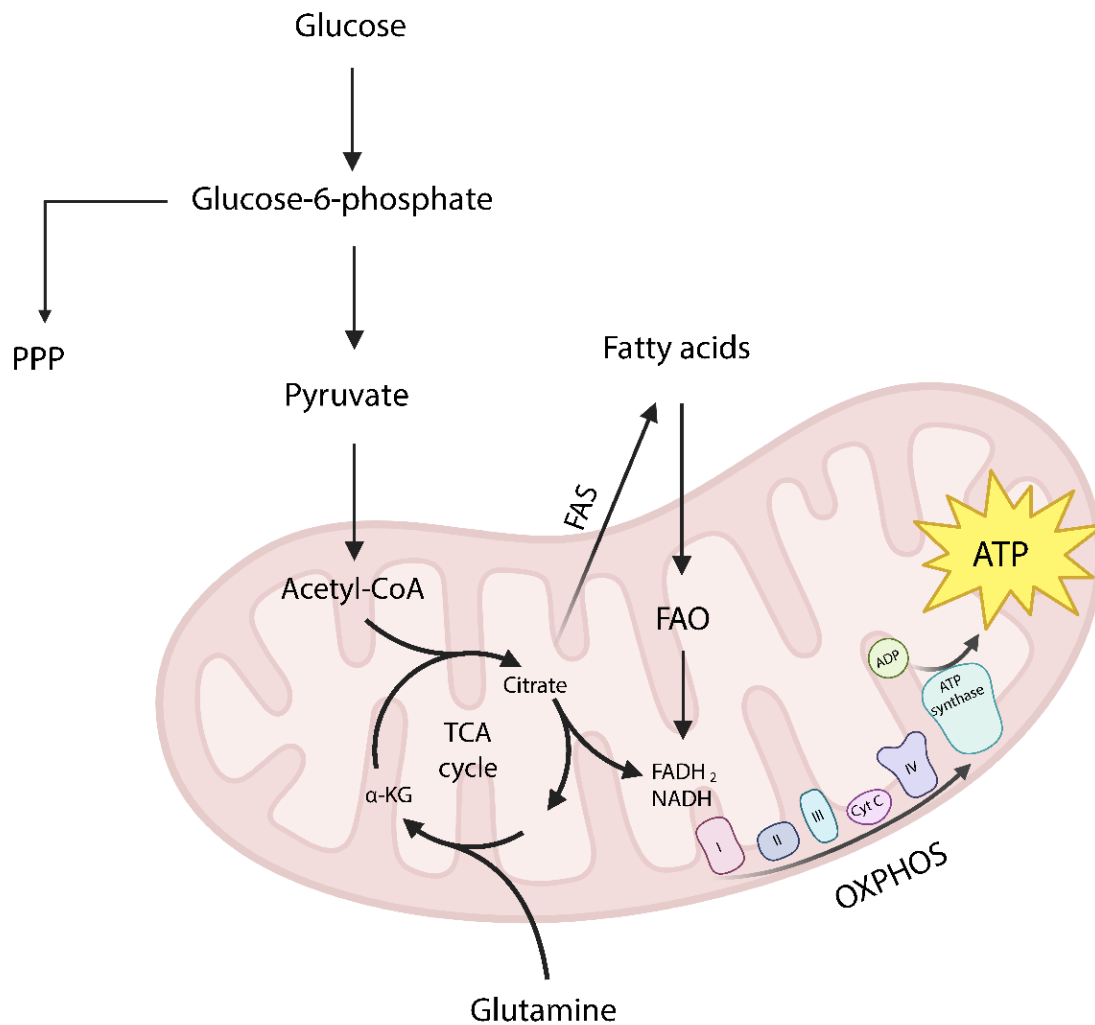


Figure 1.4. Mitochondrial metabolism and NK cells.

Illustration of mitochondrial metabolic pathways. Nutrients such as glucose-derived pyruvate, fatty acids, and amino acids like glutamine can feed the TCA cycle, producing $NADH$ and $FADH_2$, which are used as electron donors by the ETC. The ETC generates a proton gradient in the IMM, which is harnessed for ATP synthase-driven formation of ATP from ADP. TCA intermediates can also be used in the synthesis of fatty acids. Illustration made using BioRender.

1.3.4 Redox balance and redox reactions.

Highly reactive chemicals, such as ROS and reactive nitrogen species, have important roles in regulating immune cell survival and function [108-110]. However, the inability to control ROS production in cells outside of manageable physiological conditions may lead to oxidative stress, perturbed immune function, and cell death [111-114]. ROS come from many sources and include non-radical

hydrogen peroxide (H_2O_2) and free radicals such as superoxide anions, hydroxyl radical, lipid radicals, and nitric oxide [115, 116]. ROS accumulation in cells can be driven by both intracellular and extracellular factors. Intracellular ROS is driven mostly by mitochondrial dysfunction, NADPH oxidases, the endoplasmic reticulum (ER), peroxidases, cyclooxygenases, and xanthine oxidase [117]. Mitochondrial dysfunction is largely appreciated as the main source of total cellular ROS in cells [118-120]. The ETC performs aerobic respiration in mitochondria and is responsible for the huge production of superoxide anions, particularly by complex I [121, 122]. Interestingly, calcium ions (Ca^{2+}) have been extensively reported to induce mitochondrial ROS accumulation through the induction of ETC activity [123]. Increases in mitochondrial ROS lead to decreases in ATP production and a subsequent feedback leading to further ROS production and mitochondrial damage [124].

The mitochondrial permeability transition pore (mPTP) regulates the transport of Ca^{2+} , ROS and other signaling molecules from the mitochondria to the cytosol [125]. Short-term opening of mPTPs is thought to protect the mitochondria from oxidative stress; however, long-term opening from prolonged oxidative stress exposure can lead to ATP depletion, inner mitochondrial membrane (IMM) permeabilisation, resulting in proton leakage, membrane potential collapse, and cell death [126, 127]. Thus, mitochondrial dysfunction can generate large amounts of ROS capable of leaking into the cytosol and inducing cell death (Figure 1.5). Indeed, oxidative stress in NK cells may be detrimental to their cell survival and anti-cancer functions. For example, kynurenine, which is a cancer metabolite, was shown to induce ROS-mediated apoptosis in NK-92 cells, while NADPH oxidases were proposed to downregulate NK cell activity in murine melanoma models, leading to enhanced metastasis [114, 128].

However, immune cells have developed multiple methods to combat ROS-induced events. As described earlier, one of the products of the PPP is NADPH, a powerful reducing factor with roles in redox control. NAD kinase (NADK) phosphorylates NAD^+ to NADP^+ which can then be converted to NADPH in reactions such as those in the PPP [129]. Two isoforms of NADK exist, NADK, which resides in the cytosol, and NADK2, which is retained within the mitochondria, offering the ability of cells to produce compartmentalised NADP^+

[130, 131]. Similarly, superoxide dismutase (SOD) isoforms are compartmentalised in the cytosol (SOD1) and in the mitochondria (SOD2) and are essential in controlling ROS-induced damage [132, 133]. Thioredoxin 1 (TXN) is another essential antioxidative enzyme that has been extensively studied in immune cells and is associated with improved NK and T cell tolerance to oxidative stress, as well as enhanced proliferation and anti-tumour capabilities [134-138]. Other enzymes, such as catalase (CAT) and peroxiredoxins (PRDXs), have been extensively studied for their antioxidant effects; however, the most important antioxidant molecule in immune cells is glutathione [139-141]. Glutathione is formed in the cytosol from glutamate and cysteine to initially make γ -glutamylcysteine via glutamate cysteine ligase, followed by glycine addition by glutathione synthetase (GSS) [142]. Glutathione can also exert its effects in the mitochondria through mitochondrial glutathione importers such as SLC25A39 [143]. Glutathione has been extensively studied in various adaptive and innate immune cells, with roles in antioxidant regulation, immune cell priming and crosstalk, anti-tumour defence, and even in β -glucan-induced training [144-147]. Furthermore, disrupted glutathione metabolism has recently been shown to drive redox imbalance in NK cells from leukemia patients, with glutathione supplementation rescuing dysregulated NK cell disease-associated mitochondrial metabolism [148].

One key regulator of antioxidant defense is the transcription factor NRF2, whose activity is triggered by redox imbalance. The antioxidant response element (ARE) is in the promoter region of many antioxidant defense genes and is targeted by NRF2 for transcription of cytoprotective proteins under conditions of oxidative stress. NRF2-regulated genes such as NAD(P)H:quinone oxidoreductase1 (NQO1), the iron-responsive ferritin light (FTL) and heavy (FTH) chains, and heme oxygenase-1 (HO)-1 are all involved in immune cell cytoprotection and anti-inflammatory responses [149-153]. Activation of NRF2 has been shown to be effective at improving NK cell antitumour activity and effector functions during viral infection [154, 155]. Furthermore, NRF2 has been demonstrated to induce translation of PPP and glutathione synthesis-associated proteins [156, 157]. It is clear there is an essential role for redox balancing factors in immunity, and the

inability to manage ROS can be detrimental to NK cell function, particularly in the context of cancer.

1.3.5 Amino acids and fatty acids in NK cell metabolism.

There are hundreds of amino acids in nature, but only 20 of them are the fundamental building blocks of proteins [158]. However, the role of amino acids stretches beyond protein synthesis, and they represent important sources of nitrogen and carbon for metabolic signalling, redox metabolism, and fueling mitochondrial metabolism [159]. As a signalling molecule, glutamine uptake through the amino acid transporter SLC7A5 was shown to be essential for Myc activation in murine NK cells, which supported functional responses [61]. Similarly, in murine NK cells, leucine uptake after IL-18 treatment was shown to activate mTORC1, while inhibition of the amino acid transporter associated protein SLC3A2 (CD98) abrogated mTORC1 activation [160]. Amino acid metabolism is central to redox homeostasis [158]. Cysteine, glutamine, and glycine are all amino acids required for glutathione synthesis, with thiol groups on the cysteine residue available for accepting and donating electrons, an important feature of redox reactions [161, 162]. Metabolic pathways such as one-carbon metabolism and glutaminolysis support glutathione formation, also through serine, glycine, and glutamate synthesis in the mitochondria [142, 163, 164]. Glutamine can also be taken up by mitochondria, feeding the TCA cycle and driving OXPHOS and ATP generation [165]. In macrophage differentiation to an anti-inflammatory phenotype (M2), glucose and galactose restriction led to an increase in glutamine-fueled TCA cycle, indicating immune cells can use amino acids for energy carrier synthesis under conditions of nutrient deficiency [166]. Similarly, β -glucan-trained monocytes rely on glutamine-fueled anaplerosis, which is supplementation of the TCA cycle, for sustaining a trained immunity program, and inhibition of mitochondrial glutaminolysis during training abrogated enhanced secondary responses [167]. In human decidual NK cells, mitochondrial metabolism was also shown to be vital for cell differentiation via glutamine-derived α -KG fed TCA cycle and subsequent OXPHOS [168].

Fatty acids have many roles in cells, including as building blocks in cell membranes, as signaling molecules, and as an energy source. Fatty acid synthesis (FAS) is essential for providing structural lipids for cell growth, cell division, and mitochondrial biogenesis and dynamics [169]. De novo fatty acid synthesis occurs from acetyl-CoA derived from glycolysis or through the CMS via ACLY conversion of citrate to cytosolic acetyl-CoA [170]. Conversely, FAO involves the breakdown of fatty acids and is an additional source of NADPH, but importantly, can act as another source of energy through NADH and FADH₂ generation and supplementation of the TCA cycle through acetyl-CoA synthesis [171] (Figure 1.4). Studies have shown that fatty acids are a necessary fuel for murine NK cells, and their uptake is essential for NK cell responses and mitochondrial function in viral infection and cancer models [172, 173]. Fatty acids have also been shown to promote the expansion of the NK-92 cell line, again through supporting energy metabolism [174, 175]. In the case of T cells, induction of FAO was demonstrated to increase CD8⁺ T cell reactivity to tumours and improve PD-1 checkpoint inhibition therapy in mice [176]. PD-1 ligation in human CD4⁺ T cells resulted in a metabolic shift from glycolysis to FAO of exogenous fatty acids as well as upregulation of OXPHOS and spare respiratory capacity, suggestive of enhanced cell survival [177]. However, increased fatty acid uptake was shown to lead to loss of function in human NK cells, while lipid uptake from ascites fluid drives metabolic dysfunction in NK cells from patients with ovarian cancer [178, 179]. Thus, fatty acids are a double-edged sword for NK cells in that they can act as a mitochondrial fuel, but their uptake can also have immunosuppressive effects, resulting in NK cell dysfunction and exhaustion.

1.3.6 Iron metabolism and NK cells.

Iron is an essential metal important for immune function and regulation. Many cellular functions, such as DNA synthesis, mitochondrial biogenesis, and ATP production, rely on or are influenced by the presence of iron [180]. The transferrin receptor, CD71, mediates the cellular uptake of transferrin-bound iron through endocytosis and is present on various immune cells, depending on their cell type and activation status [180, 181]. Ferritin functions as an iron-storing protein composed of heavy (FTH) and light (FTL) chains, which reduce free iron in the

cell to prevent it from inducing lipid peroxidation and subsequent ferroptosis (iron-mediated cell death) [182, 183] (Figure 1.5). Iron can induce ROS generation in cells through the Fenton reaction, whereby iron reacts with hydrogen peroxide to form hydroxyl radicals [184, 185]. Iron plays a critical role in macrophage polarisation. Elevated intracellular iron levels tend to promote a pro-inflammatory M1 phenotype, whereas stimulation of M2 macrophages is associated with iron release, which facilitates cellular remodelling and proliferation [186, 187]. In lymphocytes, ferritin is essential for T cell survival, and CD71 mutations have been associated with functional T cell defects culminating in combined immunodeficiency disease [188, 189]. NK cells significantly upregulate CD71 expression upon activation, and iron deprivation inhibits NK cell-mediated responses against the Friend retrovirus infection in murine models. Similarly, diet-induced iron deficiency in mice resulted in increased viral replication, with iron supplementation rescuing NK cell dysfunction and decreasing viral loads [61, 190, 191]. However, iron overload is detrimental to NK cells, as shown by iron supplementation reducing NK-92 cytotoxicity towards breast cancer cells [192]. Similarly, fibroblasts associated with cancer can export labile, or free iron, into the tumour microenvironment (TME), promoting human NK cell and NK-92 iron overload, which induces ferroptosis [193]. Therefore, iron is central to NK cell survival and function but can be detrimental in disease states through induction of oxidative stress and cell death via ROS-mediated lipid peroxidation.

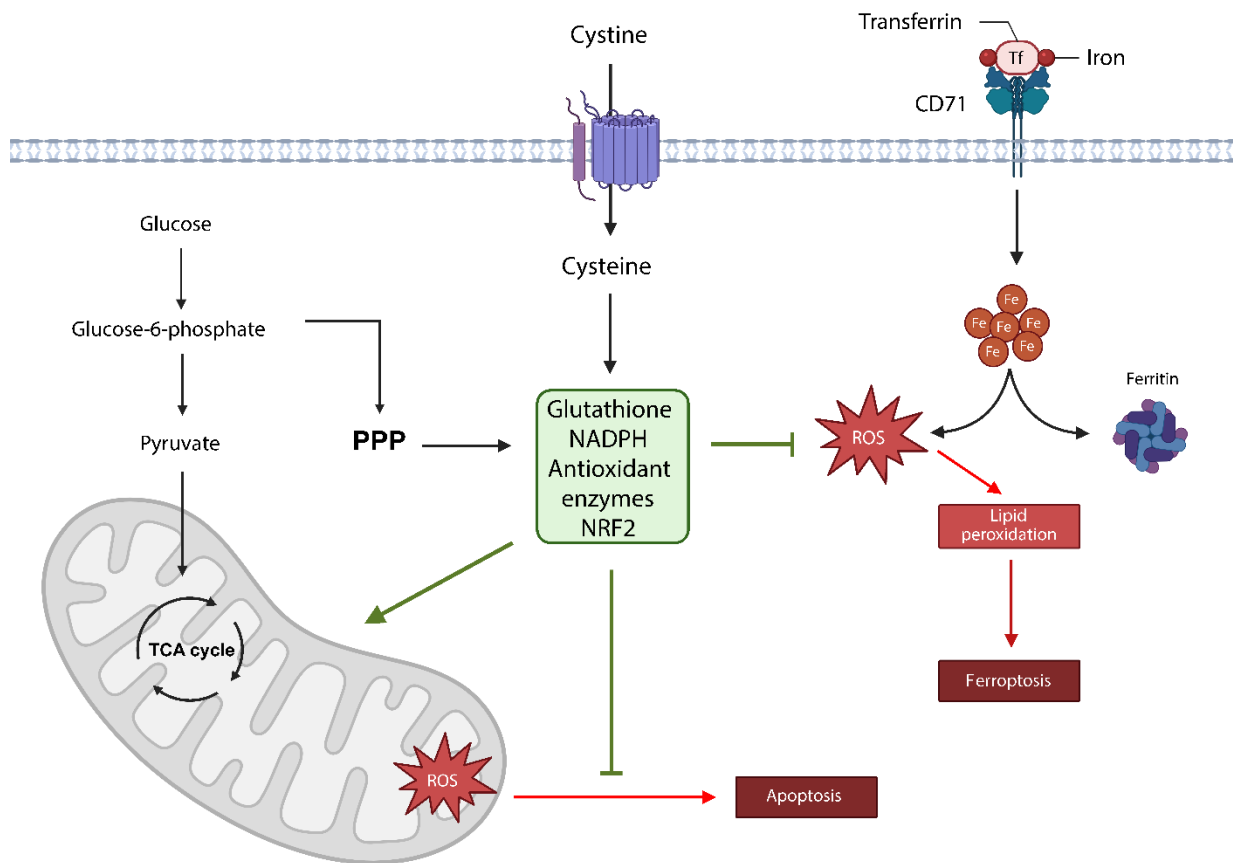


Figure 1.5. ROS sources and management.

Illustration of cellular sources of ROS and mechanisms of management. Transferrin-bound uptake of iron can generate labile iron that can lead to ROS, which induces lipid peroxidation and ferroptosis. Mitochondrial ROS accumulation can lead to events that induce cell dysfunction and apoptosis. These events are mitigated by ferritin activity, glutathione synthesis supported by cysteine and other amino acid uptake/synthesis, NADPH production by pathways like the PPP, antioxidant enzyme activity, and ARE binding transcription factors like NRF2. Many of these factors are also involved in mitochondrial survival and protection. Illustration made using Biorender.

1.4 Trained immunity.

In addition to the innate and adaptive immune systems, an intermediary form of immunity has been identified, known as “trained immunity”, linking traits from both the innate and adaptive immune systems. Trained immunity is defined as the ability of an innate immune cell to acquire adaptive, memory-like features, where exposure to a primary challenge and subsequent return to quiescence elicits an increased functional response to a non-specific secondary challenge [194]. This effect was first identified in response to *Bacillus Calmette–Guérin* (BCG) vaccination against *Mycobacterium tuberculosis* (TB) where inoculation resulted in improved mortality rates owing to the nonspecific immunity conferred by the vaccination, independent of TB resistance [195]. Furthermore, BCG vaccination was shown to result in a heightened *ex vivo* immune response to the fungal pathogen, *Candida albicans*, elicited by monocytes in humans. Murine models consolidated that this was mediated by the innate immune system by replicating enhanced innate responses in mice with adaptive immune deficiencies [196]. This discovery opened a whole new appreciation for innate immune cells and revised the old dogma that only the adaptive wing of immunity could mount a memory response. Innate immune training has been described mostly in monocytes and macrophages, but also in NK cells, in both *in vivo* and *in vitro* settings. In order for an innate immune cell to be defined as a “trained” cell, certain criteria must be met, such as enhanced responses and long-lasting effects. However, it is epigenetic modifications and metabolic reprogramming that are core to generating and sustaining the trained immunity phenotype [194] (Figure 1.6).

1.4.1 Epigenetic modifications and trained immunity.

Epigenetics was first described by Conrad Waddington in 1942, who conceptualised how gene expression can be changed without altering the DNA sequence itself [197]. The defining premise of trained immunity is the ability of innate cells to retain information about past exposures and mount a recall response, which is mediated by imprinted epigenetic modifications. These epigenetic changes result in transcriptional modulations that drive cell physiology and do not arise from permanent genetic alterations [194]. Two key markers of

epigenetic remodelling have been well defined in trained immunity and involve acetylation of histone 3 lysine 27 (H3K27ac) at distal enhancer regions and trimethylation of histone 3 lysine 4 (H3K4me3) at promoter regions [194]. Histone acetylation is broadly associated with transcriptional competency, resulting in more accessible chromatin restructuring, while histone methylation is associated with both repressive and permissive chromatin, depending on the location and type of modification on histone residues [198-200]. Addition and removal of these modifications is elicited by families of enzymes such as histone methyltransferases (KMT), histone demethylases (KDM), histone acetyltransferases (HAT), and histone deacetylases (HDAC) [201]. Epigenetic modulation has been extensively studied in canonically trained cells, such as monocytes and macrophages trained with *Candida albicans*, and its cell wall-located β -glucan particle. These cells show H3K4 trimethylation and extensive H3K27ac marking of distal elements [196, 202]. In human NK cells, epigenetic control was shown to be important for the emergence, proliferation, and memory characteristics of an adaptive NK cell phenotype specific to human cytomegalovirus (HCMV) infection. These adaptive NK cells show enhanced recall ability owing to epigenetic remodeling of the conserved non-coding sequence (CNS) 1 at the *IFNG* locus [203]. HCMV-induced NK cells in humans epigenetically parallel adaptive immune cells like cytotoxic T cells due to their DNA methylation profile and antigen specificity, but also show trained-like qualities through non-specific cytotoxicity against cancer, likely to be driven by downstream consequences of epigenetic events after HCMV infection [204, 205].

1.4.2 Metabolic reprogramming and trained immunity.

The importance of metabolism in cellular immunity is well established; however it has also been appreciated as a core regulator of innate immune training. The process of innate immune cells undergoing metabolic changes to meet the energetic and biosynthetic demands of an epigenetically divergent trained cell is known as metabolic reprogramming. This metabolic reprogramming not only supports the energetic and anabolic demands of trained cells but can also be responsible for inducing and supporting the epigenetic changes needed for sustaining the trained cellular program [206]. In β -glucan-trained monocytes,

mTOR and HIF-1 α -regulated glycolysis was found to be essential for inducing training, and in BCG-induced monocyte training, a metabolic shift toward glycolysis was also found to be essential for enhanced secondary functions [207, 208]. Furthermore, integration of glycolysis and glutaminolysis resulted in fumarate accumulation in the TCA cycle of β -glucan-trained monocytes, which was shown to be essential for KDM5 histone demethylase inhibition, allowing for the retention of epigenetic marks like H3K4me3 [167]. Other amino acids, such as arginine, have also been implicated in metabolic reprogramming, with arginine depletion and arginase inhibition decreasing OXPHOS activity and secondary responses in β -glucan-trained monocytes [209]. OXPHOS and mitochondrial reprogramming have also been shown to be central to inducing oxidised low-density lipoprotein (oxLDL) trained macrophages, with inhibition of ATP synthase and mitochondrial-associated energy metabolism pathways preventing training [210]. This is also supported by a study demonstrating that the training of murine hematopoietic stem cells with *Mycobacterium avium* results in bone marrow-derived macrophages (BMDM) that have enhanced bacterial clearance abilities that show increased basal and maximal mitochondrial respiration [211]. Regarding NK cells, it has also been shown that HCMV adaptive NK cells have increased glycolysis and OXPHOS compared to HCMV seronegative donors, which is likely to have a role in supporting expansion and long-term survival of adaptive NK cells in humans [212].

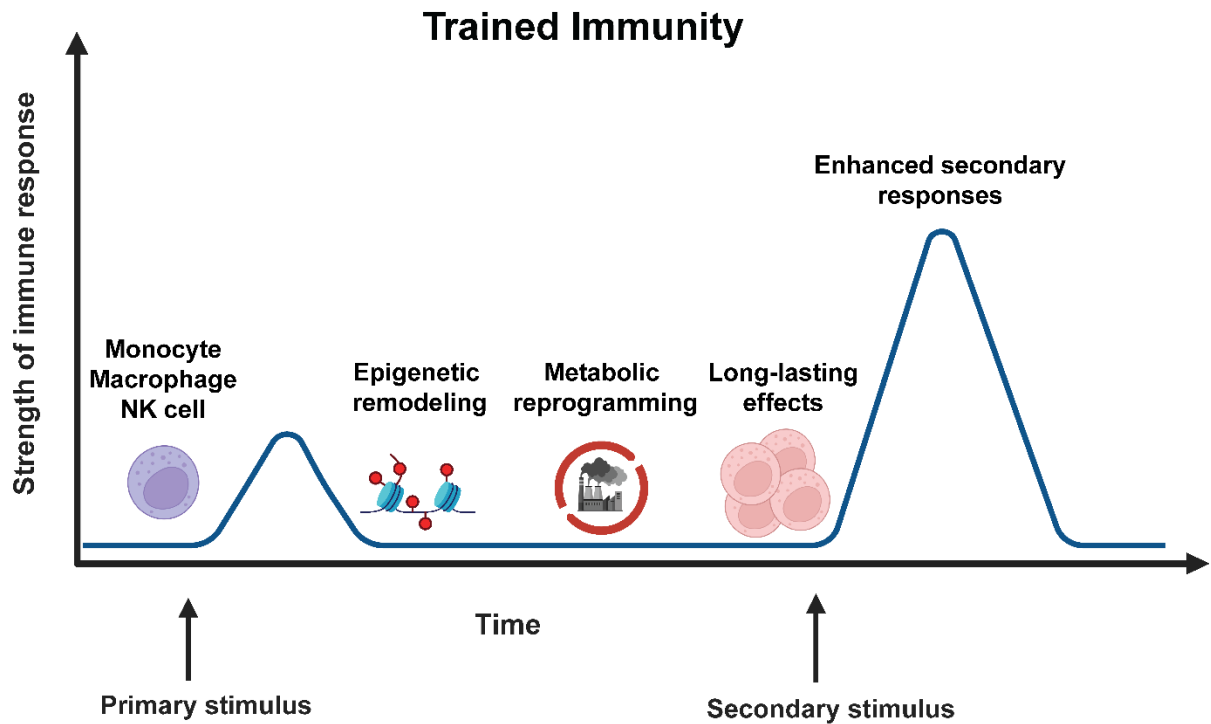


Figure 1.6. Innate immune cell training.

Illustration of the innate immune training dogma. Innate immune cells such as monocytes, macrophages and NK cells undergo an initial stimulus or exposure which results in epigenetic remodelling, metabolic reprogramming, long-lasting effects and enhanced secondary responses, the main criteria for the induction of innate immune training. Illustration made using Biorender.

1.5 Cytokine-induced memory-like NK cells.

Cytokine-induced memory-like (CIML) NK cells are a type of trained NK cell that can be generated *in vitro* from both mice and humans. Unlike adaptive NK cells that arise from viral infection *in vivo* and show specific recall responses, CIML NK cells are generated *in vitro* using a combination of cytokines, usually IL-12, IL-15, and IL-18, and display non-specific secondary responses (Figure 1.7). CIML NK cells were first defined in murine cells, where they were found to have increased capacity for IFN γ production after restimulation, and long-lasting effects that were both inheritable traits [213]. These CIML NK cells were then similarly generated from human donors, and were also associated with increased IFN γ production after restimulation and again showed an inherited ability of enhanced cytokine production in cellular progeny [214] (Figure 1.7). Human CIML NK cells have constitutively higher expression of granzyme B and cell surface markers associated with increased cytotoxicity and function, such as NKG2D, the NCRs NKp46, NKp44, and NKp30, and the high-affinity IL-2 receptor, IL2RA (CD25) [215]. This enhanced cytotoxic potential, along with their non-specific recall traits and long-lasting effects, made them interesting candidates for immunotherapy. Indeed, CIML NK cells have been investigated for their anti-cancer properties, and it was discovered that they are effective at mounting enhanced cytotoxic responses against myeloid leukemia *in vitro* when compared to untrained NK cells. Furthermore, CIML NK cells exhibited enhanced effector function against ovarian cancer *in vivo*, indicating they have cytotoxic potential against both hematological cancers and solid tumours [216, 217].

1.5.1 CIML NK cell epigenetic remodelling.

In terms of what is known regarding CIML NK cell epigenetic remodelling, the literature that exists is limited. Stimulation of NK cells with IL-12/15/18 was shown to result in the demethylation of the *IFNG* CNS1 region in both human and murine CIML NK cells, similar to that observed in HCMV-adaptive NK cells [218]. Recently, a study by Foltz et al., integrated the assay for transposase-accessible chromatin using sequencing (ATAC-seq) and single-cell transcriptomic analysis to reveal heterogeneity within the bulk CIML NK cell cohort. Within CIML NK cells exists long-lived conventional-like NK cells, a “primed” effector conventional NK

(effcNK) cell phenotype with enhanced responses but no epigenetic differences with conventional NK cells, and lastly an enriched memory-like (eML) NK cell, which showed both enhanced responses and epigenetic distinction from conventional NK cells. This demonstrates that the epigenetic landscape of CIML NK cells is indeed complex and that only the eML NK cell subpopulation is, by meeting trained immunity criteria, a bona fide trained innate immune cell [219].

1.5.2 CIML NK cell metabolic reprogramming.

There are a limited number of studies that have investigated CIML NK cell metabolic reprogramming. Upon cytokine stimulation with IL-12, IL-15, and IL-18, NK cells have increased glycolysis and upregulated OXPHOS. This increase in glycolysis is maintained and observed upon CIML NK cell generation 7 days later, as is the observed increase in mitochondrial mass and overall oxygen consumption rate, but notably, there is a lower maximal mitochondrial respiration and lower spare respiratory capacity in these cells [218, 220]. CIML NK cells also exhibit increased expression of CD71, the amino acid chaperone protein, SLC3A2 (CD98), and the glucose transporter, GLUT1 [221]. The importance of glucose in CIML NK cell metabolic reprogramming was further supported by glycolysis inhibition experiments using 2-Deoxy-D-glucose (2-DG), which demonstrated that glycolysis is essential for both induction of CIML NK cell training as well as upregulation of secondary effector responses [221, 222]. In terms of mitochondrial dynamics, CIML NK cells showed no difference in terms of mitochondrial length and cristae, suggesting they are no different from untrained NK cells in terms of mitochondrial fission or fusion [223].

CIML NK cell training and enhanced responses

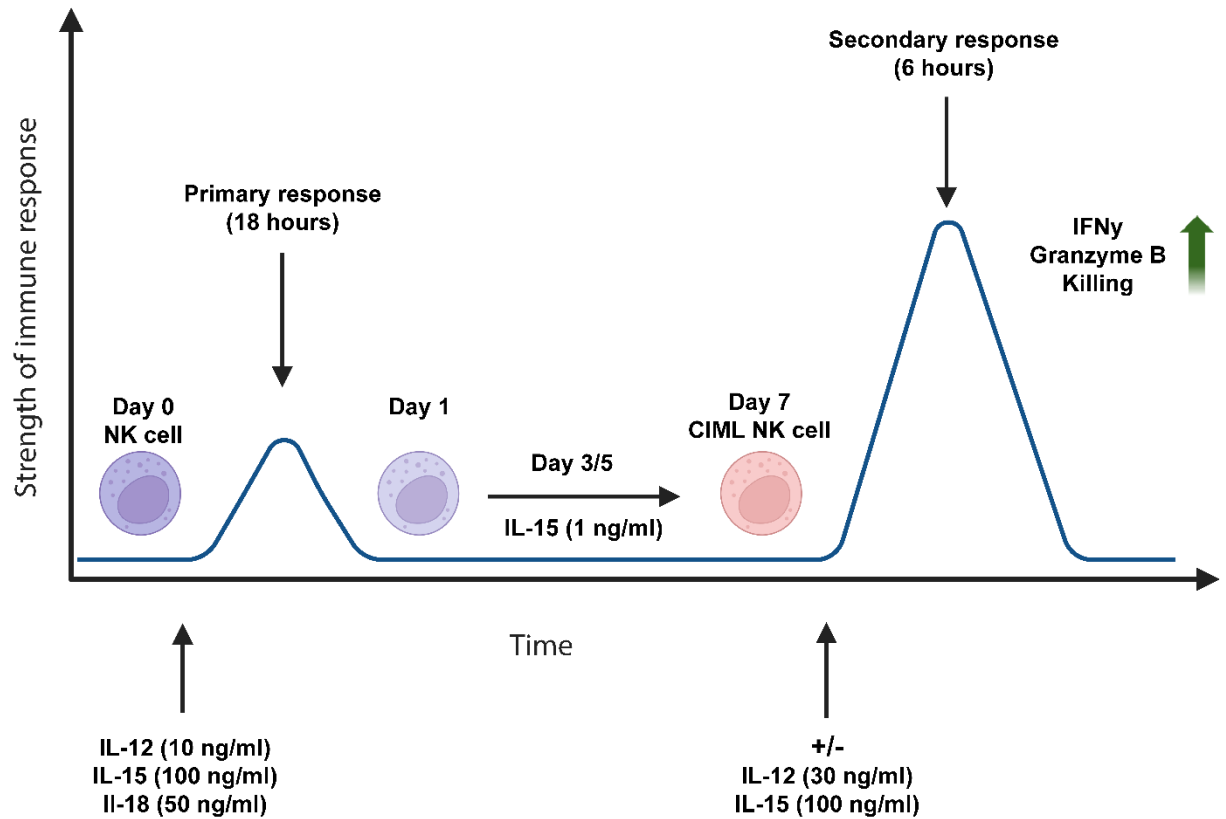


Figure 1.7. CIML NK cell training protocol and enhanced secondary functions.

Human NK cells are isolated from PBMC and stimulated for 18 hours with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml). The next day, NK cells are washed in PBS to remove the stimulus and resuspended in IL-15 (1 ng/ml) supplemented media. On days 3 and 5, the IL-15 (1 ng/ml) supplemented media is replaced. On day 7, CIML NK cells are generated and can be analysed at rest or used for further experiments, such as restimulation with IL-12 and IL-15 for measurement of increased functional responses such as IFN γ and granzyme B production. Illustration made using Biorender.

1.6 NK cells and cancer.

As described earlier, NK cells are evolutionarily equipped to detect and eliminate cancer cells, and their effectiveness has been extensively investigated *in vitro*. More importantly, however, it is in humans during cancer disease states that NK cells have been shown to be essential in mitigating progression. Patients with metastatic prostate cancer have harder to predict prognoses; however, it has been reported that patients with NK cells exhibiting high expression of NCRs have a better prognosis than those that do not [224]. In another prostate cancer study, NK cells were shown to make up a substantial portion of the total immune infiltrate into tumours, and this was associated with improved outcomes in both primary and metastatic disease [225]. Similarly, resected tumours from patients with squamous cell lung cancer that had higher numbers of tumour-infiltrating NK cells had much better survival rates than those with fewer [226]. In bladder cancer, CD56^{bright} NK cells were found to be the more prevalent tumour-infiltrating NK cells, and again, this was associated with improved prognosis in patients [227]. In patients with advanced-stage colorectal cancer, those who showed higher NK cell activity, indicated by IFN γ production, and increased circulating CD56^{bright} NK cells 30 days post-surgery, had better clinical outcomes [228]. Furthermore, peripheral NK cell counts in breast cancer patients predict response and prognosis with neoadjuvant chemotherapy. Patients with higher NK cells in the periphery had higher overall survival and relapse-free survival [229]. Patients with optimal responses to checkpoint inhibitor and chemotherapy combinations also had increased peripheral NK cell counts compared to baseline, and in non-responders with extensive-stage small lung cancer [230]. Finally, in cervical cancer, those with increased circulating NK cells in the periphery had a higher overall survival rate and efficacy of postoperative radiotherapy [231].

Although it is evident that NK cells have evolved a plethora of strategies to eliminate cancer, tumour cells can still employ strategies to evade immunosurveillance. Tumour cells have been found to upregulate FASL and display resistance to FAS-mediated apoptosis. This resistance to and upregulation of FASL promotes both tumour survival and the ability to kill immune cells, leading to the apoptosis of FAS-sensitive NK cells infiltrating the TME [232]. An absence of NKG2D ligands on chemotherapy-resistant leukemia stem cells

has been suggested as a mechanism of relapse in acute myeloid leukemia (AML) patients, as stem cells expressing NKG2D ligands were cleared by NK cells in supporting mouse models, improving survival [233]. Neuroblastoma cell lines display low to no expression of cell surface HLA molecules, which makes them ideal targets for NK cell killing; however, it has been shown that human neuroblastoma cells can effectively evade NK cells by the upregulation of the soluble MICA (sMICA) protein. Downregulated NK cell NKG2D in neuroblastoma patients can be attributed to the high levels of tumour-derived sMICA in their blood sera [234]. Overexpression of TGF β can accelerate angiogenesis, the metastasis of cancer, and dampen NK cytolytic activity by downregulating numerous NK cell activating receptors [17, 235, 236]. Tumour cells can also use platelets as an additional source of TGF β and provide steric hindrance and a physical barrier to NK cells [237, 238]. With regards to apoptosis pathways, certain cancers have been shown to upregulate decoy death receptors, which can be secreted by the tumours to bind and inhibit NK cell FASL and TRAIL [239, 240]. Anti-apoptotic proteins that are used to regulate apoptosis in normal cells are upregulated by certain tumours to compete with the pro-apoptotic signals derived from NK cell death receptor ligands [241]. Many cancer cell lines are capable of evading TRAIL and FASL-mediated apoptosis by the inactivation of caspase 8 through upregulation of these anti-apoptotic molecules [242].

In addition to these mechanisms, NK cells can be impaired by metabolic alterations induced by cancer disease states. In triple-negative breast cancers, indoleamine 2,3 dioxygenase-1 (IDO1) expression, an enzyme in the tryptophan-kynurenine metabolic pathway, resulted in increased tumour infiltration and subsequent NK cell-derived IFN γ , which triggered further IDO1 expression. IDO1 was shown *in vitro* to dampen NK cell cytotoxicity toward triple-negative breast cancer, via upregulation of the HLA-G surface molecule, associated with immune evasion [243]. Kynurenine, derived from IDO1 activity, was also shown to prevent cytokine-induced activation and upregulation of activating receptors on NK cells, again mitigating their function [244]. ATP concentrations are higher in the TME compared to other tissues, and its cleavage by ectonucleotides on lymphocytes results in the formation of adenosine, which suppresses NK cell activity through adenosinergic pathways [245-247]. Due to their highly glycolytic profile, tumour

cells are known for increased lactic acid secretion, which subsequently diminishes NK cell function through intracellular acidification and inhibition of nuclear factor of activated T cells (NFAT), a transcription factor important for NK cell activation [248, 249]. Moreover, pancreatic ductal adenocarcinoma cells can evade anti-tumour functions *in vitro* via outcompeting NK cells for vitamin B6, which is essential for glycogenolysis, supporting NK cell activity [250]. Increased fatty acid levels in the TME drive FAO, suppressing NK cell function, and lipid secretion by lung mesenchymal cells are absorbed directly by NK cells, thus promoting metastasis in breast cancer models [251-253]. Other metabolites, such as prostaglandin E2 derived from tumours impair NK cell production of chemokines such as CCL5, preventing the recruitment of other cells, such as dendritic cells, to the TME, which also promote cancer immunity [254]. Thus, it is clear that NK cells are critical mediators of anti-tumour immunity and disease progression, but cancers have evolved mechanisms of evasion targeting NK cytotoxic traits through various mechanisms that directly result in worsened disease outcomes.

1.7 NK cell-based immunotherapies.

NK cells are emerging as attractive candidates for cell immunotherapy (Figure 1.8) because of their innate ability to detect and eliminate cancer cells. There are currently many different cellular immunotherapeutic approaches using NK cells that are being investigated, such as NK cell line therapy, engineered NK cells, chimeric antigen receptor (CAR) NK cells, and cytokine-based NK cell therapies using autologous and allogeneic-derived human NK cells [255]. Other NK cell-specific strategies include antibody-based approaches such as immune checkpoint inhibitors and NK cell engager therapy that rescue cytotoxicity and function in exhausted NK cells in patients without removing the cells from the body [256, 257]. NK cells can also be generated from umbilical cord blood and particularly from induced pluripotent stem cells (iPSCs), which are easy to genetically engineer and avoid donor-dependent variability [258, 259]. NK cell lines such as NK-92 NK cells are easy to expand and do not express inhibitory KIRs but do lack CD16, and due to their malignant origin, must be irradiated before infusion [260, 261]. Genetically engineered NK cells are becoming more

relevant due to the ability to circumvent impairment of cell activity that arises from TME factors, such as transduction with a dominant-negative TGF β receptor, which blocks inhibitory signals induced by TGF β [262, 263]. Cytokine-based endogenous strategies involve pretreatment of allogeneic or autologous NK cells with cytokines such as IL-2 and IL-15 *in vitro* to promote survival and proliferation *in vivo* after administration, and CIML NK cells fall under this heading also [264-268].

1.7.1 CAR NK cell therapy.

One of the most promising NK cell-based immunotherapies is CAR NK cells, which were inspired by the revolutionary effect CAR T cells had on the field of cellular immunotherapy [269, 270]. CAR NK cells have their intrinsic anticancer functions paired with a CAR specific for a tumour antigen. The CAR is a fusion protein whose main architecture consists of a single-chain variable fragment (scFv), which binds antigens of interest, a hinge domain that allows for flexibility in CAR length needed for varying distances of antigen epitopes on target cells from the CAR, costimulatory domains, a transmembrane domain, and intracellular signaling domains, which propagate the activation signal induced upon CAR-antigen binding [271]. NK cells are commonly transduced with CAR-encoding genes using viral vectors, but NK cell transduction has proven much harder than in T cells [272]. CAR NK cells do offer advantages over CAR T cells, particularly their ability to avoid graft versus host disease (GVHD), cytokine-associated toxicity, and neurotoxicity, which have been observed in CAR T cell immunotherapies [273]. Recent clinical trials have demonstrated that CAR NK cells have reassuring safety profiles and good clinical outcomes against CD19-positive B cell lymphoma, showing superiority to the CD19 CAR T therapy [274]. CD33-targeted CAR NK cells were reported to have comparable efficacy to CD33-CAR T cells, with no reported cases of neurotoxicity or GVHD, again showing efficacy and safety in the treatment of hematological cancers [275]. Although effective against hematological malignancies, challenges remain regarding the use of CAR NK cell therapy to treat solid tumours; however, multiple clinical trials are ongoing targeting pancreatic (NCT06478459) and unresectable solid tumours (NCT06572956).

1.7.2 CIML NK cell therapy.

CIML NK cells are also being investigated in the clinic, with one active trial and 9 recruiting trials, mostly in patients with hematological cancers, but also in solid tumours such as ovarian, renal cell carcinoma, and urothelial carcinoma (Table 1.1). Adoptive transfer of haploidentical autologous CIML NK cells into patients was found to be safe, and expansion/persistence of the CIML NK cells correlated with transient disease control when used in combination therapy with an IL-15 super agonist [276]. In relapsed AML patients, CIML NK cell transfer also induced remission when given as a combination therapy. These cells retained their transcriptional signature, persisted without exogenous cytokines, and displayed enhanced effector functions at least 4 weeks post-adoptive transfer [277]. CIML NK cell products currently being investigated in preclinical trials, such as the healthy donor-derived, allogeneic WU-NK-101, have also been shown to be safe, demonstrating expansion, increased cytotoxicity receptors, and chemokine receptors, as well as persistence in lymph nodes, the spleen, and other secondary organs outside of the periphery in AML patients [278]. Finally, autologous CIML NK cells generated from patients with multiple myeloma exhibited increased functionality *in vitro* and in xenograft models [279].

Interestingly, the CIML NK cell training protocol can be incorporated with CAR NK cell technology, generating an innate trained cell with enhanced non-specific effector functions and killing capacity, armed with an antigen-specific CAR, resulting in a highly cytotoxic immunotherapy. CIML-CAR NK cells targeting mesothelin, a protein found on a range of cancer cells, showed increased cytotoxicity markers indicative of the CIML NK cell phenotype, metabolic fitness, successful CAR transduction, and effective clearance of mesothelin-positive AML cell lines [280]. Furthermore, another study where CIML NK cells were armed with a CAR specific for ovarian cancer-associated mesothelin, displayed enhanced tumour control and prevention of metastasis in a preclinical xenograft model, suggesting they may also be effective in solid tumour cancers [281]. Indeed, it is clear that CIML NK cells are a promising cell therapy with a good safety profile and effective compatibility with other NK-based cell therapies.

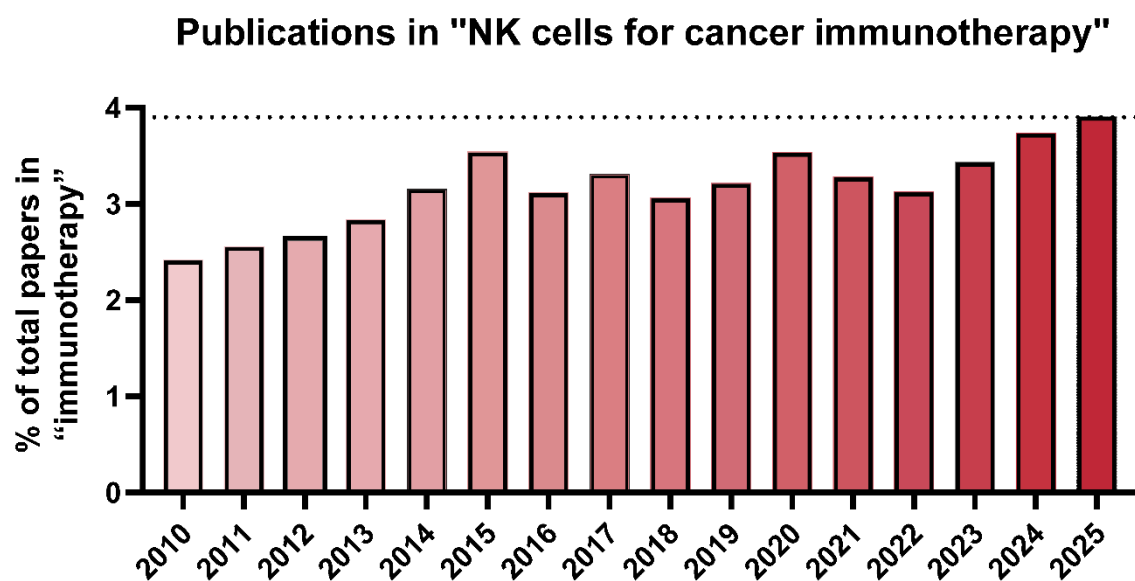


Figure 1.8. NK cells and cancer immunotherapy.

PubMed search for "NK cells for cancer immunotherapy" by year (2010-2025) as a percentage of total papers published in "immunotherapy" as of December 2025.

NCT #	Description	Status
NCT04024761	A Phase 1 Trial of CIML NK Cell Infusion for Myeloid Disease Relapse After Hematopoietic Cell Transplantation	Active
NCT05580601	Cytokine-Induced Memory-Like Natural Killer Cells (CIML-NK) for Relapsed & Refractory Acute Myeloid Leukemia (AML)	Recruiting
NCT06321484	Intraperitoneal Cytokine-Induced Memory Like (CIML) NK cells in Recurrent Ovarian Cancer	Recruiting
NCT06138587	Preemptive CIML NK Cell Therapy After Hematopoietic Stem Cell Transplantation	Recruiting
NCT06152809	CIML NK Cells With Venetoclax for AML	Recruiting
NCT06318871	Memory-like Natural Killer (NK) Cell Therapy in Patients With Renal Cell Carcinoma or Urothelial Carcinoma	Recruiting
NCT03068819	Cytokine Induced Memory-like NK Cell Adoptive Therapy for Relapsed AML After Allogeneic Hematopoietic Cell Transplant	Recruiting
NCT07011004	A Study of Natural Killer Cells in Combination With Atezolizumab People With Acute Myelogenous Leukemia	Recruiting
NCT02782546	Cytokine Induced Memory-like NK Cell Adoptive Therapy After Haploidentical Donor Hematopoietic Cell Transplantation	Recruiting
NCT05629546	Memory-Like Natural Killer Cells With Nivolumab and Relatlimab Advanced or Metastatic Melanoma After Progression on Checkpoint Inhibitors	Recruiting

Table 1.1. CIML NK cell utilising clinical trials.

Table of currently ongoing and recruiting clinical trials using CIML NK cells as a therapeutic intervention as of September 2025. Obtained from clinicaltrials.gov. (<https://clinicaltrials.gov/search?intr=CIML%20NK>).

1.8 Thesis hypothesis and aims.

As outlined above, CIML NK cells are an attractive immunotherapy that have enhanced persistence, effector functions, a good clinical safety profile, and are effective against hematological and potentially solid cancers. However, cancer has evolved to negatively regulate NK cell activity, particularly through manipulation of their environment, producing metabolically unsuitable conditions for NK cell survival and function. This project intends to decipher the specific metabolic requirements that underpin CIML NK cell function. A clearer understanding of these requirements may inform strategies to optimise their therapeutic potential in the clinic, particularly against solid tumours that have suboptimal conditions in the surrounding TME and where CIML NK cells have yet to show clinical efficacy.

This thesis tests the hypothesis that CIML NK cells undergo distinct and sustained metabolic reprogramming compared to conventional NK cells, and that these metabolic changes mechanistically underpin their enhanced functions. Furthermore, it is hypothesised that metabolic heterogeneity exists between CIML NK cell subsets, contributing to functional diversity within the population. To test this hypothesis, this thesis characterises the metabolic state of human CIML NK cells using complementary proteomic, metabolomic, and flow cytometric approaches. By defining both basal and functionally induced metabolic programmes, this thesis seeks to identify the metabolic pathways that support CIML NK cell function and enhanced effector capacity.

The specific aims are as follows:

1. To understand basal CIML NK cell metabolic reprogramming.
2. To investigate the metabolic mechanisms used by CIML NK cells to support enhanced secondary responses.
3. To explore the metabolic heterogeneity of CIML NK cell subsets.

Chapter 2. Materials and methods.

2.1 Materials.

2.1.1 General reagents.

Reagent	Supplier
Roswell Park Memorial Institute (RPMI) 1640 Medium + L-Glutamine	Gibco
Penicillin/Streptomycin (Pen/strep)	Gibco
Fetal Bovine Serum (FBS)	Sigma
Bovine Serum Albumin (BSA)	Sigma
L-Glutamine	Sigma
Dulbecco's Phosphate Buffered Saline (PBS)	Gibco
Hanks' Balanced Salt Solution (HBSS)	Gibco
Water, sterile filtered	SLS
Dimethyl Sulfoxide (DMSO)	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Golgi Plug	BD
Cytofix/Cytoperm Fixation and Permeabilization Buffer	BD
Permash	BD
Foxp3 / Transcription Factor Staining Buffer Set	Invitrogen
RBC Lysis Buffer (10X)	BioLegend
Lymphoprep	Stemcell
Trypan Blue	Sigma
Rotenone	Fisher
Oligomycin	Sigma
6-aminonicotinamide	Sigma
Devimistat (CPI-613)	Medchem express
Formaldehyde	Polysciences

Table 2.1 General reagents.

2.1.2 Flow Cytometry Antibodies.

Antibody	Fluorochrome	Clone	Dilution factor	Catalogue number	Supplier
Fc block	N/A	Fc1	100	14-9161-71	BD
CD3	PerCP-Cy5.5	OKT3	100	45-0037-42	BD
CD3	BV605	SK7	100	563219	BD
CD3	Pacific Blue	HIT3a	100	300330	BioLegend
CD25	PE-cy7	M-A251	100	560920	BD
CD39	PE-Dazzle 594	A1	100	328224	BioLegend
CD56	PE-Cy7	NCAM	100	318318	BioLegend
CD56	BV786	NCAM16.2	100	564058	BD
CD56	BV510	NCAM16.2	100	563041	BD
CD57	eFluor 450	TB01	100	48-0577-42	Thermo
CD71	APC	CY1G4	100	334108	BioLegend
CD98	PE	UM7F8	50	556077	BD
CD117	BV650	104D2	100	313222	BioLegend
GLUT1	Alexa Flour 647	EPR3915	100	ab195020	Abcam
Granzyme B	BV421	GB11	100	563389	BD
Granzyme B	PerCP	QA18A28	100	396416	BioLegend
IFN- γ	Alexa Flour 700	4S.B3	100	502520	BioLegend
IFN- γ	PerCP-Cy5.5	4S.B3	100	502526	BioLegend
Ki-67	PE	Ki-67	100	350504	BioLegend
NKG2A	APC/Fire 750	S19004C	100	375116	BioLegend

Table 2.2. Flow cytometry antibodies.

2.1.3 Dyes and stains.

Reagent	Detection channel	Dilution factor	Catalogue number	Supplier
LIVE/DEAD Fixable Dead Cell Stain Kit	Near-IR	1000	L10119	Thermo
LIVE/DEAD Fixable Viability Dye	eFlour 506	1000	65-0866-14	BD
Acradine Orange 10-Nonyl Bromide	FITC	10000	A1372	Invitrogen
Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM)	TMRM/PE	25000	T668	Invitrogen
MitoSOX™ Mitochondrial Superoxide Indicator	MitoSOX/PE	2500	M36008	Invitrogen
CellIROX™ Cellular ROS indicator	CellIROX/FITC	2500	C10444	Invitrogen

Table 2.3. Dyes and stains.

2.1.4 Uptake Assay Reagents.

Reagent	Detection channel	Final concentration	Catalogue number	Supplier
Aminoguanidine	N/A	10mM	81530	Cayman Chemical
Sodium Ascorbate	N/A	10 mM	134-03-2	Sigma
Copper (II) Sulfate Pentahydrate	N/A	1 mM	7758-99-8	Sigma
tris-hydroxypropyltriazolyl - methylamine (THPTA)	N/A	1 mM	CCT-1010	Click chemistry tools
AZDye 488 Alkyne	Alexa Fluor 488	5 μ M	CCT-1277	Click chemistry tools
O-propargyl-puromycine (OPP)	N/A	20 μ M	CCT-1407	Click chemistry tools
Homoharringtonine (HHT)	N/A	2 μ g/mL	SML1091-10MG	SLS
Transferrin conjugate	Alexa Flour 647	5 μ g/mL	T23366	Invitrogen
Holo-Transferrin	N/A	500 ug/mL	T4132	Invitrogen

Table 2.4. Uptake assay reagents.

2.1.5 Software.

Graphpad Prism 10.0.1 was used for generating plots, formatting data, and performing statistical analysis. FlowJo software was used for the analysis of flow cytometry data. RStudio was used for the analysis of proteomic data and RNA-sequencing data. Graphical figures were created using Biorender, an illustration software.

2.2 Methods.

2.2.1 Isolation of peripheral blood mononuclear cells (PBMCs) from fresh whole blood.

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors via density gradient centrifugation using Lymphoprep (Stemcell). The fresh whole blood was diluted 1 in 2 with PBS and layered on top of 15 mls of Lymphoprep before centrifugation for 30 minutes (300 g, brake off). The buffy coat layer was then isolated and washed in 50 mls of PBS. Red blood cells were lysed using Red Cell Lysis Buffer (diluted 1 in 10 with water). Isolated PBMCs were then resuspended at a concentration of 5×10^6 cells/ml in RPMI Glutamax medium (10% fetal calf serum, 1% Pen/Strep).

2.2.2 Training protocol for generating CIML NK cells.

Day 0

PBMC isolation was carried out from healthy whole blood followed by flow cytometric sorting of NK cells. Purified NK cells were seeded at 5×10^6 cells/ml in a 96-well plate and stimulated with IL-12 (10 ng/ml) and IL-15 (100 ng/ml), and IL-18 (50 ng/ml). Control cells were given a survival dose of IL-15 (1 ng/ml) only. Cells were then placed in the incubator at 37°C, 5% CO₂ for 18 hours.

Day 1

After 18 hours, control and CIML NK cells were washed three times in PBS and resuspended in 200 µl of media supplemented with IL-15 (1 ng/ml) to allow for survival. On days 3 and 5, the IL-15-supplemented media were replenished in both control and CIML NK cells.

Day 7

At day 7, CIML NK cells were assessed at rest or else restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours for functional analyses.

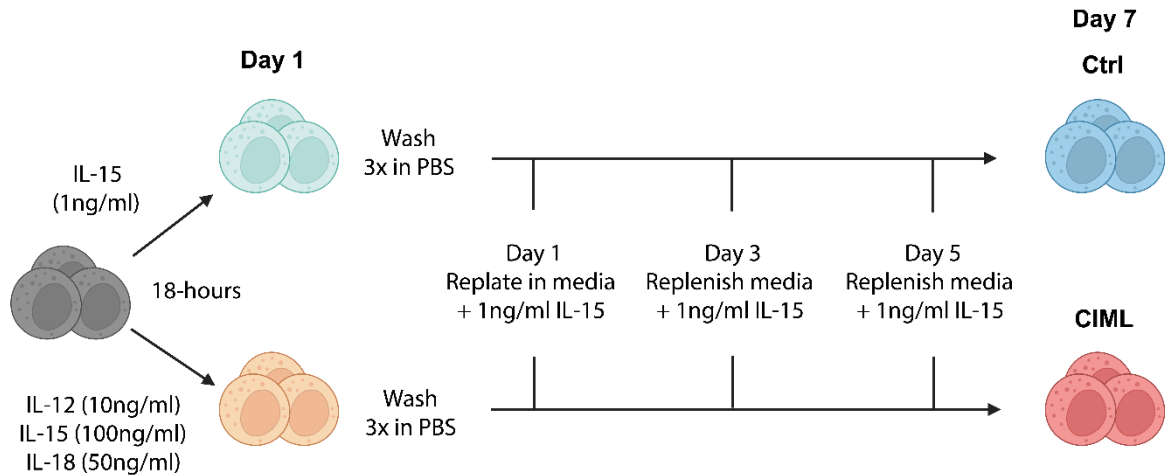


Figure 2.1. CIML NK cell training protocol.

Illustration describing protocol for generating CIML NK cells. On day 0, purified NK cells were plated at 5×10^6 cells/ml in a 96-well plate and stimulated for 18-hours with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml), and then washed three times in PBS the next day. Comparatively, control (Ctrl) cells were given only a low-dose survival feed of IL-15 (1 ng/ml) and washed three times the next day (day 1). After washing, NK cells are resuspended in IL-15 (1 ng/ml) supplemented media, which is replaced on days 3 and 5. On day 7, CIML NK cells have been generated and were ready for analysis or further experiments. Illustration made using biorender

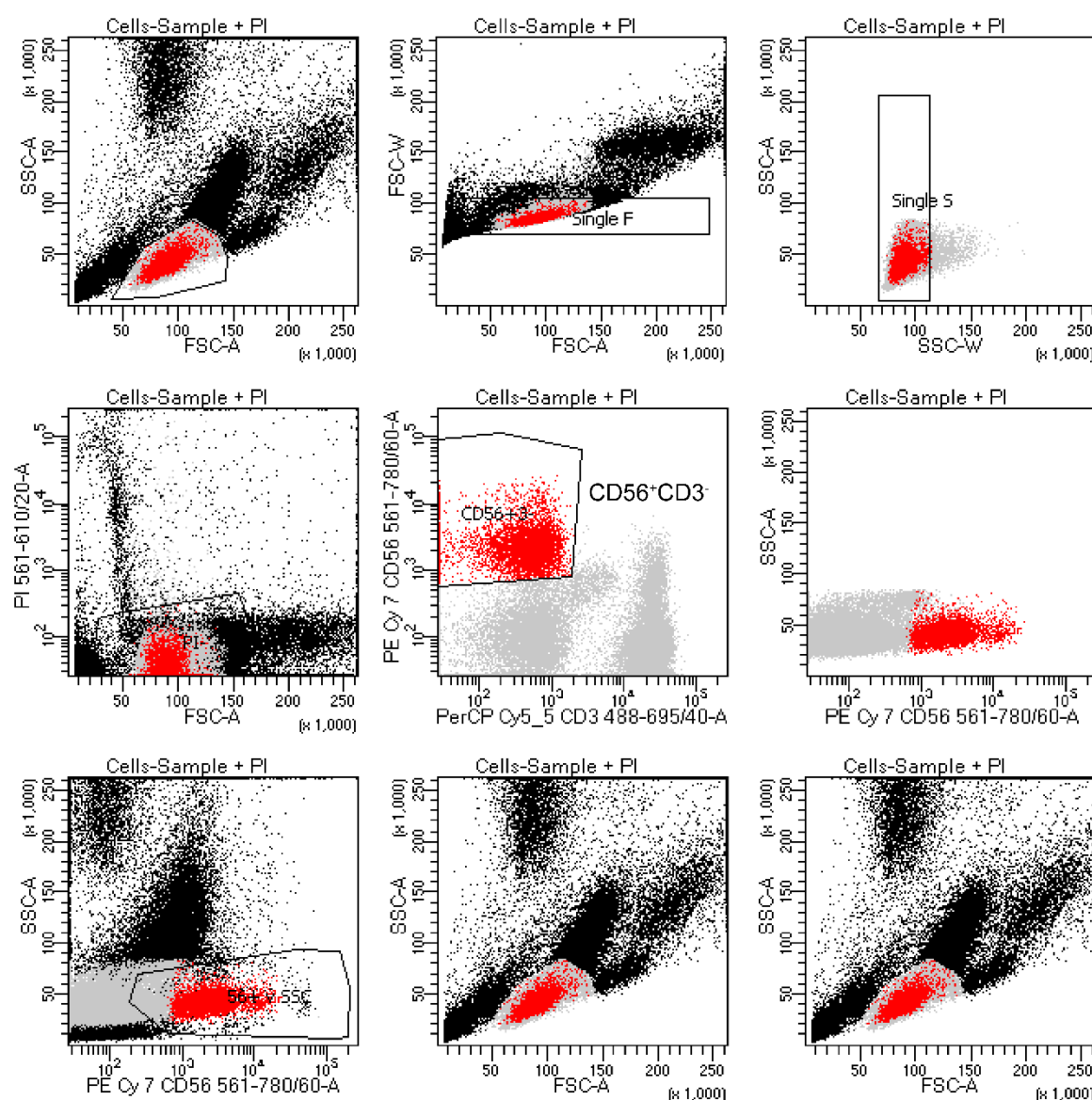
2.2.3 Flow Cytometry.

For analysis of surface markers on PBMCs or purified NK cells, cells were washed in cold PBS and stained with a viability dye (diluted 1 in 1000, PBS) for 15 minutes on ice in the dark. Fc blocking (diluted 1 in 100) was also carried out during this step to prevent unspecific binding of antibody when surface staining. Cells were then washed once in cold PBS and were then stained for extracellular surface markers (diluted 1 in 100) at 4°C in the dark for 20 minutes in FACS buffer (PBS, 2% FBS). After staining, cells were either washed in FACS buffer and analysed immediately or were fixed for 15 minutes in cytofix/cytoperm (BD) in the dark at 4°C. Intracellular antibody staining was performed using 1x Perm Wash (BD) for 30 min at 4°C in the dark. For intranuclear staining, FOXP3/transcription

factor staining buffer kit (eBiosciences) was used according to the manufacturer's instructions.

2.2.3.1 Isolation of NK cells from PBMCs by FACS sorting.

PBMCs were isolated from whole blood as described above and transferred to a 5 ml FACS tube for staining. Cells were incubated with Fc block for 10 minutes before being washed and then surface-stained for CD56 and CD3 for 20 minutes in the dark on ice. Cells were then strained through a 40-micron filter to prevent clumping in the cytometer. Cells were washed then resuspended in 1 ml of FACS buffer. PI was added before acquisition and sorting on the FACS Aria Fusion sorter. NK cells were identified as the CD56⁺, CD3⁻ lymphocyte population and sorted from the rest of the PBMCs into a 15 ml falcon tube. These cells were then briefly acquired to confirm purity. After sorting, cells were immediately spun and washed in RPMI and plated for subsequent experiments. Analysis carried out using BD FACSDiva™ Software and Flowjo Software.



Tube: Sample + PI			
Population	#Events	%Parent	%Total
■ All Events	100,000	####	100.0
▣ Cells	43,031	43.0	43.0
▣ Single F	42,851	99.6	42.9
▣ Single S	42,087	98.2	42.1
▣ PI-	41,918	99.6	41.9
■ CD56+3-	6,000	14.3	6.0
■ 56+ v SSC	5,994	99.9	6.0

Figure 2.2. NK cell isolation gating strategy.

Lymphocytes were identified based on forward (FSC) vs side scatter, and single cells were further identified by FSC area vs FSC width. Dead cells were gated out using Propidium Iodide staining, and NK cells were identified as the CD56+ CD3- population. NK cells were sorted into FACS buffer. Sorting performed on FACSaria and data acquired using BD FACSDiva 9.4 software.

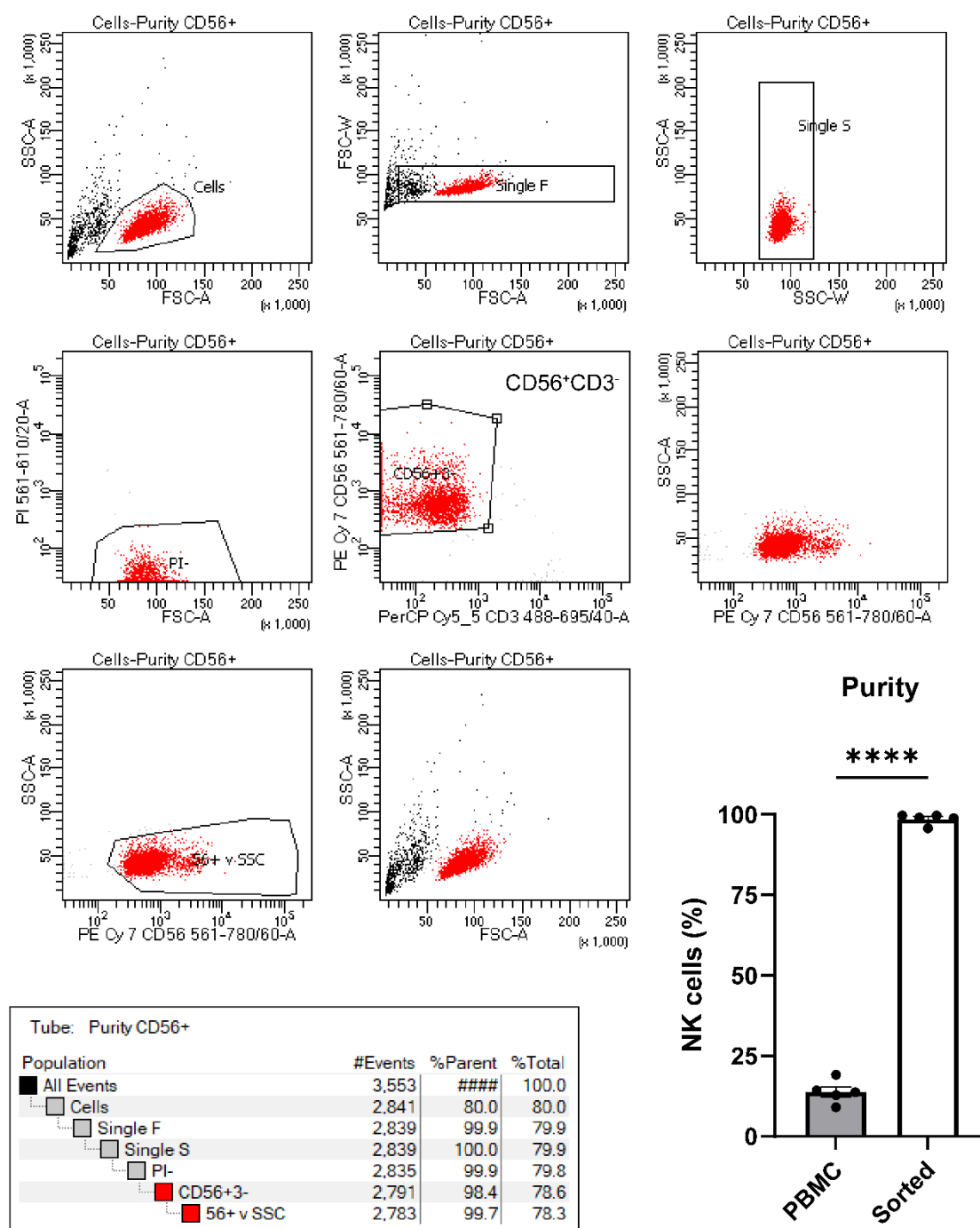


Figure 2.3. NK cell purity post-sorting.

NK cell purity was checked post-sorting. NK cell purity was at a minimum of 97% across all sorts. Bar graph depicts % of NK cells in PBMCs (unsorted) and sorted cells. Pooled data is represented as mean $\pm$ SD of 5 independent experiments (N=5).

2.2.3.2 Mitochondrial staining.

10-nonyl acridine orange (NAO) (Sigma) was used to measure mitochondrial mass. NK cells were stained with 50 nM of NAO and incubated at 37°C for 30 minutes. Tetramethylrhodamine methyl ester (TMRM, Invitrogen) was used to measure changes in mitochondrial membrane potential. NK cells were stained with 3 nM TMRM and incubated at 37°C for 30 minutes. Oligomycin (1 μ M) was added to a separate stained well as a positive mitochondrial membrane potential control. MitoSOX Red (ThermoFisher) dye was used to measure mitochondrial ROS. NK cells were stained with 2 μ M mitoSOX and incubated at 37°C for 30 minutes. Rotenone (20 μ M) was added to a separate well during mitoSOX staining as a positive mitochondrial ROS control. All staining/incubation was carried out at the same time. Cells were then washed and stained for extracellular surface markers and ran on Cytex Aurora unfixed.

2.2.3.3 Puromycin uptake and click chemistry.

O-propargyl-puromycin (OPP) was used to measure protein synthesis in NK cell populations. NK cells were stained with surface markers as described above. Following staining, NK cells were resuspended in RPMI and incubated at 37°C for 10 minutes. OPP was then added for 30 minutes at a final concentration of 20 μ M to the samples. Control cells were treated with 2 μ g/ml of homoharringtonine (HHT), a protein synthesis inhibitor, to act as a background control. Following incubation with OPP, cells were fixed using PFA (1% final concentration) for 20 minutes at room temperature in the dark. Cells were then washed twice in PBS and subsequently permeabilised in permeabilisation buffer (PBS +1% BSA + 0.01% saponin) for 20 minutes. Cells were washed twice in PBS and the click chemistry reaction was then carried out as described below.

Click chemistry is a technique in which a small reactive chemical group (handle) on a biomolecule can be ligated to a detectable group such as a fluorophore. Small molecules such as amino acids, lipids or other nutrients can be modified to have a reactive handle, usually an azide or alkyne group, which can be attached to a fluorophore with an opposing handle.

After permeabilisation cells were washed twice with PBS and the click reaction mixture was prepared as follows. 10 µl of copper sulfate (CuSO₄) (1 mM final concentration), sodium ascorbate (NaAsc) (10 mM final concentration), tris-hydroxypropyltriazolylmethylamine (THPTA) (100 mM final concentration), PBS and bio-orthogonal fluorophore (AZDye-488) (5 mM final concentration) were added together in a sequential order. After the cells were washed, all remaining PBS was removed to ensure a dry pellet and 30 µl of click reaction mix was added, and cells were left to incubate at room temperature in the dark for 30 minutes. Cells were washed three times in PBS and resuspended in FACS buffer and acquired on the BD LSR Fortessa.

2.2.3.4 Transferrin uptake.

Transferrin uptake was measured in cells using Alexa fluor-647-conjugated transferrin. Cells were washed in RPMI (0.5% BSA) to remove media containing FBS containing transferrin and subsequently incubated in RPMI (5% BSA) for 1 hour at 37°C. Cells were then spun and incubated with or without 5 µg/ml Alexa fluor-647-conjugated transferrin in RPMI (no additions) for 10 minutes at 37°C. As competitive and cold controls, cells were incubated with 500 µg/ml Holo-transferrin or uptake was performed on ice, respectively. Cells were then washed with acid wash (PBS + 150 mM NaCl and 20 mM citric acid, pH 5) to cease the uptake assay and then washed with ice-cold RPMI (0.5% BSA). Cells were then extracellularly stained as described above and analysed by flow cytometry.

2.2.4 Metabolomics.

2.2.4.1 13-Carbon (¹³C) nutrient tracing and metabolomics.

Glucose-free media was supplemented with ¹³C D-glucose (D-Glucose-¹³C₆, Sigma), 10% dialysed FBS, and 1% Pen/Strep. NK cells were purified from PBMCs by FACS Aria sorting and replated at 5x10⁶ cells/ml in a 96-well plate. Control and CIML NK cells were generated as described above, and on day 6 were washed three times in warm PBS and incubated with ¹³C glucose-supplemented media for 24 hours. The next day, cells were spun down, and supernatants were removed into safe-lock Eppendorf tubes and placed on ice. The remaining cell pellets were resuspended and transferred to safe-lock Eppendorf tubes and washed three times in cold PBS, aspirating all the remaining supernatant off to ensure a dry pellet. Cell pellets and supernatants were then snap frozen using liquid nitrogen and kept at -80°C until shipping for metabolomic analysis and isotopologue tracing via mass spectrometry by the EMBL, Heidelberg.

2.2.4.2 Sample preparation for metabolomics.

Metabolites were extracted via addition of 1 ml 0% Methanol v/v and homogenisation was carried out on dry ice with a bead beater (FastPrep-24; MP Biomedicals, CA, USA) at 6.0 m/s (3 x 30 s, 5 min pause time) using 1.0 mm zirconia/glass beads. After centrifugation for 10 min at 15,000g and 4°C with a 5415R microcentrifuge (Eppendorf) the supernatants were collected and dried under a stream of nitrogen. Dried samples were reconstituted in 100 µl 80% methanol, vortexed for 5 minutes, and transferred to analytical glass vials. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was initiated within one hour after the completion of the sample preparation.

2.2.4.3 Mass spectrometry processing for metabolomics.

LC-MS/MS analysis was performed on a Vanquish UHPLC system coupled to an Orbitrap Exploris 240 high-resolution mass spectrometer (Thermo) in positive and negative electrospray ionisation mode. Chromatographic separation was carried out on an Atlantis Premier BEH Z-HILIC column (Waters, MA, USA; 2.1 mm x 100 mm, 1.7 μ m) at a flow rate of 0.25 ml/min. The mobile phase consisted of water:acetonitrile (9:1, v/v; mobile phase phase A) and acetonitrile:water (9:1, v/v; mobile phase B), which were modified with a total buffer concentration of 10 mM ammonium acetate (negative mode) and 10 mM ammonium formate (positive mode), respectively. The aqueous portion of each mobile phase was pH-adjusted (negative mode: pH 9.0 via addition of ammonium hydroxide; positive mode: pH 3.0 via addition of formic acid). Column temperature was maintained at 40°C, the autosampler was set to 4°C and sample injection volume was 5 μ L. Analytes were recorded via a full scan with a mass resolving power of 120,000 over a mass range from 60 – 900 m/z (scan time: 100 ms, RF lens: 70%). To obtain MS/MS fragment spectra, data-dependant acquisition was carried out (resolving power: 15,000; scan time: 22 ms; stepped collision energies [%]: 30/50/70; cycle time: 900 ms). Ion source parameters were set to the following values: spray voltage: 4100 V (positive mode) / -3500 V (negative mode), sheath gas: 30 psi, auxiliary gas: 5 psi, sweep gas: 0 psi, ion transfer tube temperature: 350°C, vaporizer temperature: 300°C. All experimental samples were measured in a randomised manner.

Pooled quality control samples were prepared by mixing equal aliquots from each processed sample. To assess background contamination and non-biological signals, a processed blank sample was included. The blank consisted of extraction solvent subjected to the identical sample preparation protocol (including metabolite extraction, drying, and reconstitution) but without biological material (media only). Blank peak areas were subtracted from corresponding sample peak areas when applicable to minimise residual background contribution. Features present exclusively in blank samples were excluded entirely. This filtering step reduced artefactual signals and improved confidence in biological metabolite identification. This blank was injected under identical LC–MS/MS conditions to identify signals arising from solvents, reagents, plasticware,

column bleed, and carryover. Data was processed using MS-DIAL and FreeStyle 1.8 SP2 (Thermo Fisher Scientific) and raw peak area data was normalized via total ion count for metabolite quantification [282]. Feature identification was based on accurate mass, isotope pattern, MS/MS fragment scoring and retention time matching to an inhouse library.

2.2.4.4 Metabolomic analysis.

Data was provided as “total area” or the signal intensity of detected ions throughout the acquisition time in during an MS scan. Samples ran in positive and negative ionisation modes, and data was provided for each. Individual metabolite detection in each mode depends on metabolite charge, and the optimal ionisation mode was determined and provided by EMBL Heidelberg. ¹³ carbon isotopologue tracing was given as “relative exchange” which signifies the percentage of total carbon labelling in the metabolite pool. M+1 indicating 1 carbon in the molecule containing the heavy carbon tracer, M+2 indicating 2 carbons in the molecule, and so on. Values were compared in a pairwise manner between control and CIML groups within each donor.

2.2.5 Proteomics.

2.2.5.1 Preparation of cell pellets for proteomics.

NK cells were purified from PBMCs by FACS Aria sorting and replated at 5×10^6 cells/ml in a 96-well plate. Control and CIML NK cells were generated as described above in section 2.2.2. On day 7, control and CIML NK cells were left untreated or stimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours. Cells were then washed three times in HBSS, aspirating off the remaining supernatant to ensure no BSA contamination and a dry pellet. Cell pellets and supernatants were then snap frozen using liquid nitrogen and kept at -80°C until shipping for proteomic analysis at the University of Dundee.

2.2.5.2 Mass spectrometry processing for proteomics.

Proteomic sample preparation was performed by the technicians at the FingerPrints proteomic facility in the University of Dundee, UK. Samples were processed using S-Trap Micro columns (Protifi) where proteins were reduced, alkylated, and digested for 24 hours with 0.75µg of trypsin per sample. A second digest step was performed for 6 hours the following day. HiPPR detergent removal was performed to remove any remaining SDS from the digested peptides before further analysis was carried out.

Digested peptides were run on an Orbitrap Astral mass spectrometer (Thermo) coupled to a Vanquish Neo UHPLC system (Thermo) with LC buffers comprising of 0.1% formic acid and 80% acetonitrile, 0.1% formic acid. Samples were run and peptides were eluted from a PepMap RSLC C18 column (Thermo) and raw data was acquired in Data Independent Acquisition (DIA) mode. A scan cycle comprised a full MS scan with an m/z range of 380-940, resolution of 240,000, custom Automatic Gain Control (AGC) target of 500% and a maximum injection time (IT) of 5ms. MS scans were followed by MS/MS DIA scans of dynamic window widths with an overlap of 0m/z. DIA spectra were recorded with a scan range of 150-2000m/z, a custom AGC target of 500% and a maximum IT of 3ms. Normalised collision energy was set to 25%. Data for MS scans were acquired in profile mode with MS/MS DIA scan events being acquired in centroid mode.

2.2.5.3 Proteomics analysis.

Data analysis was performed using Spectronaut v19 using directDIA. Samples were normalised using the global median. The Spectronaut output was used to calculate copy numbers using Perseus v1.6.12.0 and the Proteomic Ruler plugin. Differential expression analysis was carried out by log2 transformation of the paired data using the LIMMA package on RStudio. The mass of individual proteins was estimated using the formula: $CN \times \frac{MW}{N_A} = \text{protein mass} \left(\frac{g}{\text{cell}} \right)$, where CN is the protein copy number calculated from Perseus and the proteomic ruler, MW is the molecular weight of the protein in Daltons, and N_A is Avogadro's Constant. Total protein content per cell was calculated as the sum of the mass of all proteins detected. Total protein content of metabolic pathways and cellular

compartments was calculated using the sum of the mass of all proteins (calculated using the above formula) within a pathway or compartment. Protein lists of each pathway/cell compartments retrieved from the Gene Ontology Browser on the mouse genome informatics browser (<https://www.informatics.jax.org/>). Pathway enrichment analyses performed using Icahn School of Medicine bioinformatics resources (EnrichR, ChEA3). STRING analysis database used for the identification of protein-protein interactions in differentially expressed proteins (<https://string-db.org/>).

2.2.5.4 Removal of outlier and undetected/lowly expressed proteins.

For both metabolomic and proteomic analysis, 5 separate donors were acquired, 3 of them were paired for both proteomic and metabolomic analyses. CIML NK cells are known to have increased cellular size determined by forward scatter (FSC-A), indicative of increased protein content [221]. Of the 5 donors, 4 of CIML NK cells observed an increase in total protein content after training, whereas one donor (Donor 3) showed no change in size after training on day 7 (Control NK: 51.59 pg/cell, CIML NK: 51.50 pg/cell) (Figure 2.4A). Furthermore, Donor 3 did not show an increase in, and in some cases decreased expression of markers indicative of CIML NK cell training, such as CD25, NKG2D and CD71 (Figure 2.4B) [221, 283]. This donor was subsequently removed from all data, and as this donor had a paired metabolomic counterpart, it was also removed from the metabolomics analysis. During the processing of proteomic data, any proteins that were not detected (0 or NaN value) across at least 50% of the samples were removed. Similarly, a threshold was set to remove proteins that had an expression value of less than 200 copies.

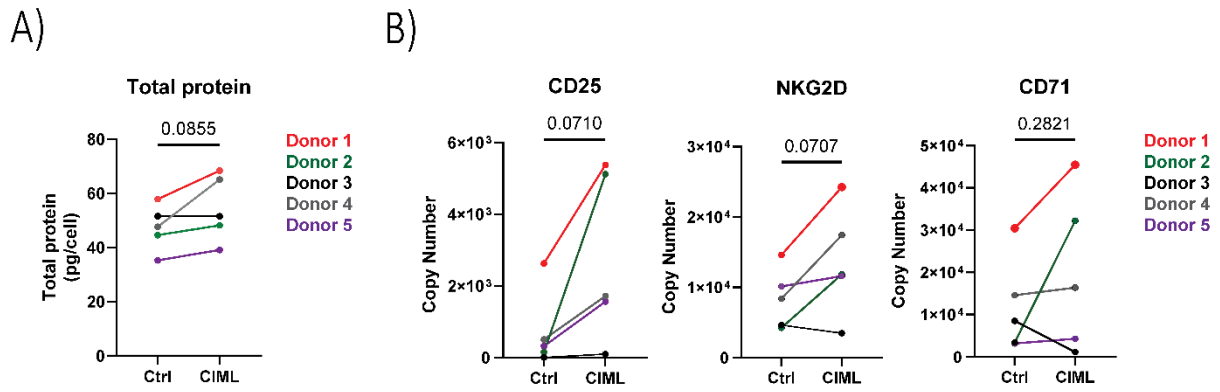


Figure 2.4. Total protein and expression of training markers in control and CIML NK cells.

(A) Total protein content and **(B)** expression of CIML NK cell training markers in donor-matched Ctrl and CIML NK cells. Total protein content per cell was calculated as $\sum ((CN \times MW)/N)$ where CN = Protein copy number, MW = Protein molecular weight, N = Avogadro's constant.

2.2.6 Single-cell RNA-sequencing data.

Data was obtained from an online, publicly available source (Foltz et al., 2024 (DOI: 10.1126/sciimmunol.adk4893)). Raw single-cell RNA-seq data were accessible at GSE264693. Processing of raw data was carried out with the help of **Dr. Conor Smith and John Scanlan**, Trinity College Dublin). RStudio was used (**with the help of Dr. Conor Smith and John Scanlan**) for differential expression analysis of proteomics and RNA-sequencing data. Analyses were conducted in R version 4.1.3 (2022-03-10) on a Windows 10 x64 platform. The Seurat pipeline and associated single-cell analyses were performed using the Seurat R package (version 4.3.0) and SeuratObject (version 4.1.3). Other packages used included ggplot2 (3.5.1) for plotting data, data.table (1.14.8) for data handling, and SeuratDisk (0.0.0.9020) for cross-format single-cell data conversion.

2.2.7 Rstudio.

Rstudio was used with the help of **Dr. Conor smith and John Scanlan** for differential expression analysis of proteomics and RNA-sequencing data processing and analysis. Packages used include. Analyses were conducted in R version 4.1.3 (2022-03-10) on a Windows 10 x64 platform. The Seurat pipeline and associated single-cell analyses were performed using the Seurat R package (version 4.3.0) and SeuratObject (version 4.1.3). Other packages used included ggplot2 (3.5.1) for plotting data, data.table (1.14.8) for data handling, and SeuratDisk (0.0.0.9020) for cross-format single-cell data conversion. Uniform manifold approximation and projection (UMAP) plots were generated as a method of dimensionality reduction for visualising transcriptional similarities. Violin plots were generated to show the distribution of gene expression between samples. Gene set enrichment analyses (GSEA) were also carried out to determine significant functional correlations between gene and protein expression.

2.2.8 Statistical analysis.

Data are expressed as mean +/- standard error of the mean (SEM) unless stated otherwise. All datasets were tested for normality using Shapiro-Wilk and skewness testing, and visual inspection of quantile-quantile (Q-Q) plots. The data met the assumptions required for parametric statistical analyses. P-values were calculated using a two-tailed Student's t-test for two-group comparisons. To compare multiple groups, one-way ANOVA with Sidak's post-hoc test comparison or two-way ANOVA with Tukey's post-test comparison was used as indicated.

Chapter 3. Proteomic and metabolomic analysis of resting human CIML NK cells.

3.1 Introduction.

CIML NK cells are an attractive immunotherapy due to their enhanced cytotoxicity and are currently being studied in numerous cancer clinical trials. Immunometabolism is also becoming a more relevant consideration in immunotherapies as the effects metabolic disorders, tumour microenvironments, and comorbidities have on immune cell metabolism are becoming more appreciated. However, the metabolism of CIML NK cells remains a largely unexplored field. As CIML NK cells are generated *in vitro*, it is important to understand what sustains their metabolic reprogramming before administration into a patient with cancer who has drastically different and constantly changing blood and tissue environments. As metabolic reprogramming is an essential factor to sustaining trained programmes in cells, it is essential to understand CIML NK cell metabolic requirements to optimise and tailor their application as an immunotherapy.

Many of the CIML NK cell metabolic studies published to date have used basic metabolic approaches, such as flow cytometric analysis of nutrient receptor expression and mitochondrial mass, or Seahorse analysis to determine extracellular acidification and oxygen consumption rates [220, 221]. More in-depth methods, such as proteomics and metabolomics, could offer more detailed insights into the global networks and systems that are activated in these cells. Indeed, proteomic analysis has proven to be a useful method to investigate metabolism in terms of the expression of pathway machinery and compartmentalisation of proteins. Metabolomics is the gold standard for untargeted global metabolite pool analysis and can be paired with an integrated heavy carbon or nitrogen nutrient tracing, which can determine the molecular fates of nutrients of interest. In this chapter, proteomic analysis and metabolomic carbon tracing were undertaken in control and CIML NK cells to examine the favoured metabolic pathways required to sustain the trained program.

Based on the established role of metabolic reprogramming in sustaining trained immune phenotypes, this chapter will test the hypothesis that CIML NK cells in a basal state exhibit a distinct and coordinated metabolic profile that supports their enhanced functional capacity. Specifically, it is proposed that CIML NK cells preferentially utilise defined bioenergetic and biosynthetic mechanisms, characterised by enhanced metabolic flux through key carbon pathways, to sustain their trained programme. Furthermore, it is hypothesised that CIML NK cells display modified mitochondrial and redox properties that support cellular fitness and effector function. Integration of proteomic and metabolomic analyses are expected to reveal metabolic alterations that underpin this metabolic reprogramming and distinguish CIML NK cells from control NK cells.

3.2 Aims.

- To characterise steady-state CIML NK cell metabolism by integrating proteomic and metabolomic analyses.
- To investigate the mitochondrial features of steady-state CIML NK cells.
- To elucidate the redox properties of CIML NK cells.

3.3 Validation of CIML NK cell generation.

Prior to sending samples for proteomic analysis, it was necessary to confirm that CIML NK cells could be successfully generated by analysing expression of markers of CIML NK cell training. Receptors such as the transferrin receptor, CD71, the glucose transporter GLUT1 and nutrient transporter chaperone protein CD98 have been demonstrated to be significantly upregulated on the surface of resting CIML NK cells [284]. Enhanced secondary responses, such as increased IFN γ expression after restimulation, are also the prototypic measurement for CIML NK cell generation in both humans and mice [213, 214]. These readouts were therefore assessed using flow cytometry post-CIML NK cell generation as outlined in Figure 2.2.2. Purified NK cells were isolated from PBMC and stimulated with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours to generate CIML NK cells, or given a low dose of IL-15 (1 ng/ml) to

generate control NK cells [284]. Enhanced secondary responses, such as increased IFN γ expression after restimulation, are also the prototypic measurement for CIML NK cell generation in both humans and mice [213, 214]. Purified NK cells were isolated from PBMC and stimulated with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) or given a low dose of IL-15 (1 ng/ml) for 18 hours to generate control NK cells. The next day, NK cells were washed three times in PBS and resuspended in media supplemented with low-dose IL-15 and left for 7 days, replacing IL-15-supplemented media on days 3 and 5 (Figure 2.2.2). On day 7, control and CIML NK cells were left untreated (at rest) or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours. As expected, CIML NK cells at rest had significantly upregulated CD98 (Figure 3.1A,B), CD71 (Figure 3.2A-C), and GLUT1 expression (Figure 3.3A,B), and upon restimulation were confirmed to have significantly increased IFN γ (Figure 3.4A-E) and granzyme B production (Figure 3.5A,B) compared to control NK cells. CIML NK cells were also revealed to be larger through forward scatter (FSC-A) (Figure 3.6A,B) measurement by flow cytometry, a proxy measurement for cell size [284]. Furthermore, CIML NK cells had increased rates of protein synthesis as demonstrated by puromycin incorporation into elongating nascent polypeptides (Figure 3.7A,B). Together, this demonstrated that CIML NK cells were generated *in vitro*, demonstrating upregulated expression of nutrient transporters, increased cell size, active synthesis of more protein at rest and characteristic enhanced secondary responses.

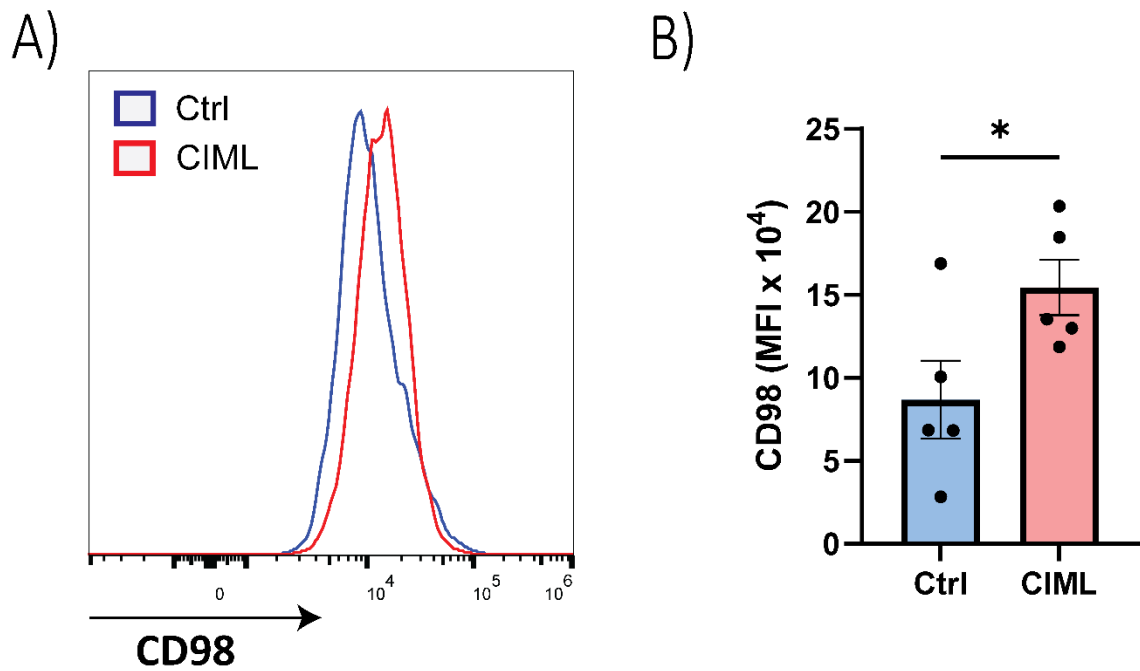


Figure 3.1. CD98 surface expression is increased on resting CIML NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. **(A,B)** Flow cytometry analysis of CD98 expression on Ctrl and CIML NK cells. **(A)** Representative histogram showing the expression of CD98 and **(B)** pooled MFI of CD98 expression on Ctrl and CIML NK cells. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=5 donors. Statistical analysis was performed using a paired t-test. ($p < 0.05$).

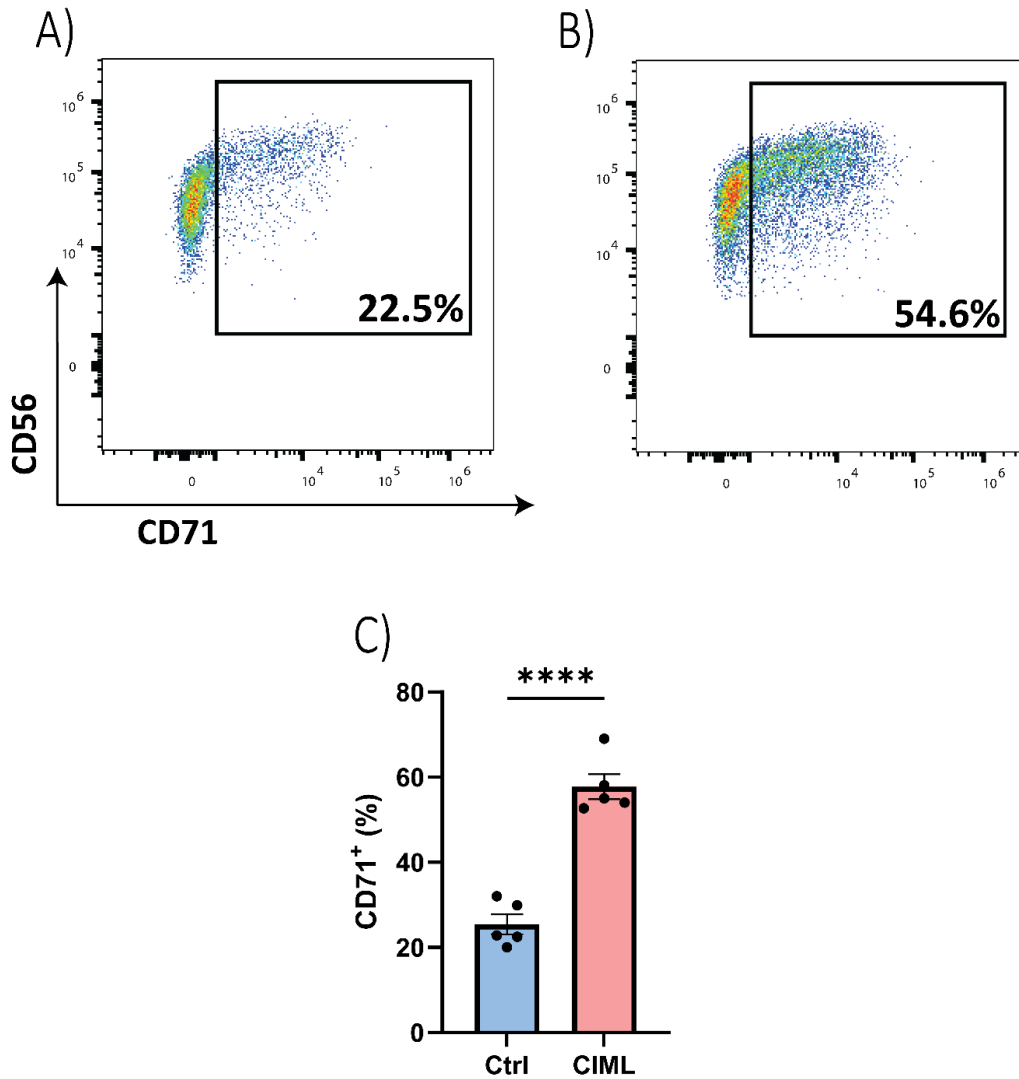


Figure 3.2. CD71 surface expression is increased on resting CIML NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. **(A-C)** Flow cytometry analysis of CD71 positivity on Ctrl and CIML NK cells. **(A,B)** Representative dot plots showing CD71 positivity in **(A)** Ctrl and **(B)** CIML NK cells. **(C)** Pooled data indicating percentage positivity for CD71 in Ctrl and CIML NK cells. Data are representative **(A,B)** or **(C)** mean $\pm$ SEM of N=5 donors. Statistical analysis was performed using a paired t-test (**** $p < 0.0001$)

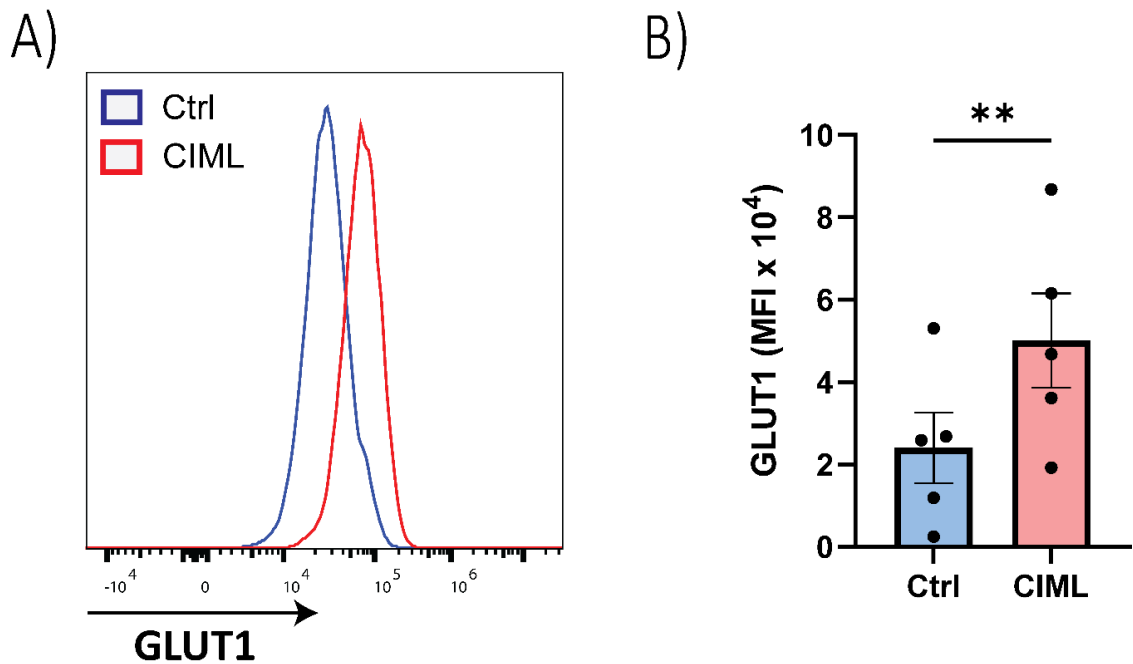


Figure 3.3. GLUT1 surface expression is increased on resting CIML NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. **(A,B)** Flow cytometry analysis of GLUT1 expression on Ctrl and CIML NK cells. **(A)** Representative histogram showing the expression of GLUT1 and **(B)** pooled MFI of GLUT1 expression on Ctrl and CIML NK cells. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=5 donors. Statistical analysis was performed using a paired t-test. (**p<0.01).

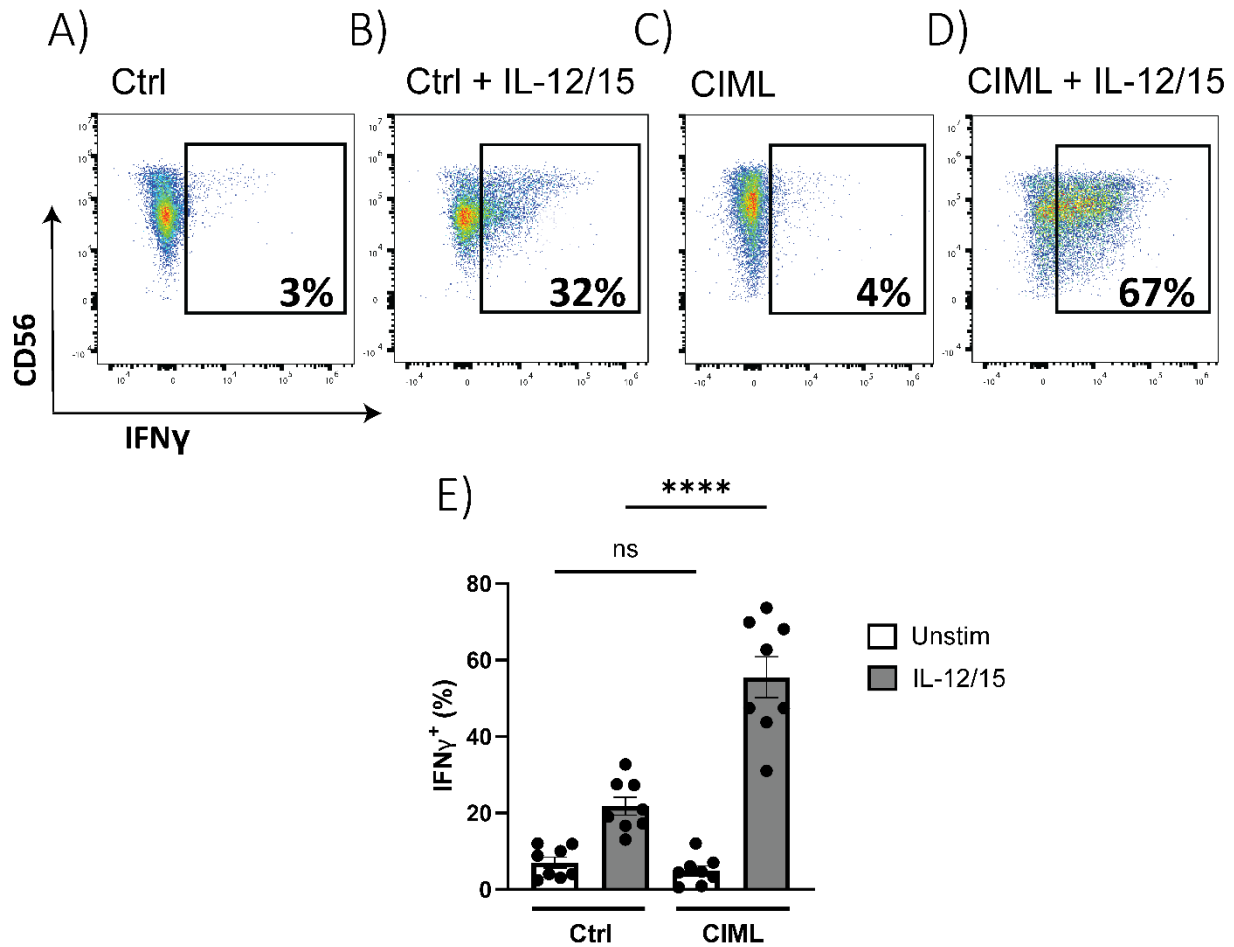


Figure 3.4. IFN γ expression is increased in CIML NK cells post-restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. **(A-E)** Flow cytometry analysis of IFN γ positivity of Ctrl vs CIML NK cells +/- IL-12/15. **(A-D)** Representative dot plots showing IFN γ positivity in **(A)** Ctrl NK cells, **(B)** Ctrl NK cells + IL-12/15, **(C)** CIML NK cells and **(D)** CIML NK cells + IL-12/15 NK cells. **(E)** Pooled data indicating percentage positivity for IFN γ in Ctrl and CIML NK cells +/- IL-12/15. Data are representative **(A-D)** or **(E)** mean +/- SEM of N=8 donors. Statistical analysis was performed using a one-way ANOVA (****p<0.0001).

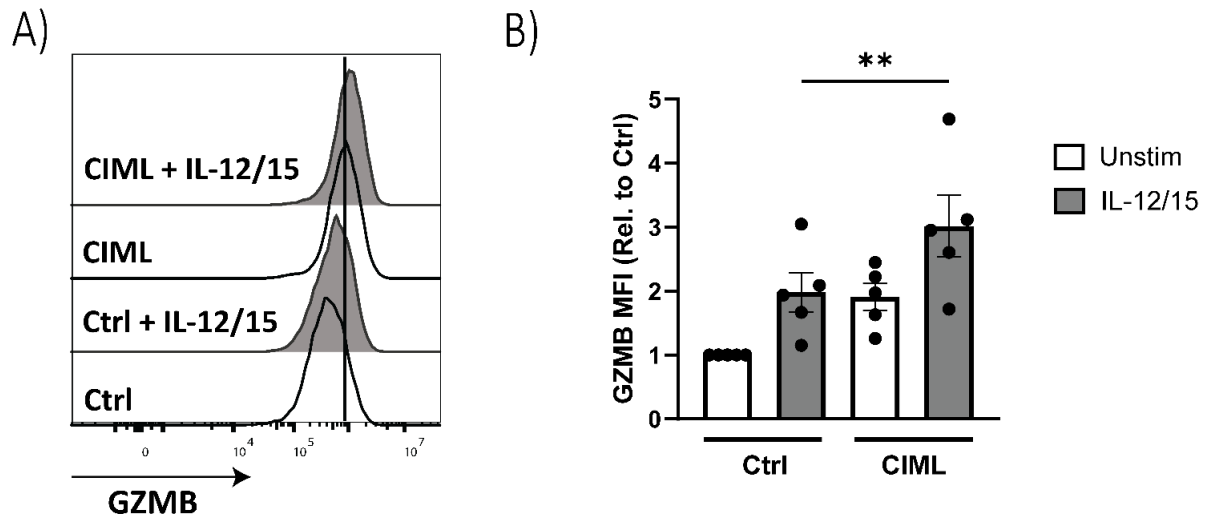


Figure 3.5. Granzyme B expression is increased in CIML NK cells post-restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. **(A,B)** Flow cytometry analysis of granzyme B (GZMB) expression on Ctrl vs CIML NK cells +/- IL-12/15. **(A)** Representative histogram showing the expression of granzyme B and **(B)** pooled MFI of granzyme B expression on Ctrl and CIML NK cells +/- IL-12/15 relative to Ctrl resting NK cells. Data are **(A)** representative or **(B)** mean +/- SEM of N=5 donors. Statistical analysis was performed using a paired t-test. (**p<0.01).

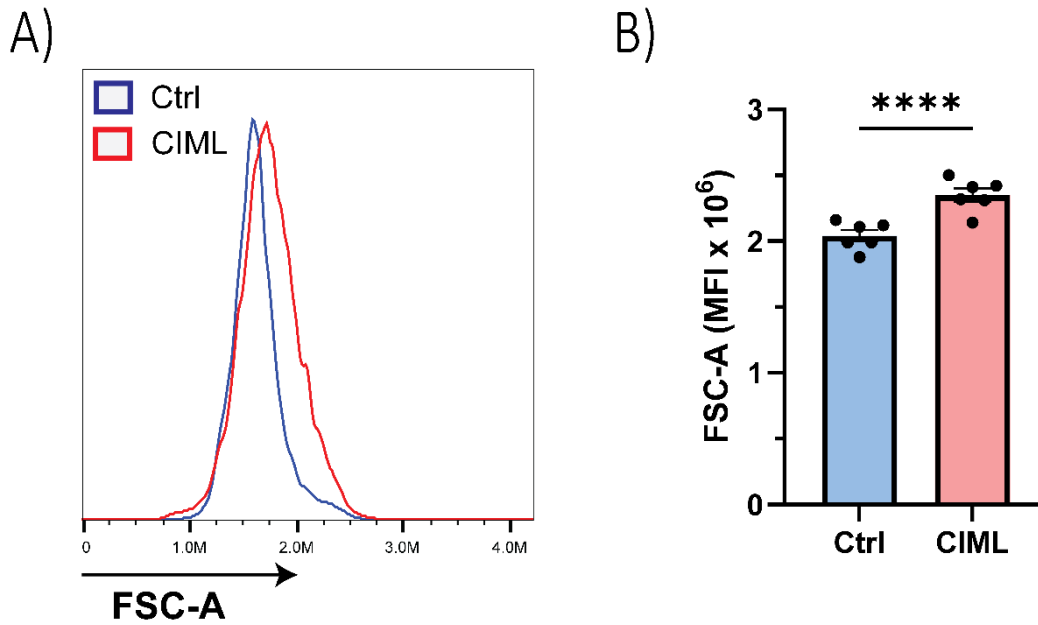


Figure 3.6. CIML NK cells have increased cell size.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. **(A,B)** Flow cytometry analysis of Ctrl and CIML NK cells size by forward scatter (FSC-A) measurement. **(A)** Representative histogram and **(B)** pooled MFI of FSC-A in Ctrl and CIML NK cells. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=6 donors. **(B)** Statistical analysis was performed using a paired t-test. (****p<0.0001).

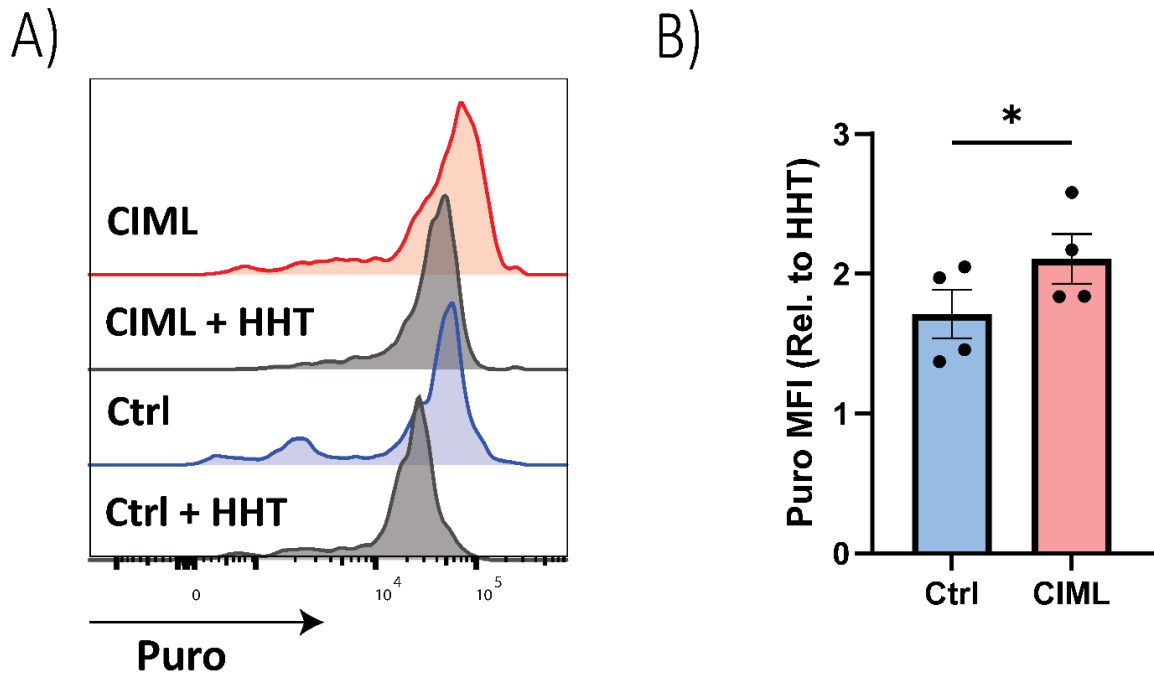


Figure 3.7. CIML NK cells have increased rates of protein synthesis.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Protein synthesis rate was determined by administering puromycin to cells and measuring incorporation into nascent polypeptide chains. Puromycin staining was performed using a click chemistry puromycin analogue (OPP) at a final concentration of 20 μ M. The click-chemistry reaction was also performed on cells treated with 2 μ g/ml of homoharringtonine (HHT), a protein synthesis inhibitor, to account for non-specific staining (grey histograms). **(A,B)** Flow cytometry analysis of puromycin (Puro) incorporation into proteins as a proxy measurement for protein synthesis rates in Ctrl and CIML NK cells. **(A)** Representative histogram showing the expression of puromycin incorporation and **(B)** pooled MFI of puromycin incorporation in Ctrl and CIML NK cells relative to Ctrl and CIML NK cells + HHT. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (* p <0.05).

3.4 Compartmentalisation of protein content in control and CIML NK cells.

NK cells make up approximately 10-15% of circulating lymphocytes in human blood. To perform proteomic and metabolomic analysis, it is important to obtain a highly pure NK cell population to avoid contaminating proteins and metabolites from other cells. To achieve this, PBMC were isolated from healthy fresh human blood and stained for CD56 and CD3, and NK cells were sorted on the FACS Aria as the CD56⁺CD3⁻ cell population (Figure 2.3). Purified NK cells were then plated and stimulated with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) or given a low dose of IL-15 (1 ng/ml) as a control for 18 hours. The next day, NK cells were washed three times in PBS and resuspended in media supplemented with low-dose IL-15 and left for 7 days, replacing IL-15-supplemented media on days 3 and 5 as outlined above (Figure 2.2.2). On day 7, control and CIML NK cells were washed three times in HBSS, and the cell pellets were snap frozen in liquid nitrogen. Control and CIML NK cells (n=4 paired) were sent to the core proteomic facility in Dundee for mass-spec (MS) analysis. Samples were digested, and subsequent peptides were run on an ultra-high performance liquid chromatography (UHPLC) coupled MS system. Data was acquired on a data independent acquisition mode (DIA), and subsequent analysis was performed using Spectronaut v19 using direct DIA and samples were normalised using the global median. Copy numbers of each protein were generated using Spectronaut outputs in the Perseus software and the Proteomic Ruler plugin. However, in this chapter, only differences in the proteomes of untreated, basal control and CIML NK cells will be compared. A total of 5103 viable proteins were detected across the samples after outliers were removed. Consistent with FSC-A and protein synthesis data, comparative global protein analysis demonstrated that there was a trend towards increased total protein in CIML NK cells compared to control NK cells (Figure 3.8).

The distribution of proteins into specific cellular compartments can give functional insights into biological pathways of importance in immune cells. To compare global differences in the proteomic landscapes of control and CIML NK cells, the subcellular distribution of proteins was investigated to better understand if cytokine-induced training resulted in altered compartmentalisation of total protein. To do this, expression of proteins from compartments such as the plasma

membrane (GO:0005885), mitochondria (GO:0005739), nuclear envelope (GO:0005635), endoplasmic reticulum (GO:0005783), cytotoxicity (GO:0042267) and motility (GO:0048870) were calculated and averaged across donors as a percentage of total cellular protein content per cell. Using this approach, modest differences in percentage distributions were observed, demonstrating marginally increased percentages of mitochondrial, nuclear envelope and cytotoxic proteins in CIML NK cells (Figure 3.9A,B). However, when compartmentalisation of proteins was compared in a quantitative pairwise fashion, CIML NK cells demonstrated a trend towards increased total protein in the plasma membrane, mitochondria, nuclear envelope, motility and cytotoxicity (Figure 3.10). This is in agreement with other CIML NK cell studies, which have identified increases in mitochondrial mass and granzyme B reservoirs [215, 221].

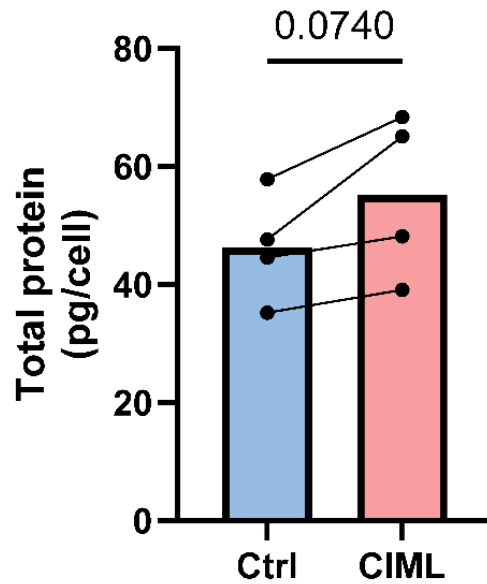


Figure 3.8. CIML NK cells exhibit a potential increase in total protein.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Quantitative proteomics was performed on resting Ctrl and CIML NK cells, analysing the total protein content per cell across individual donors. Total protein content of donor-matched Ctrl and CIML NK cells. Total protein content per cell was calculated as $\sum ((CN \times MW)/N)$ where CN = Protein copy number, MW = Protein molecular weight, N = Avogadro's constant. Data are the mean $\pm$ SEM of N = 4 donors. Statistical analysis was performed using a paired t-test.

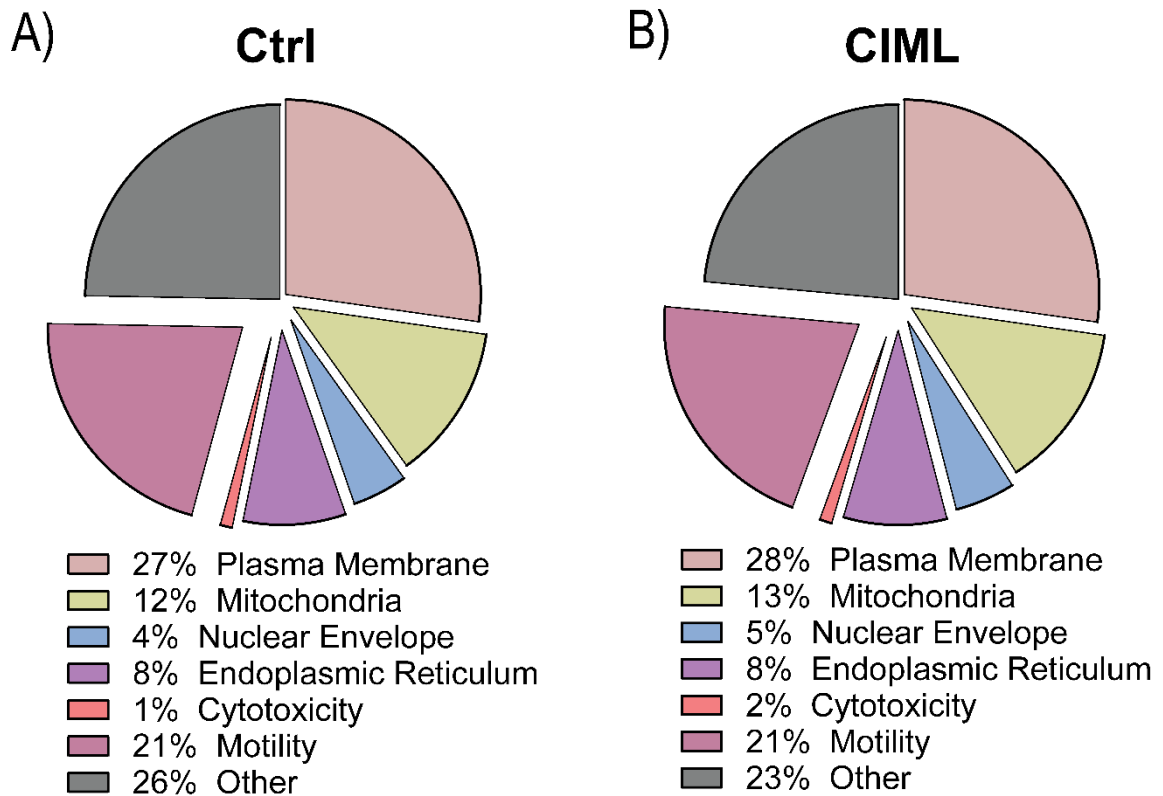


Figure 3.9. Average distribution of proteins into cellular compartments in control and CIML NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. **(A,B)** Quantitative proteomics was performed on resting Ctrl and CIML NK cells, analysing the average distribution of proteins into cellular compartments. The protein content of plasma membranes (GO:0005886), mitochondria (GO:0005739), nuclear envelope (GO:0005635), endoplasmic reticulum (GO:0005783), cytotoxicity (GO:0042267) and motility (GO:0048870) as a percentage of total protein content in **(A)** Ctrl NK cells and **(B)** CIML NK cells. N=4 donors.

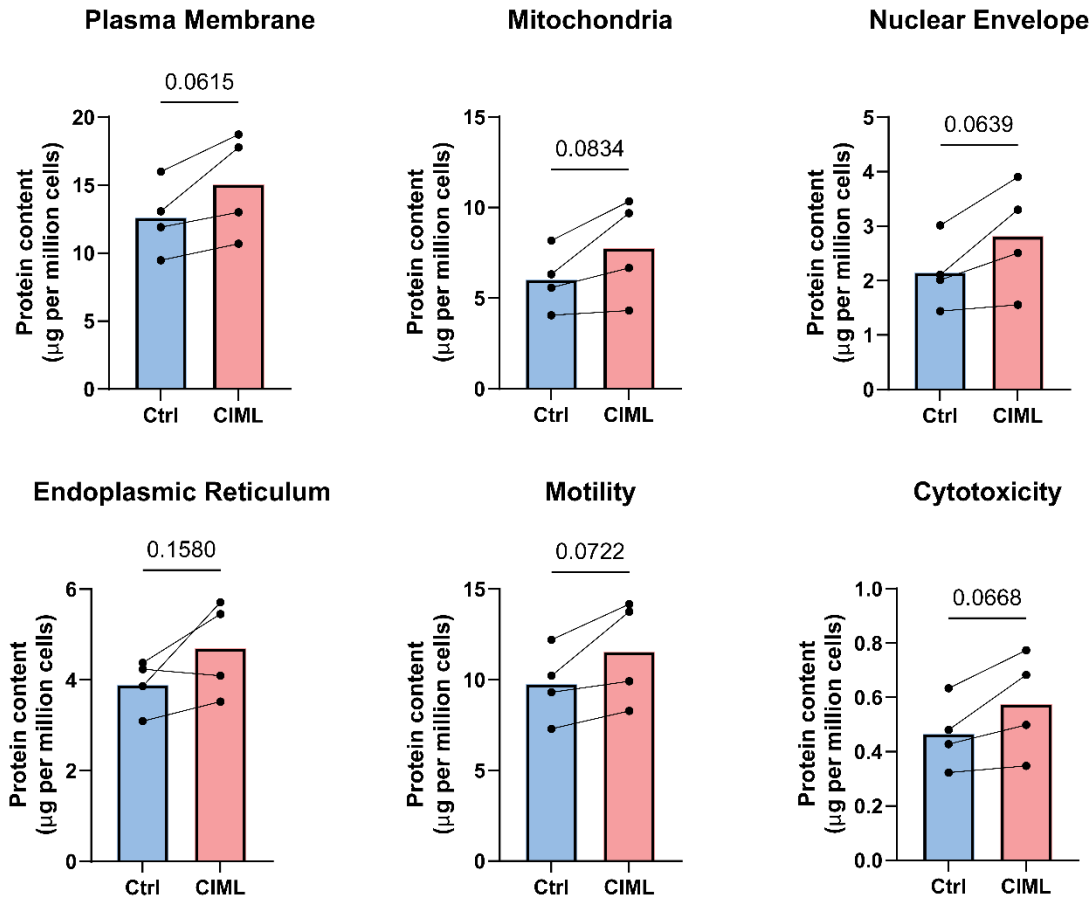


Figure 3.10. Pairwise distribution of proteins into cellular compartments in control and CIML NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Quantitative proteomics was performed on resting Ctrl and CIML NK cells, analysing the paired distribution of total proteins into cellular compartments. The protein content of plasma membranes (GO:0005886), mitochondria (GO:0005739), nuclear envelope (GO:0005635), endoplasmic reticulum (GO:0005783), cytotoxicity (GO:0042267) and motility (GO:0048870) in Ctrl NK cells and CIML NK cells. Total protein content per million cells in micrograms was calculated as $\Sigma(((CN \times MW)/N) \times 10^{12})$ where CN = Protein copy number, MW = Protein molecular weight and N = Avogadro's constant. Data are the mean $\pm$ SEM of N=4 donors. Statistical analysis was performed using a paired t-test.

3.5 Differential expression analysis reveals proteomic remodelling that favours proliferation in CIML NK cells.

To investigate proteins with significantly different expression levels between control and CIML NK cells, differential expression analysis was performed on control and CIML NK cell protein copy numbers. Differentially expressed proteins were identified as proteins with a log₂ fold change of greater than 1 or less than -1 and that were statistically significant ($p < 0.05$). This identified 388 differentially expressed proteins, 81 of which were upregulated in control NK cells and 307 were upregulated in CIML NK cells (Figure 3.11). Chip-X enrichment analysis was then used to identify which transcription factors were highly associated with differentially expressed proteins. Significantly enriched transcription factors were plotted with a significance threshold of adjusted p-value < 0.05 . The top 10 enriched transcription factors in CIML NK cells were all associated with the cell cycle, cell division, cell proliferation and DNA replication, notably half of which belonged to the E2F family (Figure 3.12). Comparatively, analysis of differentially expressed proteins in control NK cells resulted in only 4 enriched transcription factors, and these were also associated with cell cycle progression (Figure 3.12). To investigate functional pathway enrichment, the Reactome online software was used to identify enriched cellular activities with a threshold of adjusted p-value < 0.05 . Reactome pathway analysis of differentially expressed proteins in CIML NK cells again revealed enriched proliferative pathways such as cell homology directed repair through homologous recombination, cell cycle and DNA repair (Figure 3.13). Reactome analysis of differentially expressed proteins in control NK cells revealed only 4 significantly enriched pathways, which were associated with platelet function, amino acids activating mTORC1 and hemostasis (Figure 3.13).

Gene set enrichment analysis (GSEA) is a statistical method of classifying groups of genes and, in this case, proteins, that are over-represented in terms of biological process. GSEA was performed on the entire proteomes of both control and CIML NK cells to investigate if there was a total proteomic shift favouring certain functions and pathways in CIML NK cells. Supporting what was observed in pathway analysis of differentially expressed proteins, GSEA revealed that the CIML NK cell proteome was enriched in proteins regulated by the E2F family

(Figure 3.14). Furthermore, it was observed that there was a significant increase in the total number of CIML NK cells compared to control NK cells based on cell count. Finally, a significant increase in expression of the proliferation marker Ki-67 was observed in CIML NK cells, as assessed using flow cytometry (Figure 3.15A-C). Taken together, this data reveals that the CIML NK cell proteome undergoes a shift towards a proliferative phenotype.

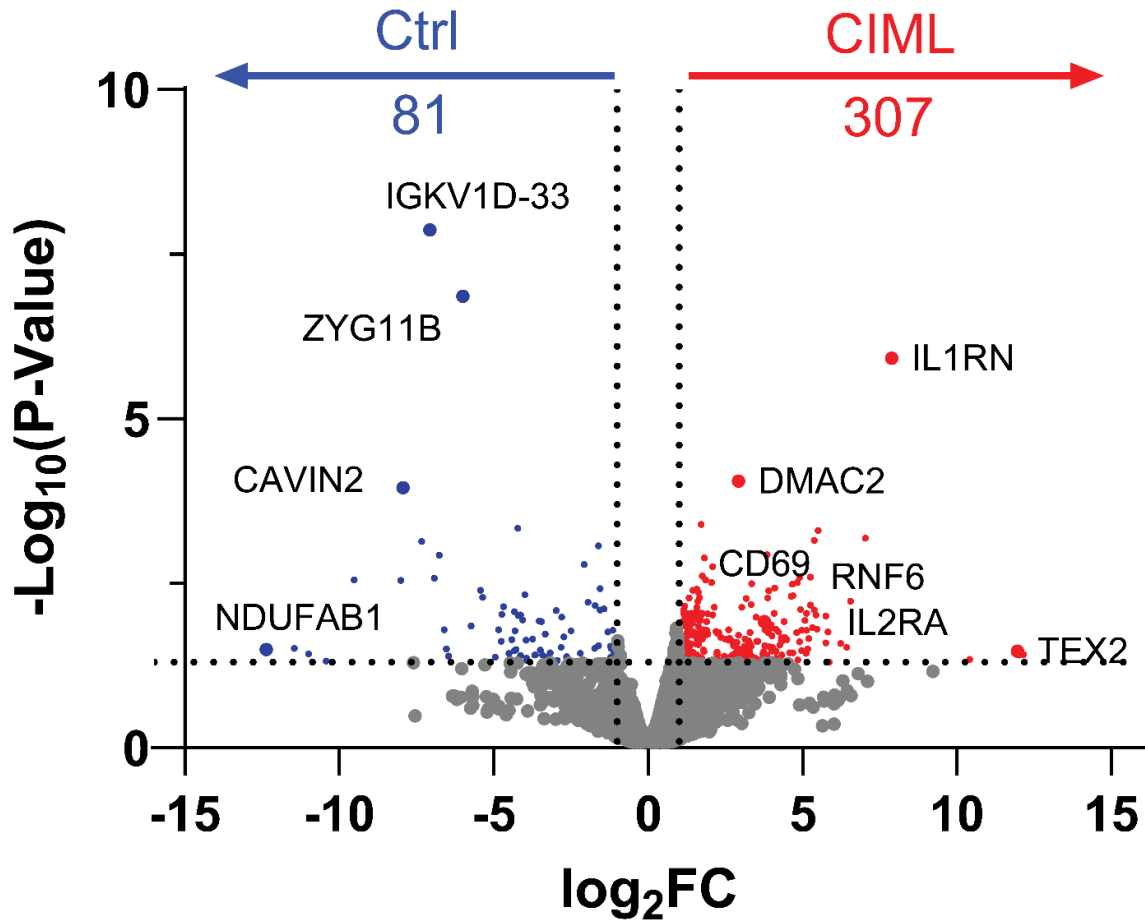


Figure 3.11. Differential expression analysis of control and CIML NK cell protein expression.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Volcano plot of 5103 total identified proteins displaying differences in relative abundance between Ctrl and CIML NK cells. The plot represents the $-\log_{10}$ of the p-value versus $\log_2$ of the fold change of protein expression. Differentially expressed proteins (red and blue) have a $\log_2$ fold change >1 or <-1 and a $-\log_{10}(\text{p-value}) > 1.35$. Proteins highlighted in red are enriched in CIML NK cells, and those highlighted in blue are enriched in Ctrl NK cells.

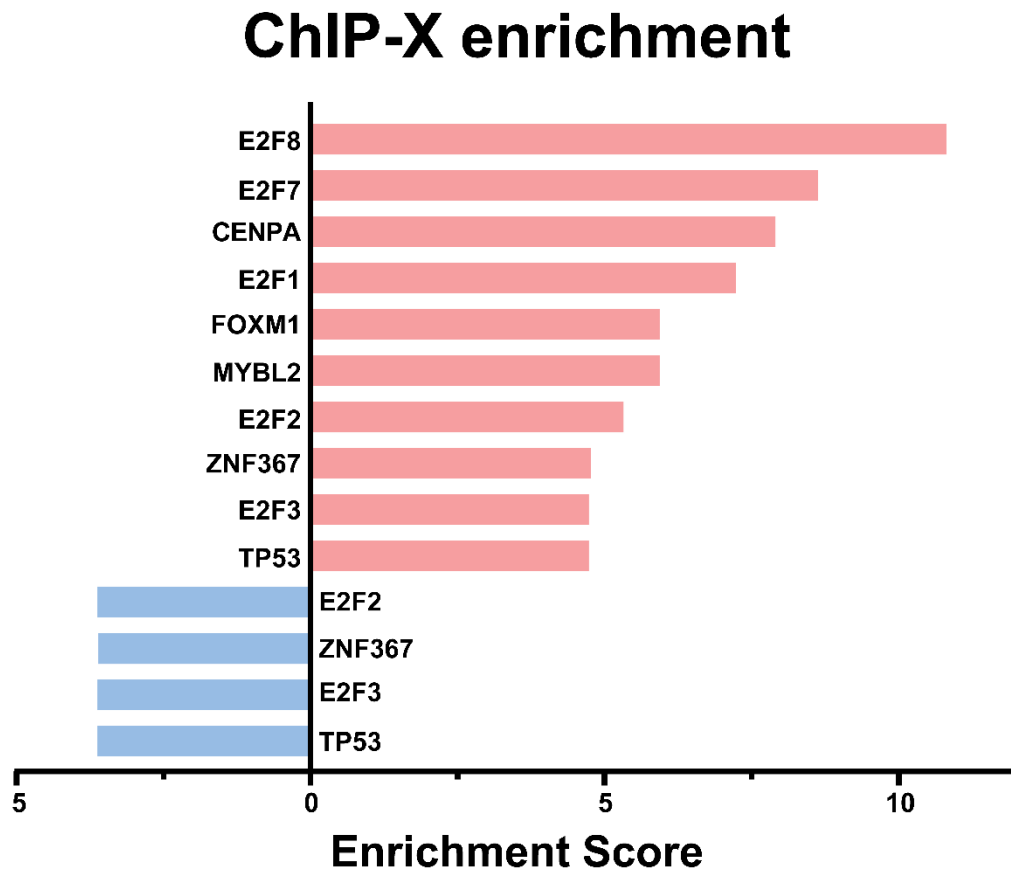


Figure 3.12. ChIP-X enrichment analysis of differentially expressed proteins in control and CIML NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Quantitative proteomics was performed on Ctrl and CIML NK cells, analysing enriched associated transcription factors. Significantly upregulated ($\text{Log}_2\text{FC} > 1$, $p\text{-value} < 0.05$) proteins in Ctrl and CIML NK cells were analysed for transcription factor enrichment using the ChIP-X enrichment database (ARCHS4 library). Transcription factors highly associated with differentially expressed proteins in Ctrl and CIML NK cells are shown in blue and red, respectively. False discovery rate cutoff = $\text{FDR} < 0.1$. Enrichment score = $-\text{Log}_{10}\text{Adj.pvalue}$. Quantitative proteomics was performed on N=4 donors.

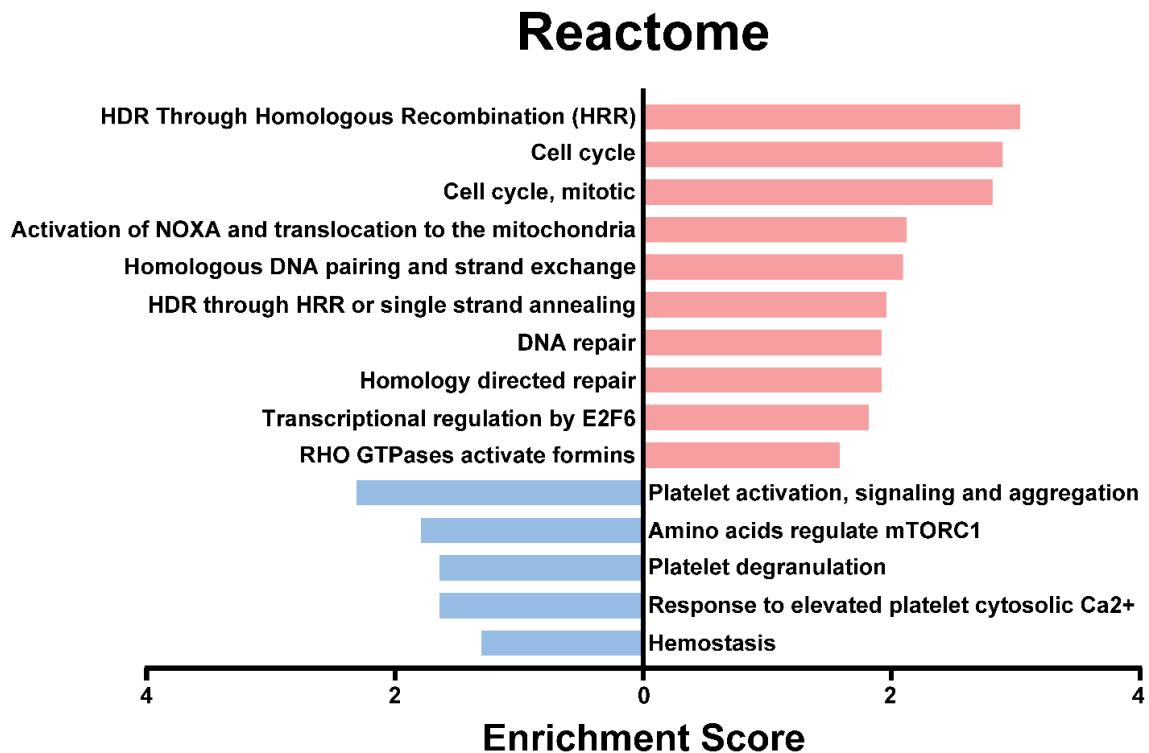


Figure 3.13. Reactome pathway analysis of differentially expressed proteins in control and CIML NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Quantitative proteomics was performed on Ctrl and CIML NK cells, analysing enriched biological pathways. Significantly upregulated ($\text{Log}_2\text{FC} > 1$, $p\text{-value} < 0.05$) proteins in Ctrl and CIML NK cells were analysed for functional enrichment using the Reactome database. Pathways highly associated with differentially expressed proteins in Ctrl and CIML NK cells are shown in blue and red, respectively. Enrichment score = $-\text{Log}_{10}\text{Adj.pvalue}$. Quantitative proteomics was performed on N=4 donors.

HALLMARK E2F Targets

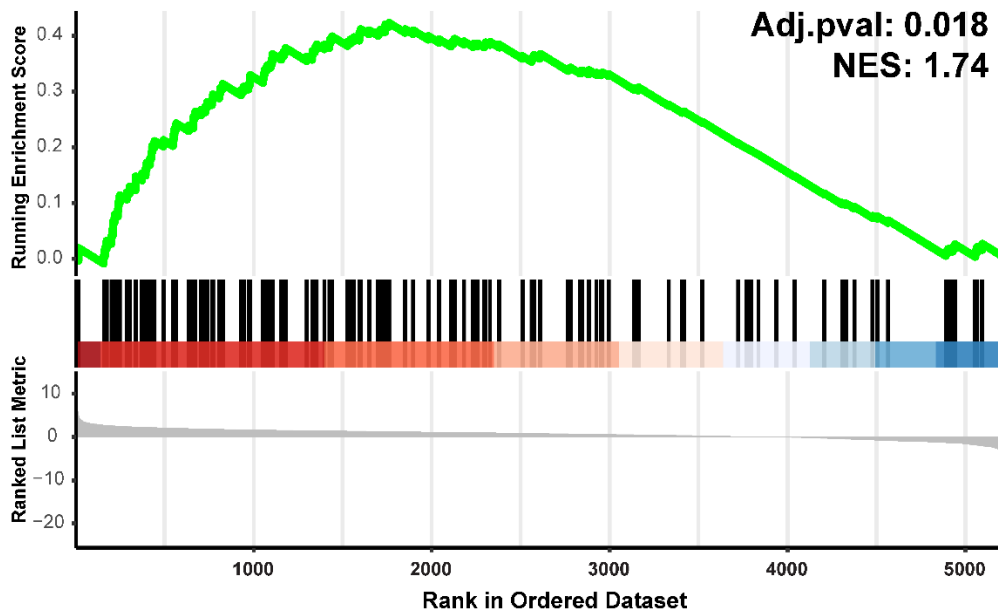


Figure 3.14. Whole proteome gene set enrichment analysis reveals upregulated E2F target pathway in CIML NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. GSEA was performed on the entire proteomic datasets to identify pathways enriched across the full range of significantly upregulated protein expression, independent of differential expression thresholds. Proteins were ranked by expression differences between Ctrl and CIML NK cells, and enrichment was assessed using predefined pathway gene sets. Normalised enrichment scores (NES) are shown, with positive values indicating pathways enriched in CIML NK cells. Enriched CIML NK cell pathways included HALLMARK E2F Targets (M5925). Pathways with an adjusted p-value of < 0.05 were considered statistically significant.

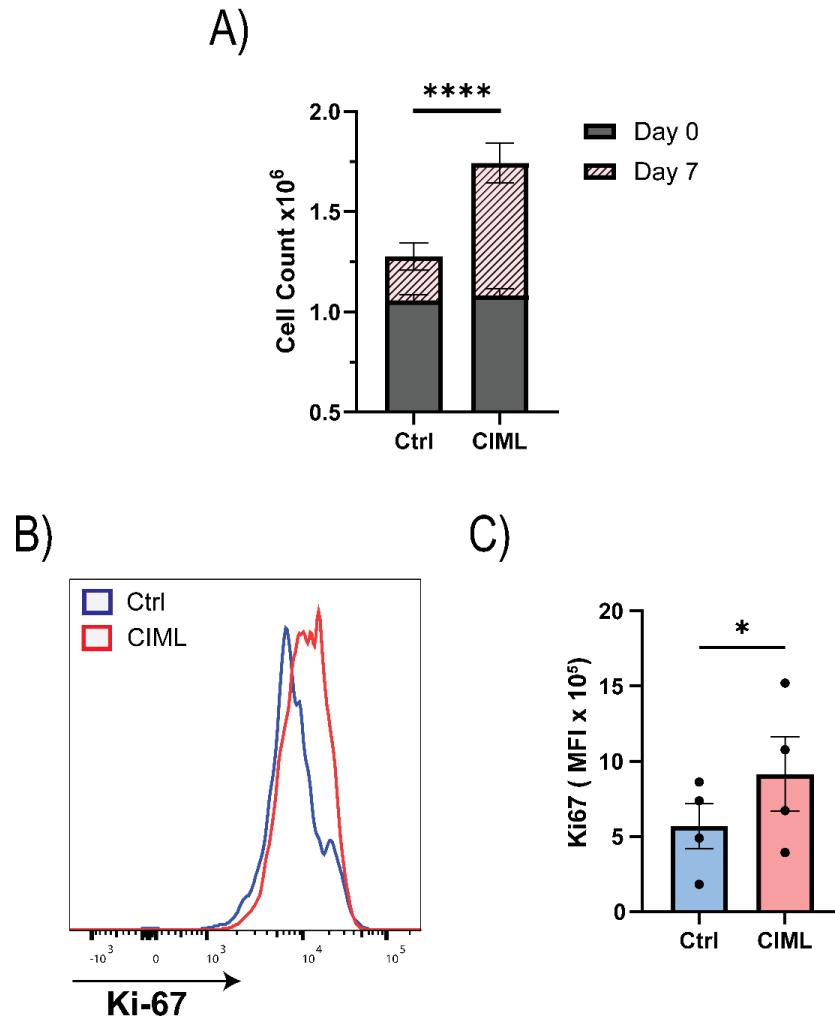


Figure 3.15. CIML NK cells have significantly increased cell counts and Ki-67 expression at rest.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. **(A-C)** Proliferation data for CIML NK cells vs Ctrl NK cells. **(A)** Cell count numbers of Ctrl and CIML NK cells on day 0 and day 7. **(B,C)** Flow cytometry analysis of Ctrl and CIML NK cells proliferation by Ki-67 expression. **(B)** Representative histogram and **(C)** pooled raw MFI values of Ki-67 expression in Ctrl and CIML NK cells. Data are representative **(B)** or mean $\pm$ SEM of N=4-8 donors **(A,C)**. Data was analysed using a paired t-test (* $p < 0.05$, **** $p < 0.0001$).

3.6 Integrated proteomics and metabolomics reveal enhanced glycolysis, pentose phosphate pathway, and purine metabolism in CIML NK cells.

Cellular metabolism is a vital cornerstone for supporting the demands of a fully functional and active immune cell. Different cell types in different states preferentially use certain metabolic pathways to sustain their survival and immune potential. As CIML NK cells are highly proliferative and clearly capable of mounting efficient and potent effector functions, an analysis was undertaken to investigate their cellular metabolism to identify what pathways are important in sustaining this phenotype. To do this, metabolic characterisation of CIML NK cells was undertaken by integrating proteomic and metabolomic analyses to distinguish important metabolic machinery/metabolite intermediates in control and CIML NK cell activity.

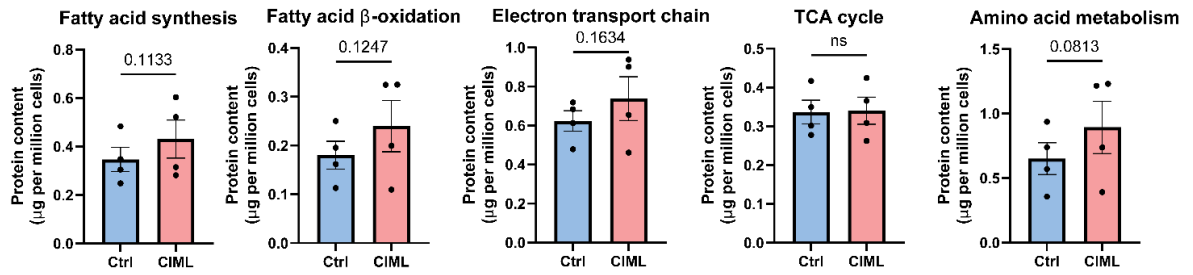
Firstly, the total protein content of the major metabolic pathways was analysed as outlined in Section 2.2.5.3. There were no significant changes in FAS (GO:0006633), FAO (GO:0006635), ETC (GO:0022900), TCA cycle (GO:0006099), and amino acid metabolism (GO:0006520) associated protein content (Figure 3.16A). However, there was a significant increase in protein content of both the glycolytic pathway (GO:0006096) and the PPP (GO:0006098) in CIML NK cells (Figure 3.16B). Comparisons of protein copy numbers demonstrated significantly increased expression of individual glycolytic enzymes such as GPI, ALDOA, TPI, PGK1, PGM1, ENO1, and LDHA in CIML NK cells (Figure 3.17). In the oxidative phase of the PPP, although there was no change in the rate-limiting PPP enzyme G6PD, there was a significant increase in PGD (Figure 3.18A) and in the non-oxidative PPP, TALDO1 was significantly upregulated and a trend towards increased expression of TKT (Figure 3.18B). To investigate this increase in glycolytic protein content further, targeted metabolomics was performed on control versus CIML NK cells. A universally labelled, heavy carbon glucose tracer (U-13C glucose) was used to validate glycolytic flux at the metabolomic level and to investigate the molecular fate of glucose as a nutrient for CIML NK cells. Control and CIML NK cells were incubated for 24 hours with U-13C glucose-supplemented media, and both supernatants and cell pellets were collected. In agreement with the proteomic data and as prior studies have also suggested, metabolomic analysis revealed

that glycolysis is upregulated in CIML NK cells. Metabolomic analysis revealed a trend towards decreased total glucose (Figure 3.19 A) and a significant increase in pyruvic acid and lactic acid (the end products of glycolysis), in the supernatants from CIML NK cells (Figure 3.19B,C). Furthermore, U-¹³C glucose tracing revealed that there was a significant increase of ¹³C labelling in the entire pyruvic acid and lactic acid pools in the supernatants from CIML NK cells (Figure 3.20A,C), indicating increased flux of ¹³C-glucose into CIML NK cells and increased expulsion of end products into the supernatants. There was also a significant increase in M+3 labelled pyruvic acid and an increase, albeit not significant, in M+3 lactic acid in supernatants from CIML NK cells (Figure 3.20B,D), indicating that they are derived from the original “tracing” glucose.

As the PPP siphons glucose-6-phosphate from glycolysis, CIML NK cells may be using glucose for PPP-associated processes. The PPP is one of the main sources of NADPH in the cell, which is a vital reducing agent that sustains redox homeostasis and promotes FAS, resulting in R5P formation, an essential intermediate in nucleotide synthesis. Metabolomic analysis of control versus CIML NK cells revealed a significant increase in intracellular nucleoside monophosphates such as AMP and GMP (Figure 3.21A), which are synthesised from IMP, a product of R5P metabolism. Furthermore, CIML NK cells had significantly increased levels of the nucleoside inosine, but not guanosine or adenosine (Figure 3.21B). The purines hypoxanthine and guanine were significantly increased in CIML NK cells, whereas no change was observed in adenine pools (Figure 3.21C). ¹³C glucose tracing also revealed significantly increased M+5 labelling of AMP and a non-significant increase in M+5 labelling of GMP and inosine in CIML NK cells. Although there were no changes observed in total adenosine pools, a trending increase in M+5 labelling was observed in CIML NK cells (Figure 3.22A). In support of this, proteomic analysis revealed a significant increase in many of the enzymatic mediators of this pathway. PRPP has a central role in nucleotide metabolism and is formed by PRPS from R5P and ATP, with subsequent reactions culminating in IMP formation. Although there was no change in PRPS1 expression, there was a trending increase in PRPS2 protein expression (p=0.065) (Figure 3.23). Enzymes downstream of this, including IMPDH2, NT5DC1 and PPP, were also found to be significantly

upregulated in CIML NK cells (Figure 3.23). These findings suggest that CIML NK cells upregulate glycolysis and the PPP, committing more glucose-derived carbons to purine biosynthesis compared to control NK cells.

A)



B)

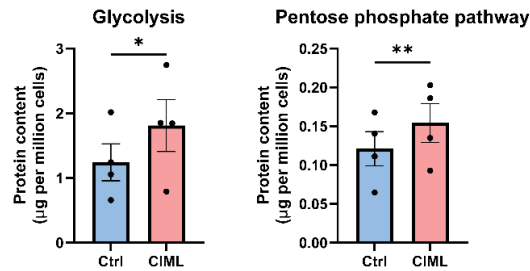


Figure 3.16. Pairwise analysis of total protein content of metabolic pathways in control and CIML NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. **(A,B)** Quantitative proteomics was performed on resting Ctrl and CIML NK cells, analysing paired total protein of metabolic pathway machinery. **(A)** non-significant protein content changes of FAS (GO:0006633), FAO (GO:0006635), ETC (GO:0022900), TCA cycle (GO:0006099), amino acid metabolism (GO:0006520), and **(B)** significant changes in protein content of glycolysis (GO:0006096) and the PPP (GO:0006098) in Ctrl NK cells and CIML NK cells. Total protein content per million cells in micrograms was calculated as $\Sigma(((CN \times MW)/N) \times 10^{12})$ where CN = Protein copy number, MW = Protein molecular weight and N = Avogadro's constant. Data is mean $\pm$ SEM of N=4 donors **(A,B)**. Statistical analysis was performed using a paired t-test. (* $p < 0.05$, ** $p < 0.01$)

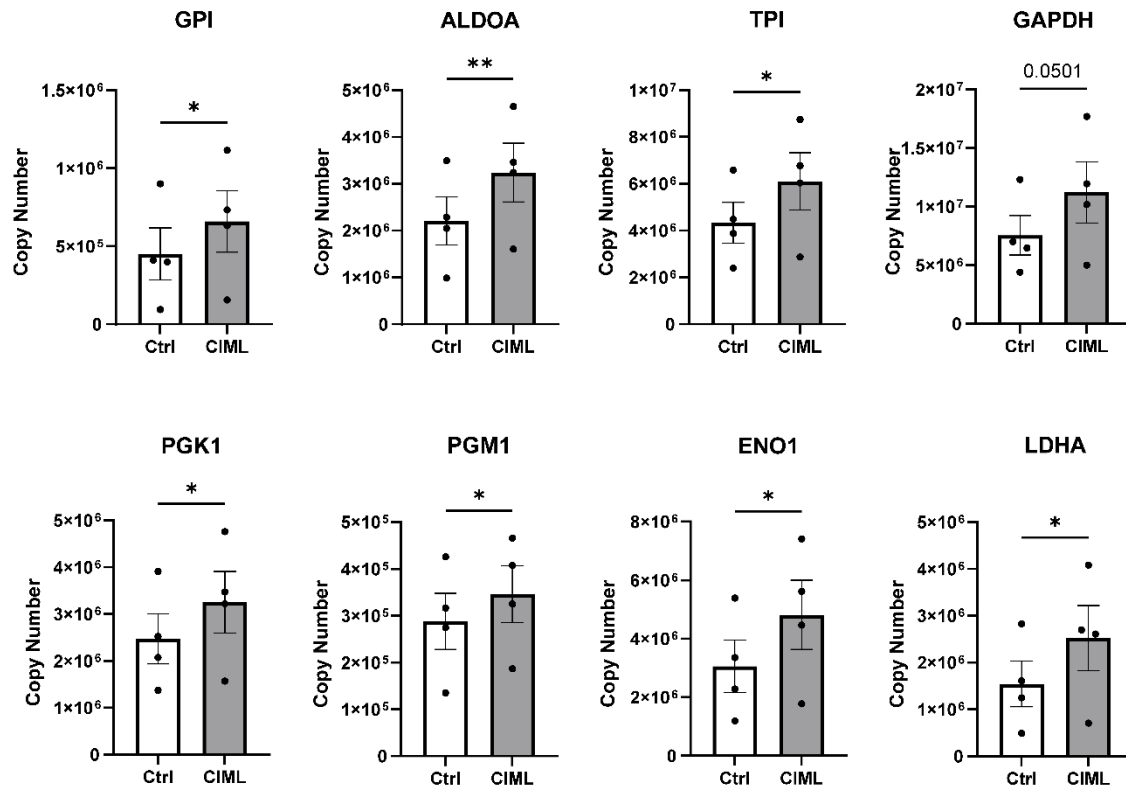


Figure 3.17. Glycolytic proteins are upregulated in CIML NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Quantitative proteomics was performed on resting Ctrl and CIML NK cells, analysing paired copy numbers per cell of glycolytic machinery. Data are the mean $\pm$ SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (*p<0.05, **p<0.001).

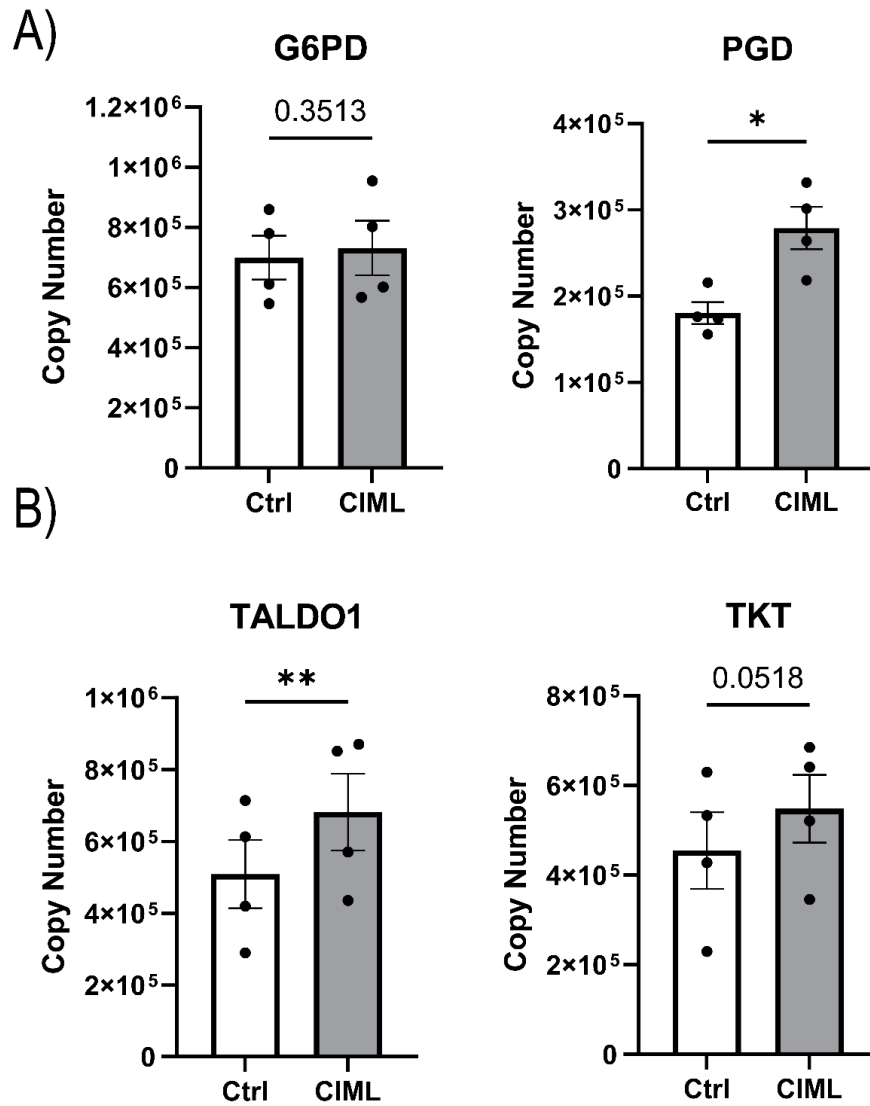


Figure 3.18. The PPP proteins, PGD and TALDO1, are upregulated in CIML NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. **(A,B)** Quantitative proteomics was performed on resting Ctrl and CIML NK cells, analysing paired copy numbers per cell of **(A)** oxidative and **(B)** non-oxidative PPP machinery. Data are the mean $\pm$ SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (* $p < 0.05$, ** $p < 0.001$).

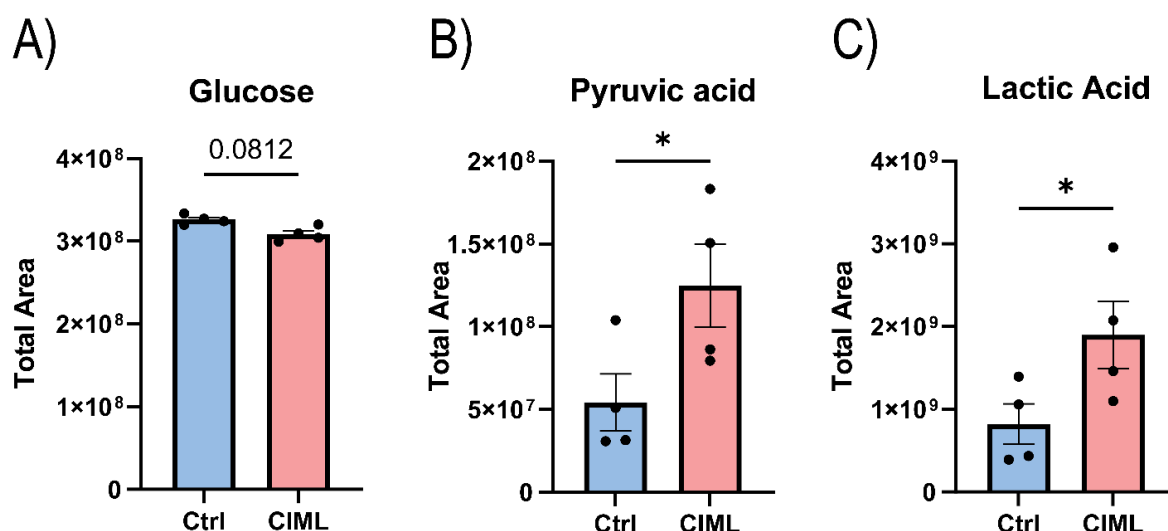


Figure 3.19. Glycolytic products are significantly upregulated in supernatants from CIML NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 6 days, the Ctrl and CIML NK cells were washed in PBS and incubated for 24 hours with U- ^{13}C glucose-supplemented media. The next day, supernatants were harvested for analysis. **(A-C)** Metabolomic analysis was carried out on the total unlabelled and ^{13}C labelled metabolite pools in supernatants from Ctrl and CIML NK cells, analysing metabolite total area intensities. Supernatant intensities of **(A)** glucose, **(B)** pyruvic acid and **(C)** lactic acid in Ctrl vs CIML NK cells. Data are the mean $\pm$ SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (* $p < 0.05$).

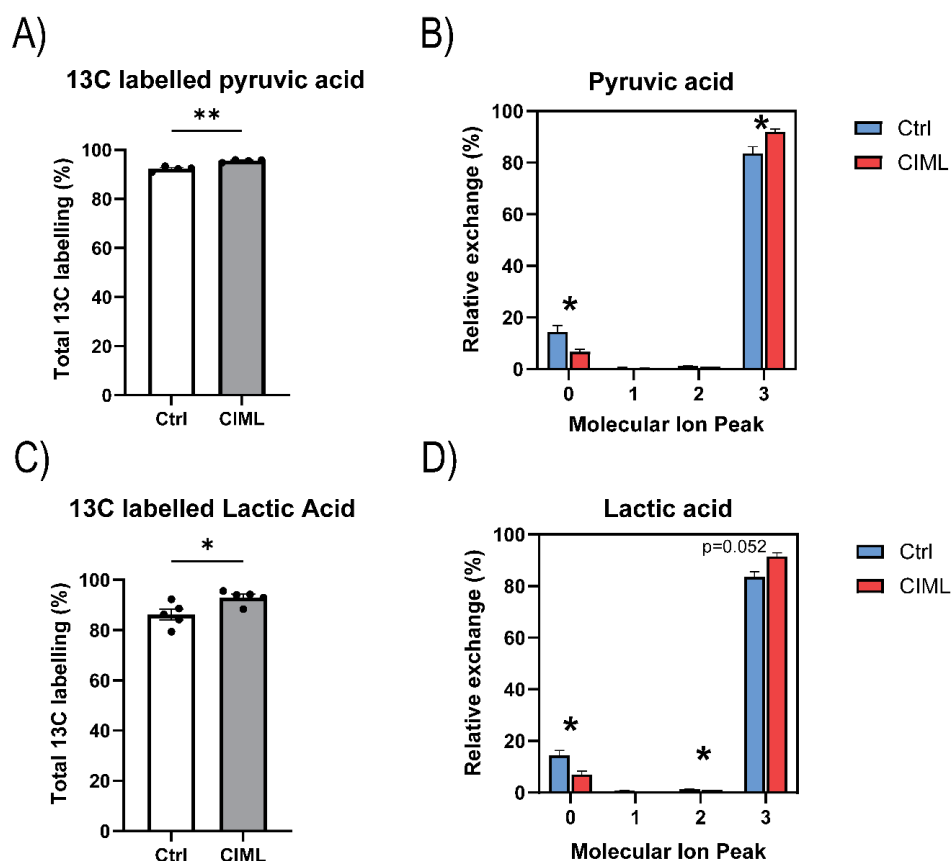


Figure 3.20. ^{13}C labelling of glycolysis products in control and CIML NK cell supernatants.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 6 days the Ctrl and CIML NK cells were washed in PBS and incubated for 24-hours with U- ^{13}C glucose-supplemented media. The next day, supernatants were harvested for analysis. **(A-D)** Targeted metabolomics was carried out, analysing ^{13}C labelling of metabolites in supernatants from resting Ctrl and CIML NK cells. Total ^{13}C labelling **(A)** pyruvic acid and **(C)** lactic acid pools from supernatants, and isotopologue ^{13}C labelling of carbons in **(B)** pyruvic acid and **(D)** lactic acid in supernatants from Ctrl vs CIML NK cells. Total ^{13}C labelling indicates the percentage of metabolite containing a ^{13}C label, and relative exchange indicates the percentage of total metabolite containing a ^{13}C label. Molecular ion Peak number indicates the number of ^{13}C atoms in the molecule. Data are the mean $\pm$ SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (* $p < 0.05$, ** $p < 0.01$)

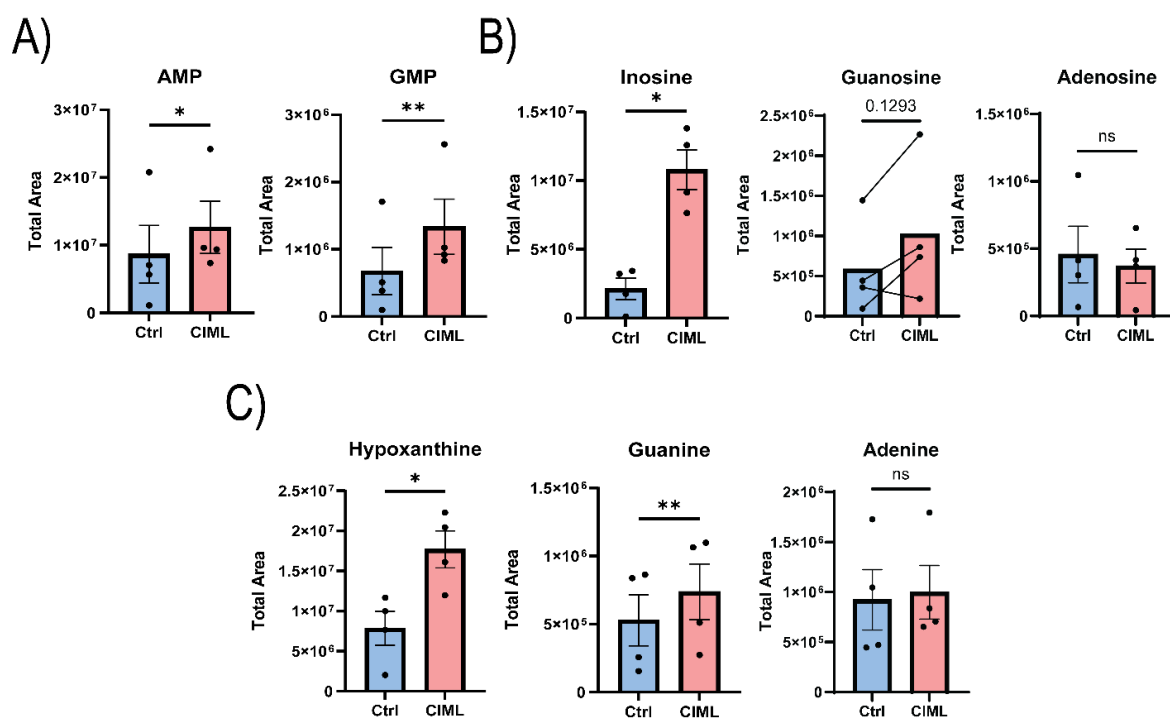


Figure 3.21. CIML NK cells have increased expression of metabolites associated with purine metabolism.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 6 days, the Ctrl and CIML NK cells were washed in PBS and incubated for 24-hours with U-¹³C glucose. The next day pellets were harvested for analysis. **(A-C)** Metabolomic analysis was carried out on the total unlabelled and ¹³C labelled metabolite pools in Ctrl and CIML NK cells, analysing metabolite total area intensities of **(A)** nucleoside monophosphates, **(B)** nucleosides and **(C)** purine bases in Ctrl vs CIML NK cells. Data is mean +/- SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (*p<0.05, **p<0.01).

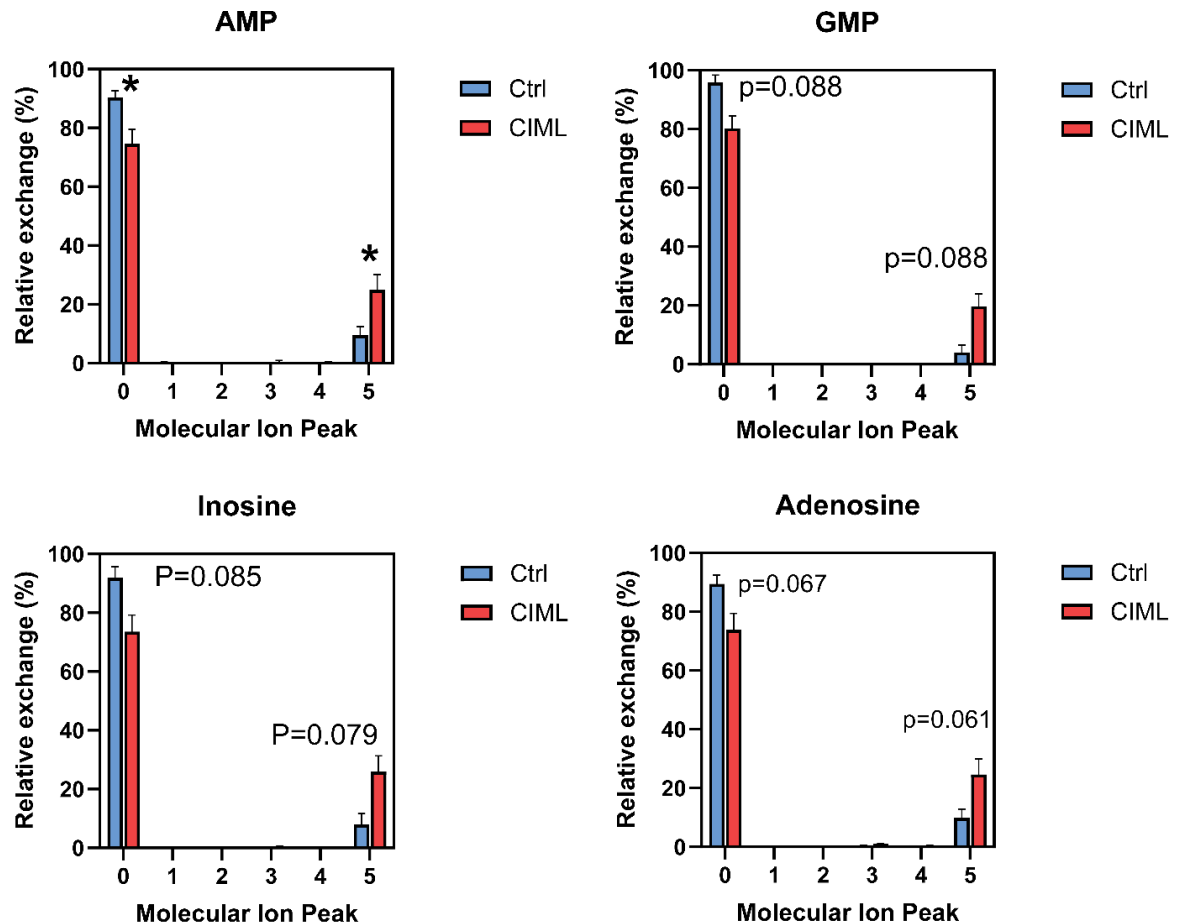


Figure 3.22. CIML NK cells have increased ^{13}C labelling of purine metabolism intermediates.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml) and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 6 days, the Ctrl and CIML NK cells were washed in PBS and incubated for 24 hours with U- ^{13}C glucose. The next day, pellets were harvested for analysis. Targeted metabolomics was carried out on resting Ctrl and CIML NK cells, analysing ^{13}C labelling. Isotopologue ^{13}C labelling of carbons in the purine metabolism pathway. Relative exchange indicates the percentage of total metabolite containing a ^{13}C label, and the molecular ion peak number indicates the number of ^{13}C atoms in the molecule. Data are the mean $\pm$ SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (* $p < 0.05$).

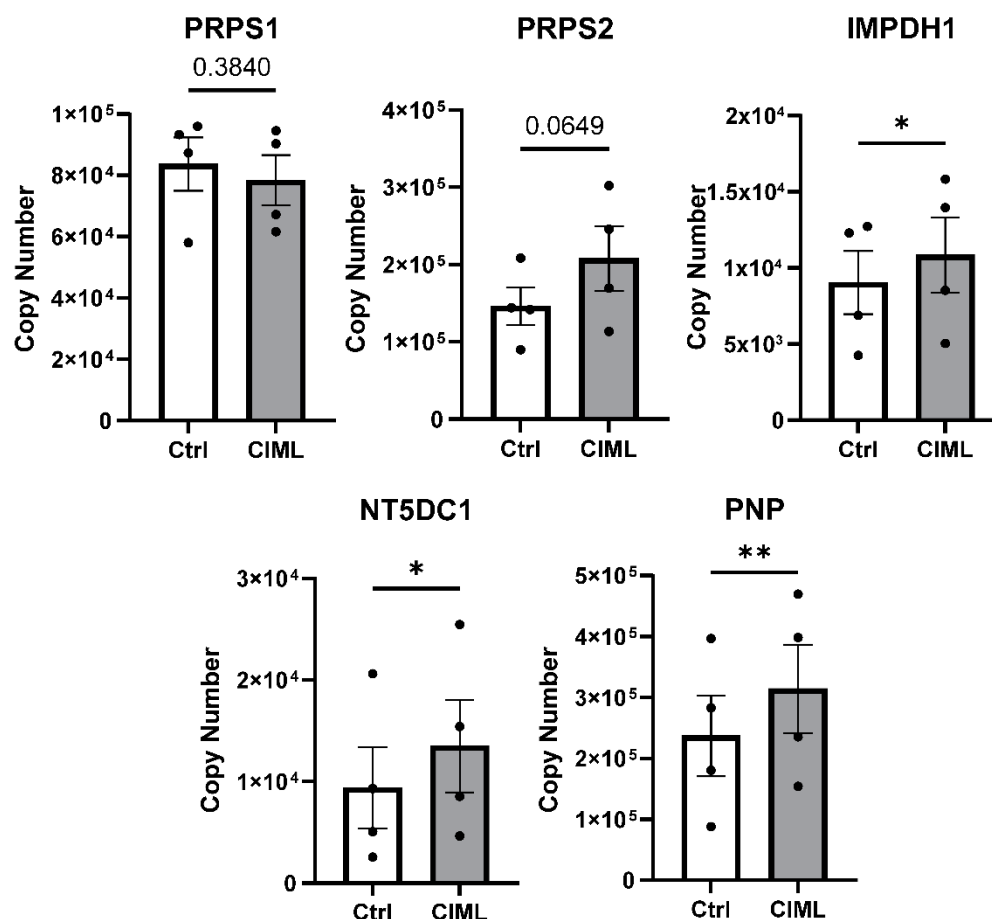


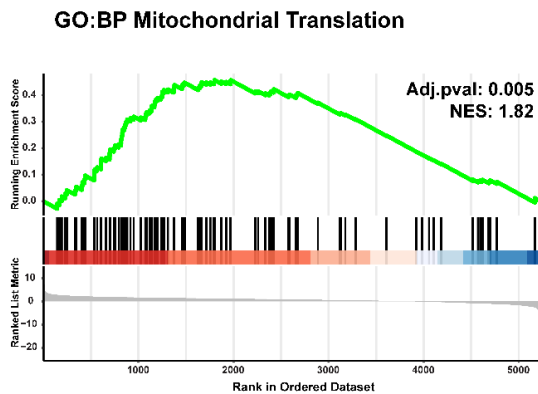
Figure 3.23. Purine metabolism enzymes are upregulated in CIML NK cells.

PBMCs were isolated from fresh healthy human blood, and NK cells were sorted by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Quantitative proteomics was performed on resting Ctrl and CIML NK cells, analysing paired copy numbers per cell of proteins involved in purine metabolism. Data are the mean $\pm$ SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (* $p < 0.05$, ** $p < 0.01$).

3.7 CIML NK cells accumulate dysfunctional mitochondria.

In addition to the E2F target gene set being enriched in CIML NK cells, GSEA analysis also revealed mitochondrial-associated biological processes, namely those associated with translation of mitochondrial proteins (Figure 3.24A,B). To investigate this further, flow cytometry-based assays were performed to measure the mitochondrial characteristics of CIML NK cells. Mitochondrial mass was measured using nonyl acridine orange (NAO), in both control and CIML NK cells, as outlined in Section 2.2.3.1. It was found that CIML NK cells have significantly higher mass at rest compared to control NK cells (Figure 3.25A,B). However, mitochondrial mass is not the only measure of activity, as mitochondria rely on polarisation of their inner membrane via accumulation of ETC-fed protons, which drive ATP synthesis through proton motive force. Furthermore, ROS generated from mitochondrial mass is a major source of total cellular oxidative stress, and its accumulation can be detrimental to cell survival and function. To measure mitochondrial polarisation or mitochondrial membrane potential and mitochondrial ROS, control and CIML NK cells were stained with tetramethylrhodamine methyl ester (TMRM) dye and mitoSOX stain, respectively and found that CIML NK cells had significantly increased mitochondrial membrane potential (Figure 3.26A,B) and mitochondrial ROS (Figure 3.27A,B) at rest compared to control NK cells. However, mitochondrial membrane potential and ROS signal can increase because increased mitochondrial mass will automatically result in increased staining for membrane potential and ROS. To account for this, mitochondrial membrane potential and ROS values were divided by the mitochondrial mass signal. When compared relative to mitochondrial mass, no changes were observed in membrane potential between control and CIML NK cells (Figure 3.28A). However, a significant increase in mitochondrial ROS was still observed in CIML NK cells relative to mitochondrial mass (Figure 3.28B). Furthermore, proteomic analysis revealed no change in total OXPHOS or TCA cycle-associated proteins (Figure 3.29). These data suggest that CIML NK cells have accumulated mitochondrial mass, but mitochondria are not bioenergetically more active compared to control NK cells. CIML NK cells also had significantly increased mitochondrial ROS relative to mass, which may result in more superoxide radical production in CIML NK cells.

A)



B)

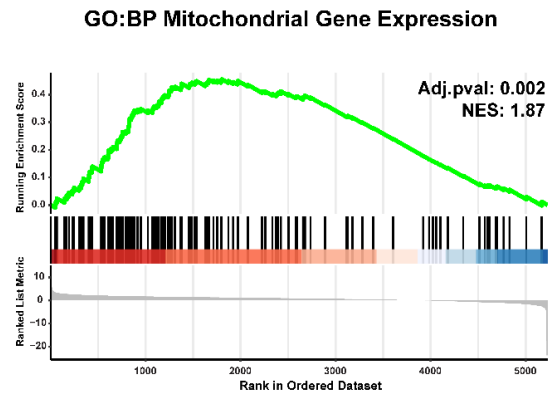


Figure 3.24. GSEA analysis reveals increased mitochondrial protein production in CIML NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. **(A,B)** GSEA was performed on the entire proteomic datasets to identify pathways enriched across the full range of significantly upregulated protein expression, independent of differential expression thresholds. Proteins were ranked by expression differences between Ctrl and CIML NK cells, and enrichment was assessed using predefined pathway gene sets. Normalised enrichment scores (NES) are shown, with positive values indicating pathways enriched in CIML NK cells. Enriched CIML NK cell pathways included **(A)** GO:BP Mitochondrial Translation (M11131) and **(B)** GO:BP Mitochondrial Gene Expression (M24915). Pathways with an adjusted p-value of <0.05 were considered statistically significant.

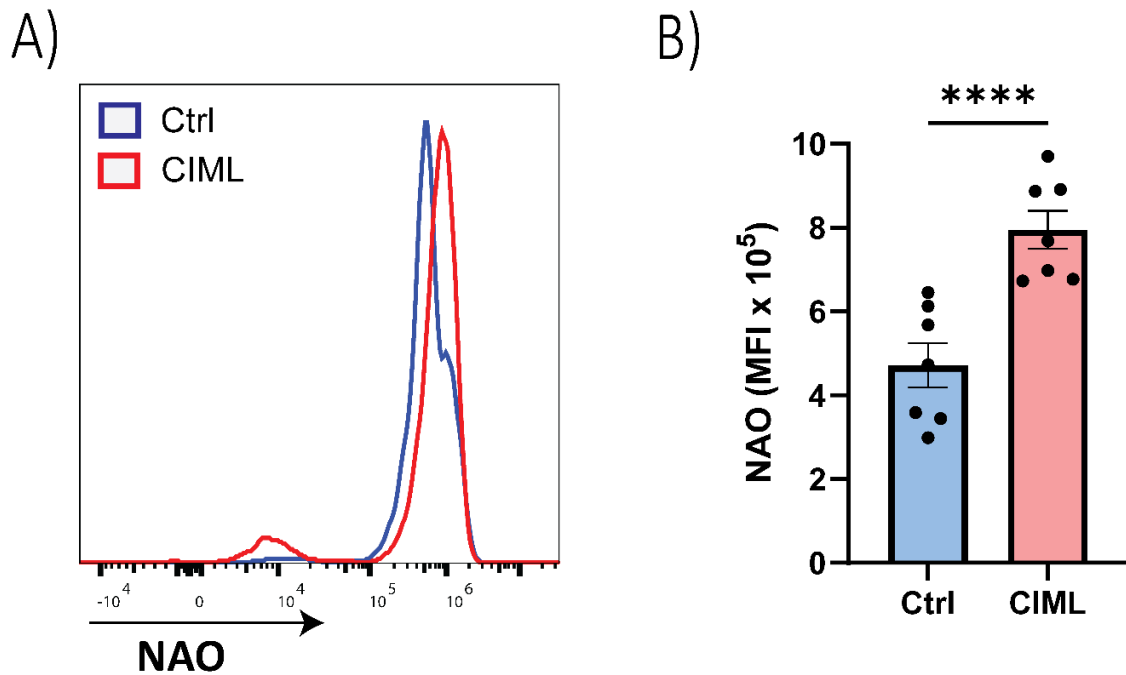


Figure 3.25. CIML NK cells have increased mitochondrial mass.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Mitochondrial mass was determined by staining with the nonyl acridine orange (NAO) probe. Cells were incubated with 50 nM NAO for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A,B)** Flow cytometry analysis of NAO staining on Ctrl and CIML NK cells. **(A)** Representative histogram showing NAO staining signal and **(B)** pooled MFI of NAO signal in CIML NK cells relative to Ctrl. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=7 donors. Statistical analysis was performed using a paired t-test. (**** $p < 0.0001$).

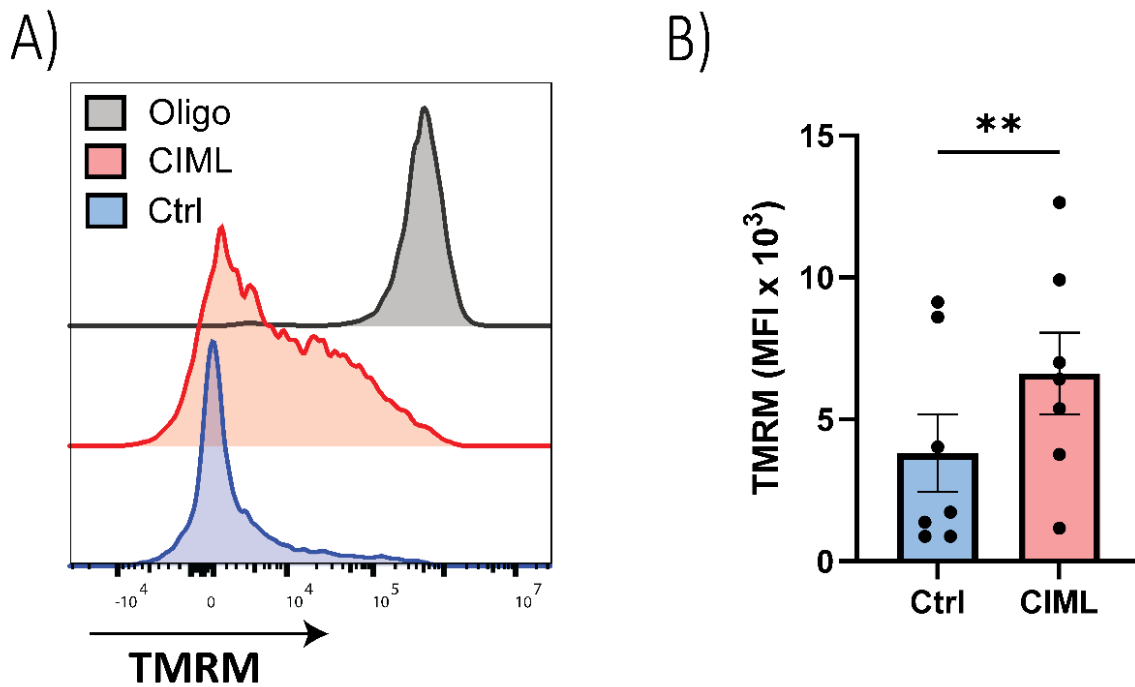


Figure 3.26. CIML NK cells have increased total mitochondrial membrane potential.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Mitochondrial membrane potential was determined by staining with tetramethylrhodamine methyl ester (TMRM) dye. Cells were incubated with 3 nM TMRM for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A,B)** Flow cytometry analysis of TMRM staining on Ctrl and CIML NK cells. **(A)** Representative histogram showing TMRM staining signal with oligomycin (1 µM) positive control in grey and **(B)** pooled raw MFI values of TMRM signal in Ctrl and CIML NK cells. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=7 donors. Statistical analysis was performed using a paired t-test. (**p<0.01).

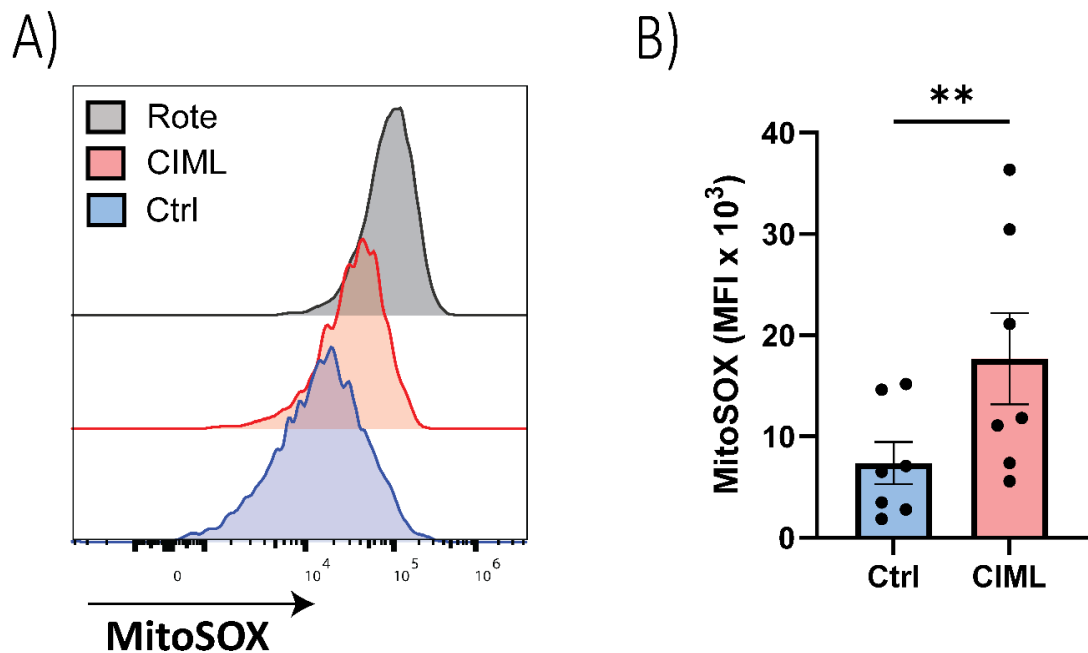
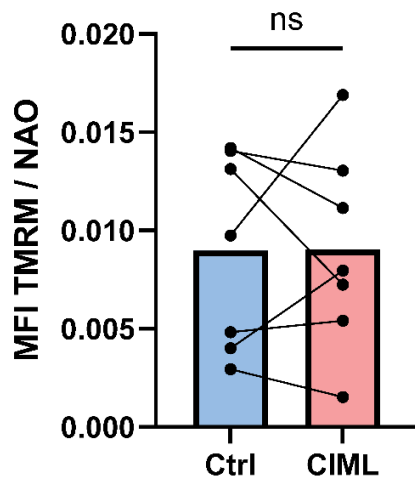


Figure 3.27. CIML NK cells have significantly increased mitochondrial ROS.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Mitochondrial ROS was determined by staining with mitoSOX dye. Cells were incubated with 2 μ M mitoSOX red dye for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A,B)** Flow cytometry analysis of mitoSOX staining on Ctrl and CIML NK cells. **(A)** Representative histogram showing mitoSOX staining signal with rotenone positive control in grey and **(B)** pooled raw MFI values of mitoSOX signal in Ctrl and CIML NK cells. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=7 donors. Statistical analysis was performed using a paired t-test. (** $p < 0.01$).

A)



B)

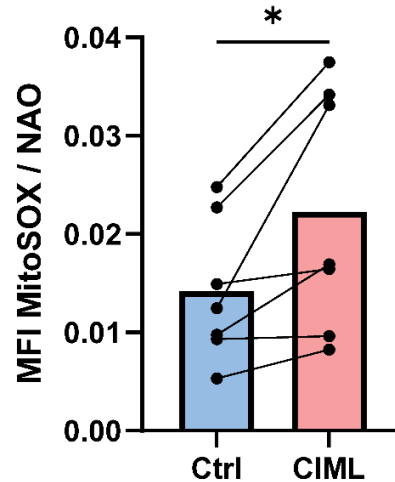


Figure 3.28. CIML NK cell membrane potential and mitochondrial ROS production relative to mitochondrial mass.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Mitochondrial ROS was determined by staining with mitoSOX dye. Cells were incubated with 2 μ M mitoSOX red, 3 nM TMRM, and 50 nM NAO for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A,B)** Flow cytometry analysis of TMRM and mitoSOX relative to NAO staining on Ctrl and CIML NK cells. **(A)** Pooled data of TMRM MFI values divided by NAO MFI values in cells and **(B)** pooled data of mitoSOX MFI values divided by NAO MFI values in Ctrl vs CIML NK cells. Data are mean $\pm$ SEM of N=7 donors. Statistical analysis was performed using a paired t-test. ($P < 0.05$).

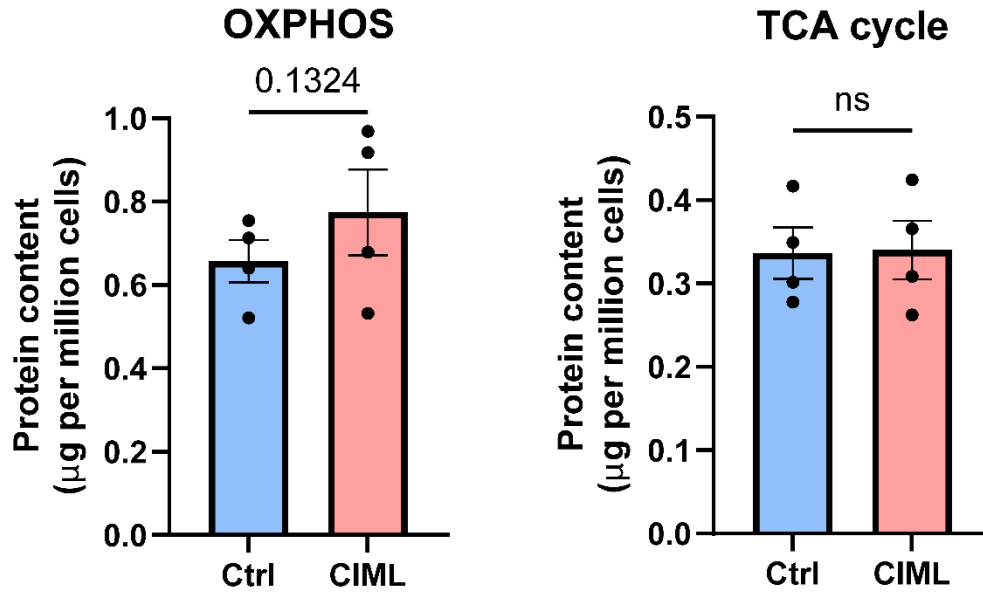


Figure 3.29. OXPHOS and TCA cycle proteins are unchanged in CIML NK cells compared to control NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Quantitative proteomics was performed on resting Ctrl and CIML NK cells, analysing paired total protein of mitochondrial respiration-associated processes. Protein content of OXPHOS (GO:0006119) and TCA cycle (GO:0006099) mitochondrial processes in Ctrl NK cells and CIML NK cells. Total protein content per million cells in micrograms was calculated as $\Sigma(((CN \times MW)/N) \times 10^{12})$ where CN = Protein copy number, MW = Protein molecular weight, and N = Avogadro's constant. Data are mean $\pm$ SEM of N=4 donors. Statistical analysis was performed using a paired t-test.

3.8 The pentose phosphate pathway mediates cellular and mitochondrial redox balance in CIML NK cells.

As discussed earlier, another function of the PPP is to generate NADPH, a reducing agent essential for ROS management. As CIML NK cells have increased mitochondrial mass-producing mitochondrial ROS, this may diffuse into the cytoplasm and cause oxidative stress. To investigate if the PPP had a role in managing CIML NK cell ROS, expression of enzymes involved in ROS management was analysed from the proteomic dataset, which revealed that CIML NK cells have significantly increased expression of proteins such as catalase (CAT), thioredoxin (TXN), peroxiredoxins (PRDX) 1, 2, and 6, which either directly or indirectly interact with NADPH (Figure 3.31). Other ROS protective enzymes were upregulated in CIML NK cells, such as methionine sulfoxide reductase A (MSRA), responsible for converting methionine sulfoxide back to methionine, and the glutathione-forming enzyme, glutathione synthetase (GSS) (Figure 3.31). This was a strong indicator that CIML NK cells have an increased redox balancing potential, which is likely driven by increased cellular ROS, potentially derived from the mitochondria.

NAD kinase (NADK) catalyses the phosphorylation of NAD^+ to form NADP^+ , used by the PPP to form NADPH. Two isoforms of NADK exist: NADK, which is active in the cytosol, and NADK2, which is retained in the mitochondria for localised NADP^+ production. CIML NK cells have no change in NADK2 expression but do have significantly increased NADK expression (Figure 3.32A,B), suggesting a preference for cytosolic NADP^+ production. Since the PPP is the main cellular pathway of producing NADPH from NADP^+ , and CIML NK cells have increased expression of ROS-managing associated enzymes such as NADK and PGD, it was hypothesised that the PPP plays a role in total cell ROS management. Indeed, inhibition of the PPP with 6-aminonicotinamide (6-AN) resulted in significantly increased cellular ROS production in CIML NK cells compared to control NK cells (Figure 3.33A,B). Furthermore, 6-AN inhibition of the PPP resulted in a trend towards increased mitochondrial ROS production (Figure 3.34A,B), suggesting that the PPP has an essential role in neutralising cellular ROS and may have a role in dampening mitochondrial ROS in CIML NK cells.

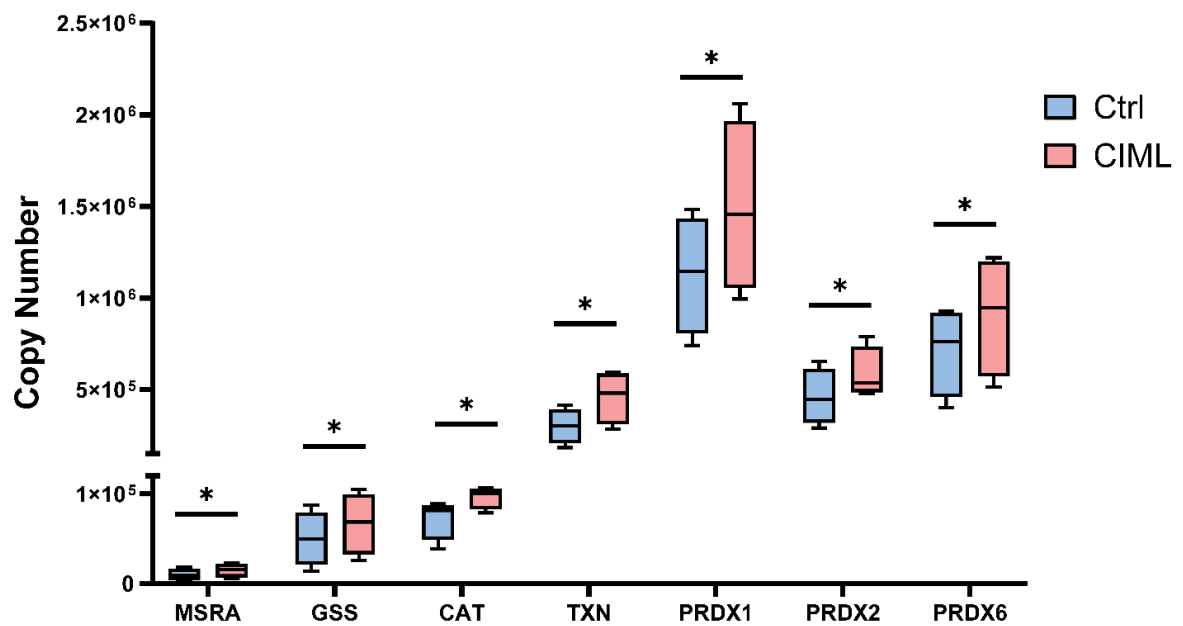


Figure 3.31. CIML NK cells upregulate ROS-managing enzymes.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Quantitative proteomics was performed on resting Ctrl and CIML NK cells, analysing paired copy numbers per cell of upregulated enzymes involved in redox homeostasis. Data are mean +/- SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (*p<0.05)

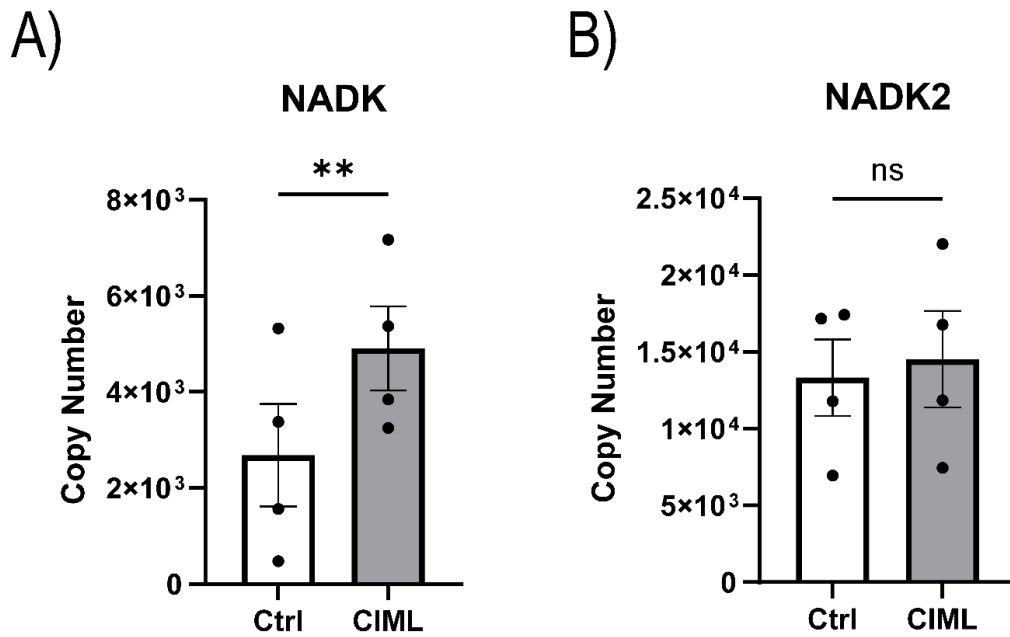


Figure 3.32. CIML NK cells have increased cytosolic NAD kinase.

PBMC were isolated from fresh healthy human blood, and NK cells were sorted by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. **(A,B)** Quantitative proteomics was performed on resting Ctrl and CIML NK cells, analysing paired copy numbers per cell of **(A)** cytosolic and **(B)** mitochondrial NAD kinase (NADK). Data are mean $\pm$ SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (** $p < 0.01$).

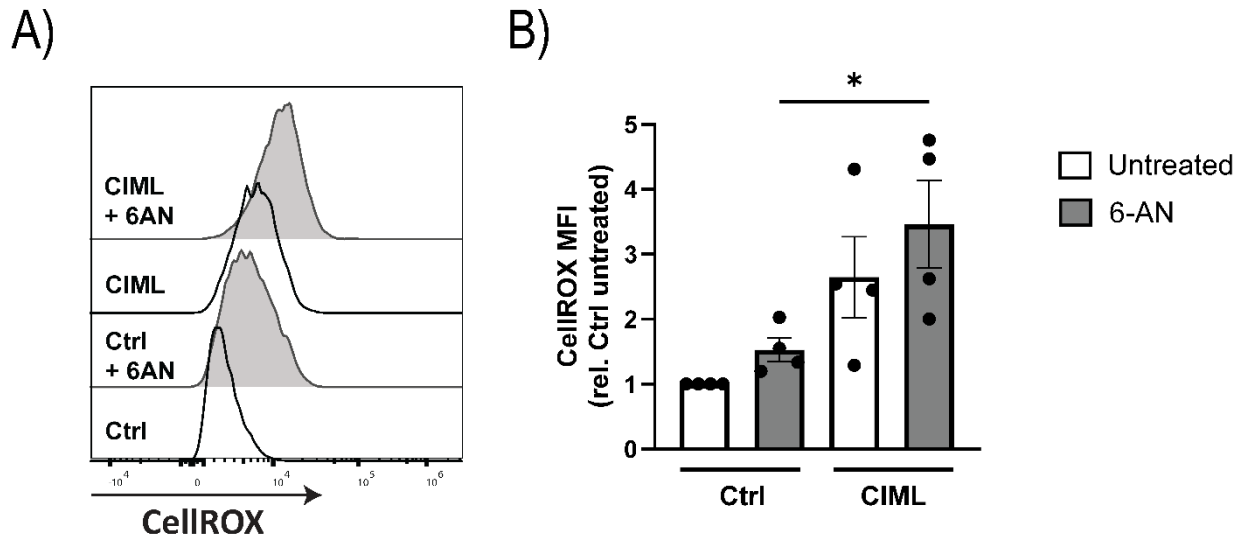


Figure 3.33. 6-AN treatment results in increased CIML NK cell cellular ROS compared to control NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Cellular ROS was determined by staining with cellROX dye. Cells were incubated with 5 μ M cellROX green dye +/- 50 μ M of the PPP inhibitor 6-AN for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A,B)** Flow cytometry analysis of cellROX staining on Ctrl and CIML NK cells +/- 6-AN. **(A)** Representative histogram showing cellROX staining signal and **(B)** pooled MFI values of cellROX signal in Ctrl and CIML NK cells +/- 6-AN relative to Ctrl untreated. Data are **(A)** representative or **(B)** mean +/- SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (* $p < 0.05$).

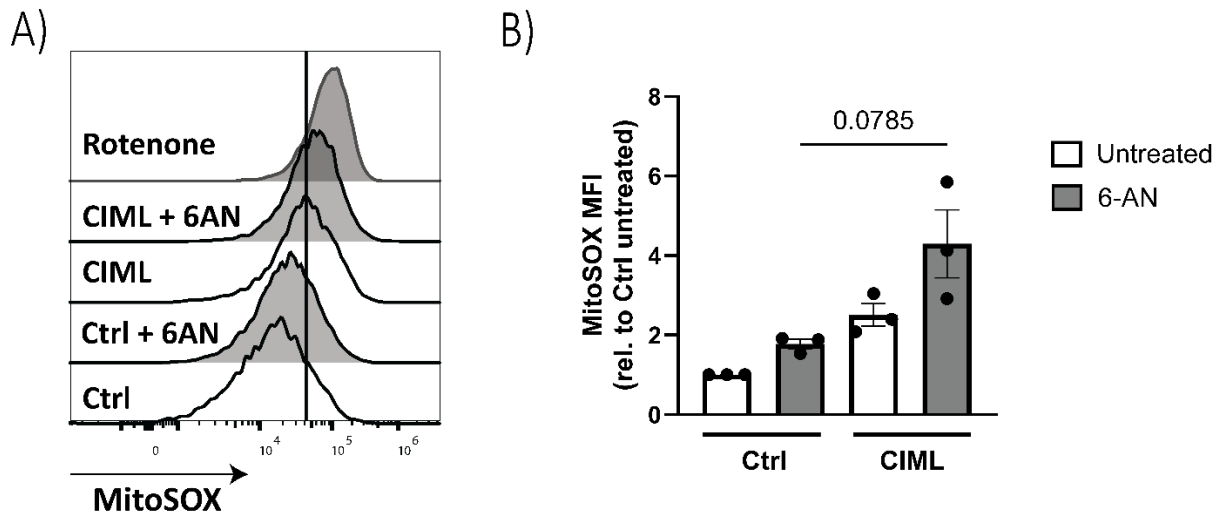


Figure 3.34. 6-AN treatment may increase CIML NK mitochondrial ROS compared to control NK cells

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Mitochondrial ROS was determined by staining with mitoSOX dye. Cells were incubated with 2 μ M mitoSOX red dye +/- 50 μ M of the PPP inhibitor 6-AN for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A,B)** Flow cytometry analysis of mitoSOX staining on Ctrl and CIML NK cells +/- 6-AN. **(A)** Representative histogram showing mitoSOX staining signal with rotenone positive control and **(B)** pooled MFI values of mitoSOX signal in Ctrl and CIML NK cells +/- 6-AN relative to Ctrl untreated. Data are **(A)** representative or **(B)** mean +/- SEM of N=3 donors. Statistical analysis was performed using a paired t-test.

3.9 Discussion

The aim of Chapter 3 was to broadly characterise CIML NK cell metabolism using advanced mass spectrometry for high-resolution identification of proteins and metabolites of interest. Many studies have used proteomics and metabolomics for understanding both large and discrete changes in metabolic profiles that have helped characterise the molecular underpinnings of cell function. Proteomics studies, such as those carried out by Cantrell et al., have given useful mechanistic insight into various T cell subset differentiation and activation [285-287]. Similarly, untargeted and targeted metabolomics have helped shape fields such as innate immune training, with studies revealing glutamine-fueled fumarate accumulation as a vital link between metabolism and epigenetic rewiring in trained macrophages [167]. The research presented in this chapter adds mechanistic depth to the current unexplored field of CIML NK cell metabolic reprogramming by identifying that CIML NK cells undergo a metabolic shift toward enhanced proliferation, glycolysis, PPP, and purine biosynthesis. This study also demonstrates that CIML NK cells accumulate dysfunctional ROS-producing mitochondria, accompanied by enhanced ROS management potential owing to upregulated antioxidant enzyme expression and PPP metabolism.

Differential expression analysis reveals that most differentially expressed proteins are harboured by CIML NK cells, and Reactome, ChIP-X databases, and GSEA analyses, as well as Ki67 expression, revealed that CIML NK cells display a highly proliferative signature. These are indicators of actively proliferating CIML NK cells that could be used as biomarkers that can be tracked through the course of immunotherapy treatments. Many studies use proliferative markers as a predictive measurement of immunotherapy outcomes. A clinical study on cancer patients receiving immunotherapy, including checkpoint inhibitors, noted that early and sustained proliferation of CD8⁺ T cells was associated with improved clinical responses and disease outcomes, highlighting immune cell proliferation as a key biomarker for predicting therapeutic efficacy [288]. In lung cancer patients receiving checkpoint inhibitors, CD8⁺ T cells also demonstrated early expression of Ki67 with retained cytotoxic activity in 70% of patients [289]. NK immunotherapies are often used in conjunction with other therapies such as chemotherapy and tyrosine kinase inhibitors [290, 291]. However, it is often the

case that these combination therapies can impact NK cell persistence in the blood and their function, ultimately affecting their efficacy as a treatment [292, 293]. Thus, it would be useful to track NK cell immunotherapy persistence via proliferative biomarkers for combination therapy tailoring. Results from this study demonstrate that E2F transcriptional regulation is enriched in CIML NK cells. E2F family members are overexpressed in many cancer types, driving proliferation, and are often used as predictors of disease progression [294-297]. This finding could be used for predictive immunotherapy outcomes with E2F family activity in CIML NK cells acting as an indicator of CIML proliferative and functional competency. Overall, these proliferative biomarkers could provide valuable insights during immunotherapy treatment in the clinic through simple peripheral blood sampling over time.

This chapter defines and expands on current knowledge by integrating both proteomic and metabolomic analysis to gain a comprehensive understanding of metabolic flux within the CIML NK cell biological system. The data, showing increased glycolytic protein content and lactate in the supernatants from CIML NK cells, support existing studies suggesting that glycolysis is an important pathway for CIML NK cell metabolism [220, 221]. Together, these studies with the data presented here, definitively demonstrate increased rates of glycolysis in CIML NK cells. A recent study by John *et al.* demonstrated that exogenous pyruvate is essential in human NK cell metabolism and that glucose-derived carbons do not contribute to endogenous pyruvate formation [298]. However, the study in question investigated the metabolism of conventional CD56^{dim} NK cells only, and experiments were mostly performed within 24 hours of isolation. In contrast, CIML NK cells exhibit a distinct metabolic profile characterised by enhanced glycolytic flux and active pyruvate export, suggesting a reduced reliance on exogenous pyruvate. This metabolic shift may arise from cytokine-induced training and the 7-day culture period. This chapter builds on the current literature surrounding CIML NK cell glycolytic metabolism by identifying the auxiliary PPP as an important metabolic pathway. Downstream of this, metabolomic analysis revealed a significant purine biosynthesis signature, with increased levels of ¹³C labelled nucleosides, monophosphate nucleosides, and purine bases. Recently, several studies have identified purine nucleotide

synthesis and salvage as an important metabolic pathway in innate cells such as macrophages for functionally relevant metabolic remodelling and also in dendritic cell-mediated regulation of autoimmunity via restriction of T cell priming [84, 299]. This data further consolidates that CIML NK cells undergo metabolic shift toward a proliferative phenotype likely to be supported by glucose-driven anabolism via pathways such as the PPP, whose role is largely ill defined in NK cell metabolism literature.

The data presented herein also suggests that CIML NK cells have, to some degree, accumulated bioenergetically dormant mitochondrial mass. Other studies have reported similar findings demonstrating increased mitochondrial mass accompanied by no change in oxygen consumption rate, or that CIML NK cells have an increased oxygen consumption rate, but lower maximal mitochondrial respiration and lower spare respiratory capacity. Also, blockade of ATP synthesis with oligomycin resulted in continued oxygen consumption, potentially indicating increased proton leakage [220, 221]. Another marker of increased mitochondrial ATP synthesis is mitochondrial fusion and condensed mitochondrial cristae. A study by Terrén *et al.* investigated the mitochondrial morphology of CIML NK cells and found no differences in mitochondrial features such as mitochondrial length and cristae density, compared to controls [223]. Furthermore, data presented in this chapter revealed that the expression of OXPHOS and TCA cycle-associated proteins remains unchanged between control and CIML NK cells. This reveals an interesting phenomenon where CIML NK cells accumulate phenotypically dysfunctional mitochondria. Mitophagy, which is the specific autophagic removal of mitochondria in cells, has been shown to be essential in the early development of other forms of NK cell memory, as observed in CMV adaptive NK cells [300]. It is possible that mitophagy is not a necessary process in the generation of the memory-like properties of CIML NK cells, as it is in other forms of NK cell memory. One potential benefit of accumulating mitochondria is for the rapid induction of OXPHOS upon reactivation, which is often observed in adaptive cells such as CD8⁺ T cells [48].

There are many sources of cellular ROS in immune cells, including NADPH oxidases, ER stress, xanthine oxidase from purine biosynthesis, and notably, mitochondrial dysfunction [117, 301]. It was hypothesised in this chapter that

mitochondria-sourced ROS may be dampened by increased PPP metabolism, which is a significant source of cellular NADPH and supported by NADK expression [66, 129]. Moon et al. reported that mitochondrial NADPH levels are sensitive to both mitochondrial and cytosolic oxidative stress, whereas the cytosolic NADPH pool was unperturbed under mitochondrial oxidative stress [302]. Redox factors generated in the cytosol, like glutathione, have been shown to be imported into mitochondria through SLC25A39, where they can exert oxidative and non-oxidative functions, including supporting iron-sulfur cluster biogenesis and mitochondrial translation in addition to redox balance [143]. The data presented in this thesis revealed that PRDX1 and PRDX6 were upregulated in CIML NK cells, which have been demonstrated to protect mitochondrial integrity via inhibition of apoptosis and support mitogenesis and OXPHOS, respectively [303, 304]. Other antioxidant enzymes upregulated in CIML NK cells have been shown to be essential in maintaining NK cell cytotoxic integrity, protecting them in environments such as the tumour microenvironment, where TXN was shown to be important [138]. PRDX1 has also been shown to increase survival and cytotoxicity when overexpressed in CAR-NK cells [305]. These enzymes use NADPH either directly or indirectly to exert their ROS protective effects, suggesting CIML NK cell mitochondria are reliant on reductive agents derived from the cytosol. This may be a potential vulnerability for CIML NK cells, as oxidative stress exposure may unravel mitochondrial ROS management, which is known to be detrimental to cell function and survival [306].

One interesting possibility that combines PPP metabolism, purine biosynthesis, and mitochondrial mass is folate metabolism [307]. Mitochondria have a role in folate metabolism where tetrahydrofolatepurine (THF) is taken from the cytosol into the mitochondria and through subsequent reactions produces formate, which is exported back into the cytosol for purine biosynthesis [308]. As revealed in this chapter, CIML NK cells have upregulated purine metabolism, which requires one-carbon metabolic pathways such as folate metabolism, and increased mitochondrial mass may provide a mechanism for providing one-carbon metabolism intermediates. Furthermore, these reactions that synthesise formate also result in the synthesis of NADPH in the mitochondria, which could contribute to mitochondrial ROS management [309]. Moreover, the PPP has a direct role in

the cytosolic portion of the folate cycle. Interestingly, NADPH, which would predominantly be supplied by the PPP, is required for both of the cytosolic enzymatic reactions required to convert folate to THF [129, 163]. Collapse in cytosolic NADPH pools and subsequent THF-mediated NADPH synthesis in the mitochondria may also explain the increase in mitochondrial ROS observed upon PPP inhibition in CIML NK cells. Furthermore, mitochondrial Serine Hydroxymethyltransferase, involved in folate metabolism, contributes to serine:glycine ratios in the cell, which is vital for glutathione synthesis [310]. A similar system has been identified in activated T cells, where mitochondrial biogenesis and subsequent one-carbon metabolism were contributing to purine biosynthesis [311]. This would imply an alternative yet useful role for the accumulation of seemingly dysfunctional mitochondria in CIML NK cells, driving purine biosynthesis supported by PPP-derived NADPH.

Overall, in this chapter, proteomic and metabolomic analyses were integrated and coupled with flow cytometry to metabolically profile CIML NK cells. This study has identified that CIML NK cells undergo a proteomic shift toward a proliferative and anabolic phenotype, underpinned by enhanced glycolysis and PPP machinery upregulation, which contributes more glucose-derived carbon to purine biosynthesis. CIML NK cells accumulate mitochondria, which seem, in part, dysfunctional, with no observed increases in membrane potential but increased mitochondrial ROS generation. CIML NK cells also have enhanced ROS management potential owed to upregulated antioxidant enzyme expression, PPP activity, and enhanced cytosolic NADK expression. CIML NK cells are particularly sensitive to PPP inhibition in terms of oxidative stress vulnerability, both in the cytosol and mitochondria, suggesting the PPP may act as a central node for CIML NK cell metabolism.

Chapter 4. Proteomic analysis of stimulated human CIML NK cells.

4.1 Introduction.

CIML NK cells are effective mediators of enhanced cytotoxic responses compared to conventional NK cells. This enhanced response is characterised by potent cytokine production, upregulated granzyme B reservoirs and increased direct killing of cancer cells upon rechallenge. CIML NK cells have been shown to be particularly effective against AML but are also being investigated in clinical trials treating multiple myeloma and solid tumours like renal cell carcinoma and ovarian cancer. CIML NK cells must meet bioenergetic and biosynthetic demands in order to carry out these effective cytotoxic functions, and this is facilitated by an upregulation of metabolic function. However, immune cells differ in what metabolic pathways they favour to meet these demands, and this remains understudied in the context of CIML NK cells.

To date, only a handful of studies have investigated what metabolic changes CIML NK cells undergo upon restimulation. These studies found that glycolysis was important for IFN γ , TNF α and CCL4 production in restimulated CIML NK cells, and inhibition of glycolysis significantly decreased specific lysis of the K562 leukaemia cell line [221]. However, these studies were limited to Seahorse and flow cytometry-based methods of metabolic analysis. In this work, proteomic analysis was undertaken in primary human control and CIML NK cells before and after restimulation, with supporting flow cytometric data, to determine favoured metabolic pathways for supporting enhanced effector functions in CIML NK cells.

This chapter will test the hypothesis that upon restimulation, CIML NK cells undergo a rapid and coordinated metabolic reprogramming that preferentially supports their enhanced secondary effector responses compared to control NK cells. Specifically, it is proposed that multidimensional proteomic analysis is expected to reveal pathway-level insights consistent with these metabolic adaptations.

4.2 Aims.

- To investigate differences in control and CIML NK metabolism post-stimulation by multidimensional proteomic analyses.
- To investigate mitochondrial phenotypes in control and CIML NK cells by flow cytometry.
- To investigate the role of the PPP and mitochondrial metabolism in CIML NK cell-enhanced secondary responses.

4.3 Proteomic validation of CIML NK cell enhanced secondary responses.

PBMC were isolated from healthy fresh human blood and stained for CD56 and CD3, and NK cells were sorted on the FACS Aria as the CD56⁺CD3⁻ cell population (Figure 2.3). Purified NK cells were then plated and stimulated with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) or given a low dose of IL-15 (1 ng/ml) as a control for 18 hours. The next day, NK cells were washed three times in PBS and resuspended in media supplemented with low-dose IL-15 and left for 7 days, replacing IL-15-supplemented media on days 3 and 5 as outlined above (Figure 2.2.2). On day 7, control and CIML NK cells were left unstimulated or stimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours. Control and CIML NK cells were then washed three times in HBSS, and the cell pellets were snap frozen in liquid nitrogen. Control and CIML NK cells (n=4 paired) were sent to the core proteomic facility in Dundee for MS analysis. UHPLC coupled MS was carried out, and data were acquired on DIA mode. Copy numbers of each protein were generated using Spectronaut outputs in the Perseus software and the Proteomic Ruler plugin. In order to confirm enhanced secondary responses in CIML NK cells at the protein level, the expression of effector molecules was assessed after restimulation. Firstly, the overall total protein content in both control and CIML NK cells was measured, as this would be expected to increase post-cytokine stimulation. Stimulation of cells with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours resulted in a significant increase in total protein in both control and CIML NK cells compared to their unstimulated counterparts (Figure 4.1A,B), however, no change in total protein was observed between stimulated control and CIML NK cells (Figure 4.1C). As expected, CIML

NK cells had significantly increased IFN γ production compared to control NK cells, and a trending increase in granzyme B production after restimulation (Figure 4.1A,C). Furthermore, there was significantly increased expression of TNF α and CCL3, a proinflammatory chemokine, in CIML NK cells after restimulation (Figure 4.2B,D). Together, this data indicated that both control and CIML NK cells were appropriately activated, and the CIML NK cells displayed enhanced effector protein levels as would be expected.

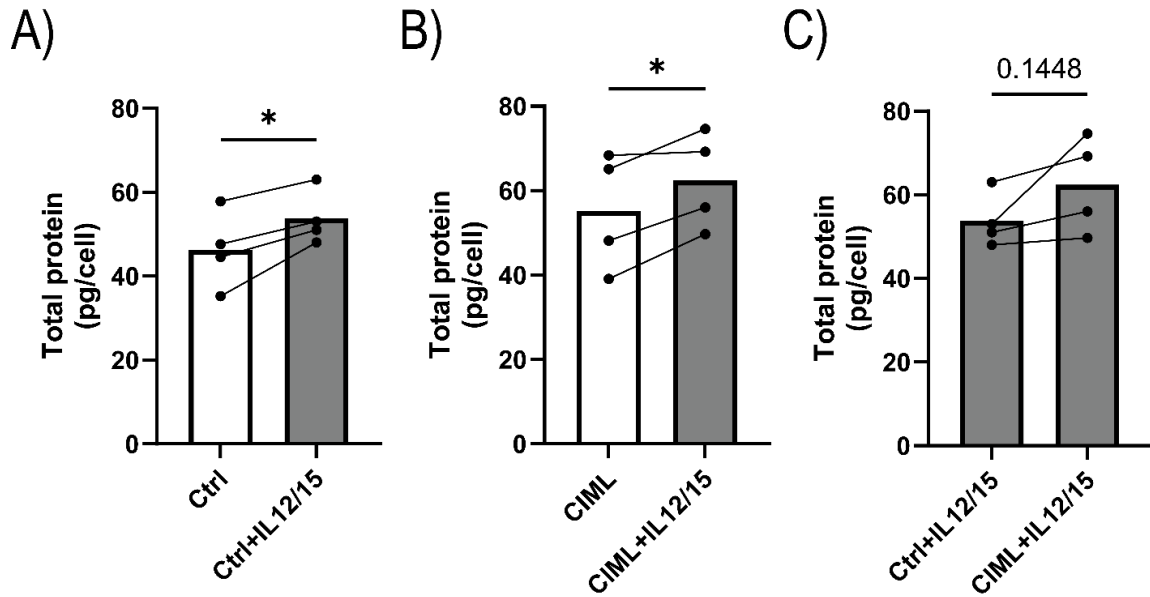


Figure 4.1. Control and CIML NK cells increase their total protein content post-stimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. **(A,B,C)** Quantitative proteomics was performed on Ctrl and CIML NK cells +/- IL-12/15, analysing total protein content. **(A)** Total protein content of Ctrl vs Ctrl + IL-12/15, **(B)** CIML vs CIML + IL-12/15 and **(C)** Ctrl + IL-12/15 vs CIML + IL-12/15. Total protein content per cell in picograms was calculated as $\Sigma((CN \times MW)/N)$ where CN = Protein copy number, MW = Protein molecular weight and N = Avogadro's constant. Data is mean +/- SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (*p<0.05).

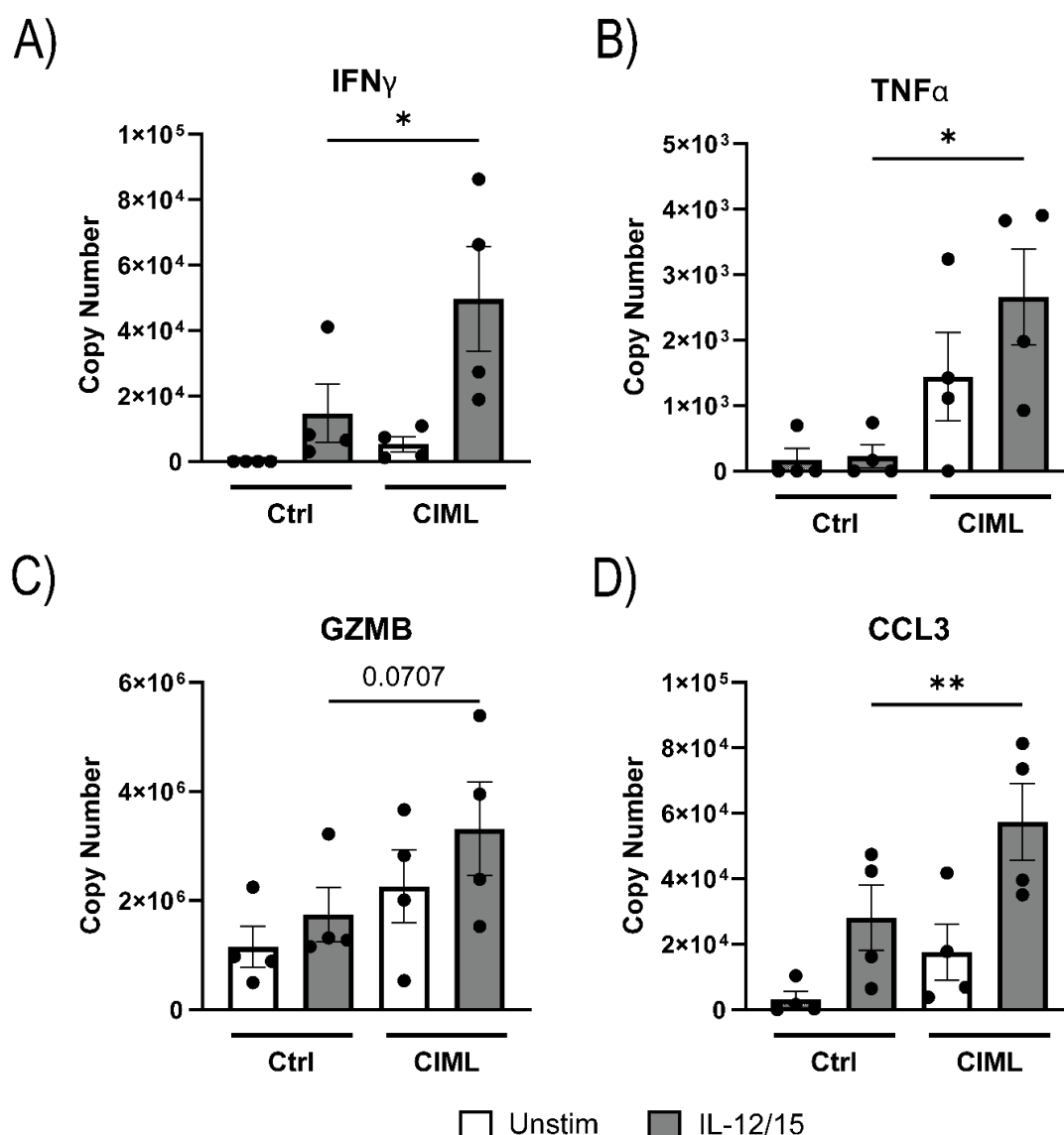


Figure 4.2. CIML NK cells have enhanced secondary responses at the protein level.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. **(A-D)** Quantitative proteomics was performed on Ctrl and CIML NK cells +/- IL-12/15, analysing paired copy numbers per cell of **(A)** IFN γ , **(B)** TNF α **(C)** granzyme B and **(D)** CCL3. Data is mean +/- SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (*p<0.05, **p<0.01).

4.4 Multidimensional proteomic analysis of CIML NK cells after restimulation.

To broadly investigate the metabolic changes in CIML NK cells after restimulation, the total protein content of the major metabolic pathways (glycolysis, OXPHOS, TCA cycle, fatty acid and amino acid metabolism) was first calculated as described in Section 2.2.5.3. No significant differences between control and CIML NK cells were observed after stimulation in these pathways. However, there was a trend towards increased expression of fatty acid β -oxidation (FAO) (GO:0006635) ($p=0.0849$) and electron transport chain ($p=0.0636$) associated proteins in CIML NK cells compared to control NK cells post-stimulation (Figure 4.3). To investigate total proteome differences further, three separate differential expression analyses were performed on control and CIML NK cells before and after stimulation. Differentially expressed proteins are identified as proteins that at least show a log2 fold change of greater than 1 or less than -1 and statistically significant ($p<0.05$). Firstly, control NK cells after stimulation were compared to resting control NK cells to investigate conventional-like NK cell protein upregulation during activation, revealing 719 differentially expressed proteins (Figure 4.4). STRING analysis was then performed on these proteins to cluster them by both functional and physical protein-protein interactions. STRING networks were generated where network edges indicate both functional and physical protein associations, and a threshold confidence interval of 0.900 was applied. Local network clustering revealed 2 clusters of associated proteins from the 719 differentially expressed proteins in stimulated control NK cells (Figure 4.5). The first cluster (Cluster 1, CL:558) was made up of 14 proteins, associated with mitochondrial ribosome and gene expression, suggesting that control NK cells rapidly induce mitochondrial protein translation after stimulation (Figure 4.6A). The second cluster (Cluster 2, CL:5665) consisted of 18 proteins, associated with RNA polymerase II activity and core mediator complex assembly (Figure 4.6B). Indeed, stimulation of NK cells results in active transcription of proteins in which RNA polymerase II and the core mediator complex are essential for their activity [312]. Furthermore, pathway analysis using the Reactome database revealed these 719 differentially expressed proteins were associated with mitochondrial translation and tRNA modifications, indicating these proteins are highly associated with active transcription (Figure 4.7A). However, pathway

analysis using the KEGG database revealed no significantly upregulated pathways associated with the 719 differentially expressed proteins in control NK cells post stimulation (Figure 4.7B).

Differential expression analysis was then performed on CIML NK cells after restimulation compared to resting CIML NK cells. Interestingly, restimulated CIML NK cells had 641 differentially expressed proteins compared to resting CIML NK cells (Figure 4.8), which was less than that observed in control NK cells after stimulation. However, STRING analysis revealed 4 local network clusters within these differentially expressed proteins (Figure 4.9). The first, and most statistically significant local network cluster (Cluster 1: CL:11065, CL:11068, CL:11069) was comprised of 14 proteins highly associated with OXPHOS (Figure 4.10A). The second (Cluster 2: CL:5665, CL:5664, CL:5667, CL:5735) and third (Cluster 3: CL:558) clusters were again associated with mitochondrial translation and RNA polymerase II/core mediator complex assembly (Figure 4.10B,C), indicating they are common to both control and CIML NK cells after stimulation. The final cluster (Cluster 4: CL:900, CL:905) was associated with preribosome and ribosomal biogenesis (Figure 4.10D), again indicative of a cell synthesising more protein. Furthermore, pathway analysis of these 641 proteins using the Reactome database revealed pathway enrichment of aerobic respiration and respiratory electron transport (Figure 4.11A). Similarly, KEGG database analysis of these proteins revealed pathway enrichment of oxidative phosphorylation (hsa00190) and non-alcoholic fatty liver disease (hsa04932) (Figure 4.11B), which has a KEGG pathway largely comprised of mitochondrial oxidative genes [313]. This indicates that although CIML NK cells upregulate fewer proteins after restimulation compared to controls, the proteins that are upregulated are more related to metabolic activation, particularly those involved in mitochondrial translation and OXPHOS.

Lastly, differential expression analysis was performed on control stimulated and CIML restimulated NK cells. This revealed 284 upregulated proteins in CIML NK cells after restimulation and 62 proteins in stimulated control NK cells (Figure 4.12). As expected, differential expression of proinflammatory factors like IFN γ and CCL3 in CIML NK cells after restimulation, as well as the glycolytic oncogene HK2 and the high-affinity IL-2 receptor (IL2RA) were observed. Notably, the

metabolic transcriptional regulator hypoxia-inducible factor 1- α (HIF1 α) and cell proliferative E-twenty-six (ETS)-like protein 1 (ELK1) were differentially upregulated in CIML NK cells after restimulation (Figure 4.12). Reactome and KEGG analysis of differentially expressed proteins in CIML NK cells after restimulation showed enrichment of cell proliferative pathways, while control stimulated NK cells showed enrichment of cell regulation by Runt-Related Transcription Factor 1 (RUNX1) (Figure 4.13A,B). To investigate more nuanced changes within the proteomes of control and CIML NK cells after stimulation, GSEA was performed on the entire protein datasets to account for all significantly upregulated proteins, not just differentially expressed ones. GSEA analysis revealed 18 enriched pathways in control NK cells after stimulation compared to restimulated CIML NK cells (Figure 4.14A), which were largely associated with peptide cross-linking, aminoglycan catabolism, chemotaxis and apoptosis-associated pathways. CIML NK cells had 54 enriched pathways after restimulation compared to control stimulated NK cells, however, the analysis was restricted to the top 20 that were most significant (Figure 4.14B). Interestingly, 10 of the 20 most enriched pathways were associated with mitochondrial activity and 5 of these were directly involved in mitochondrial aerobic respiration. These mitochondrial pathways included mitochondrial translation (M11131) (Figure 4.15A), mitochondrial gene expression (M24915) (Figure 4.15B), oxidative phosphorylation (M5936) (Figure 4.15C) and aerobic respiration (M11785) (Figure 4.15D). Other enriched pathways included ribosome biogenesis, RNA metabolism, MYC and mTORC1 signalling (Figure 4.14B). Overall, this clearly demonstrates that CIML NK cells rapidly induce mitochondrial translation of proteins, particularly OXPHOS-associated machinery, compared to control NK cells after stimulation.

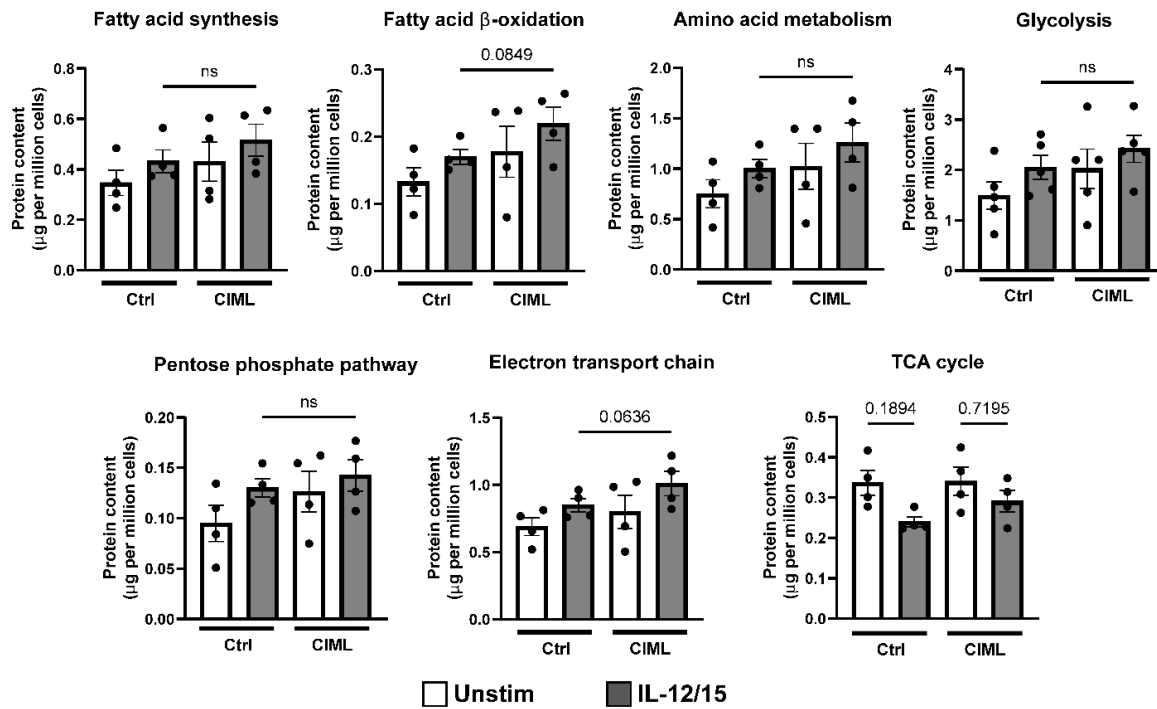


Figure 4.3. Pairwise analysis of total protein content of metabolic pathways in control and CIML NK cells after restimulation

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. Quantitative proteomics was performed on Ctrl and CIML NK cells +/- IL-12/15, analysing paired total protein of metabolic pathway machinery. Protein content changes of FAS (GO:0006633), FAO (GO:0006635), amino acid metabolism (GO:0006520), glycolysis (GO:0006096), PPP (GO:0006098), ETC (GO:0022900) and TCA cycle (GO:0006099) in Ctrl NK cells and CIML NK cells +/- IL-12/15. Total protein content per million cells in micrograms was calculated as $\Sigma(((CN \times MW)/N) \times 10^{12})$ where CN = Protein copy number, MW = Protein molecular weight and N = Avogadro's constant. Data is mean +/- SEM of N=4 donors (**A**). Statistical analysis was performed using a paired t-test and a one-way ANOVA.

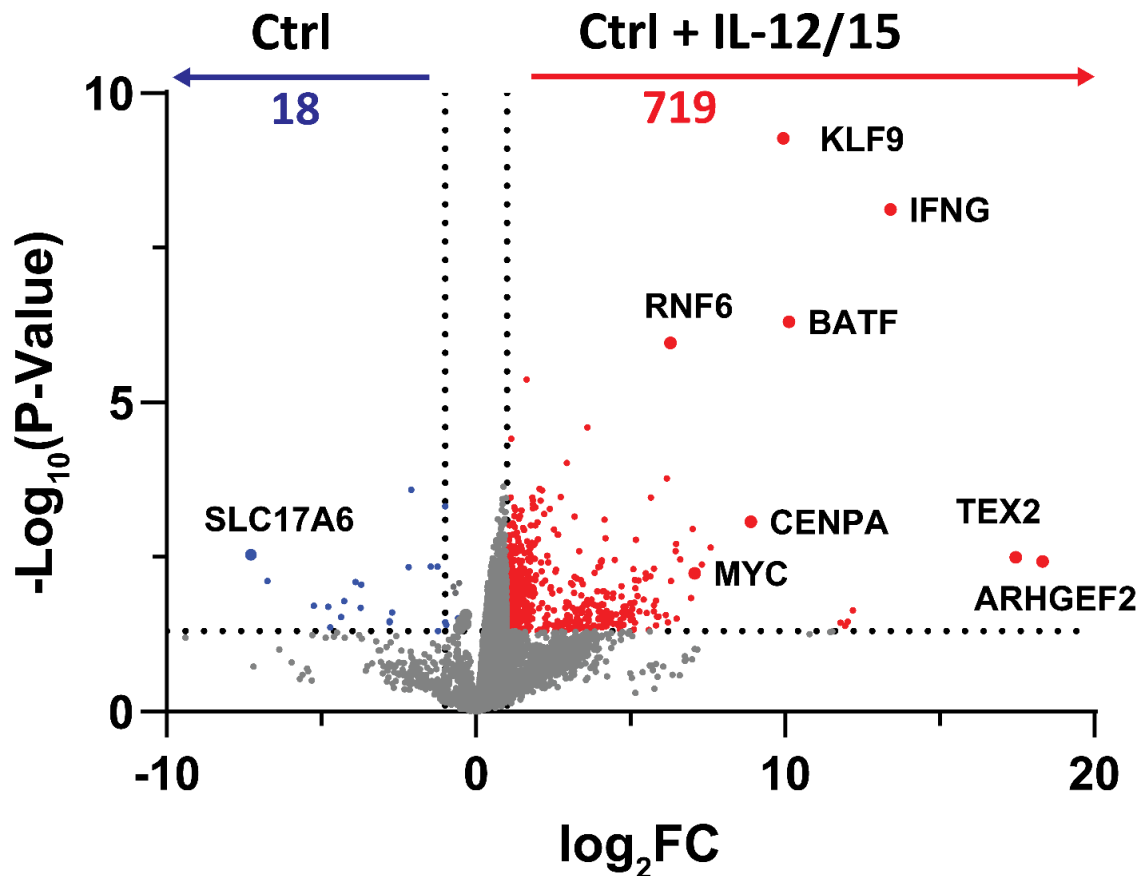


Figure 4.4. Differential expression analysis of resting control NK cells versus control NK cells + IL-12/15.

PBMCs were isolated from fresh healthy human blood, and NK cells were sorted by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. Volcano plot of 5103 total identified proteins displaying differences in relative abundance between Ctrl and Ctrl + IL-12/15 NK cells. The plot represents the $-\log_{10}$ of the p-value versus $\log_2$ of the fold change of protein expression. Differentially expressed proteins (red and blue) have a $\log_2$ fold change >1 or <-1 and a $-\log_{10}(\text{p-value}) > 1.35$. Proteins highlighted in red are enriched in Ctrl NK cells + IL-12/15, and those highlighted in blue are enriched in resting Ctrl NK cells. Quantitative proteomics was performed on N=4 donors.

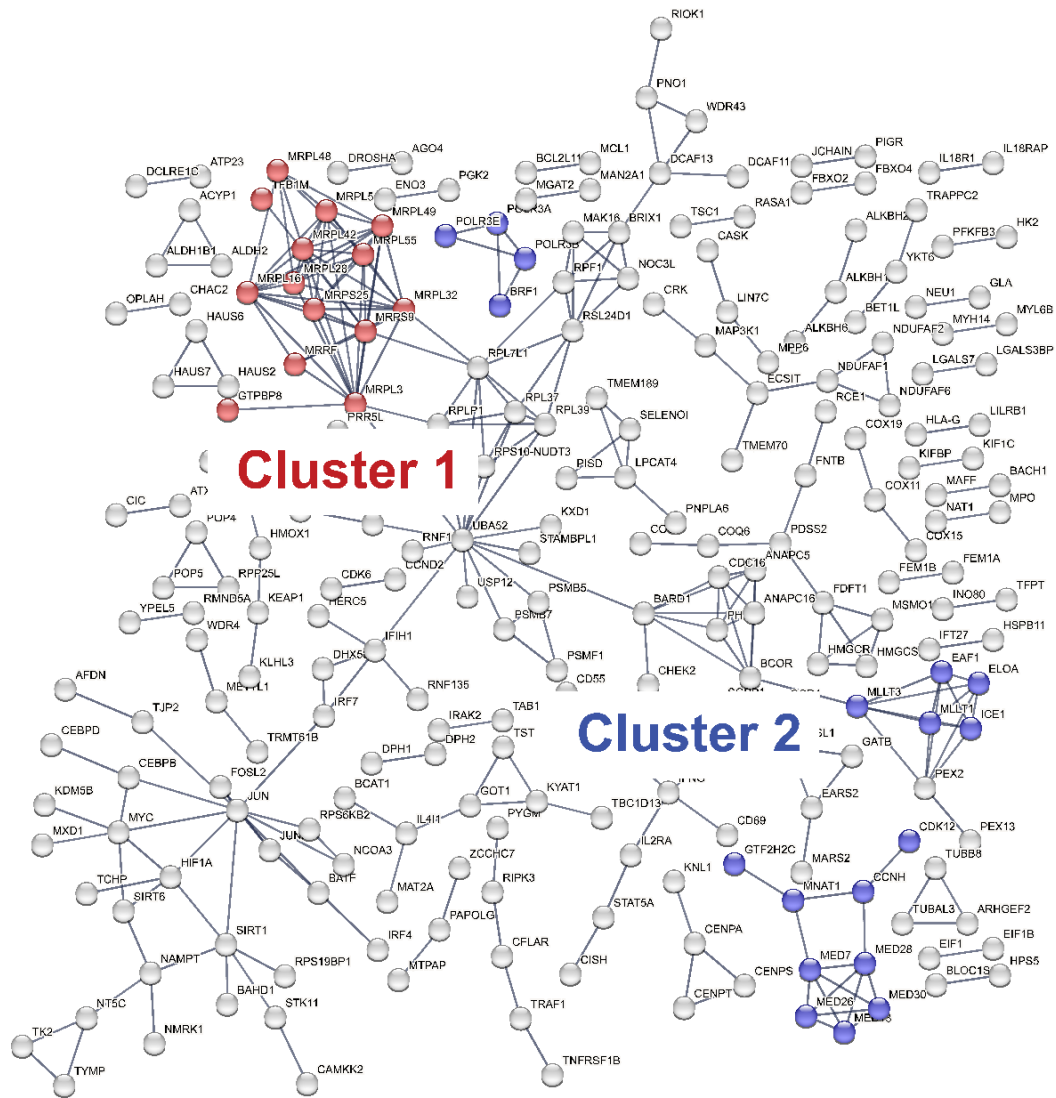


Figure 4.5. STRING analysis of differentially expressed proteins in control NK cells after restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. Protein-protein interaction network of the 719 differentially expressed proteins in Ctrl NK cells after restimulation, analysed using STRING. Full STRING network where network edges indicate both functional and physical protein associations with a minimum required interaction threshold score of the highest confidence (0.900). Local network clustering identified 2 main clusters of highly functionally and physically associated proteins. Disconnected nodes hidden for clarity.

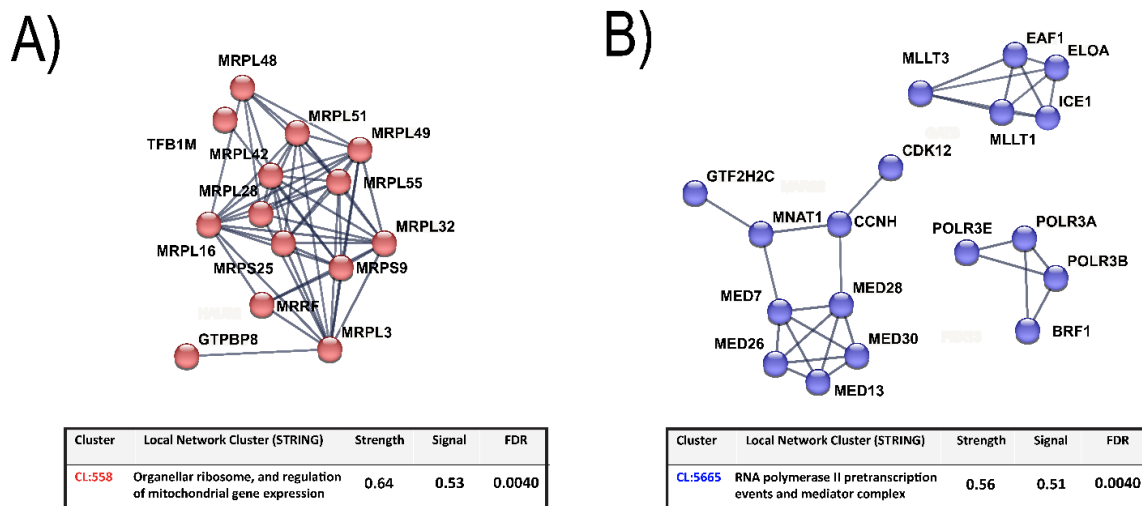


Figure 4.6. STRING clustering of highly associated proteins in control NK cells after restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. **(A,B)** Isolated STRING analysis clusters identified from the 719 differentially expressed proteins in Ctrl NK cell after restimulation: **(A)** Cluster 1 – mitochondrial ribosome and gene expression (CL:558) and **(B)** Cluster 2 – RNA polymerase II and core mediator complex (CL:5665). Isolated STRING networks with a minimum required interaction threshold score of the highest confidence (0.900). Disconnected nodes hidden for clarity.

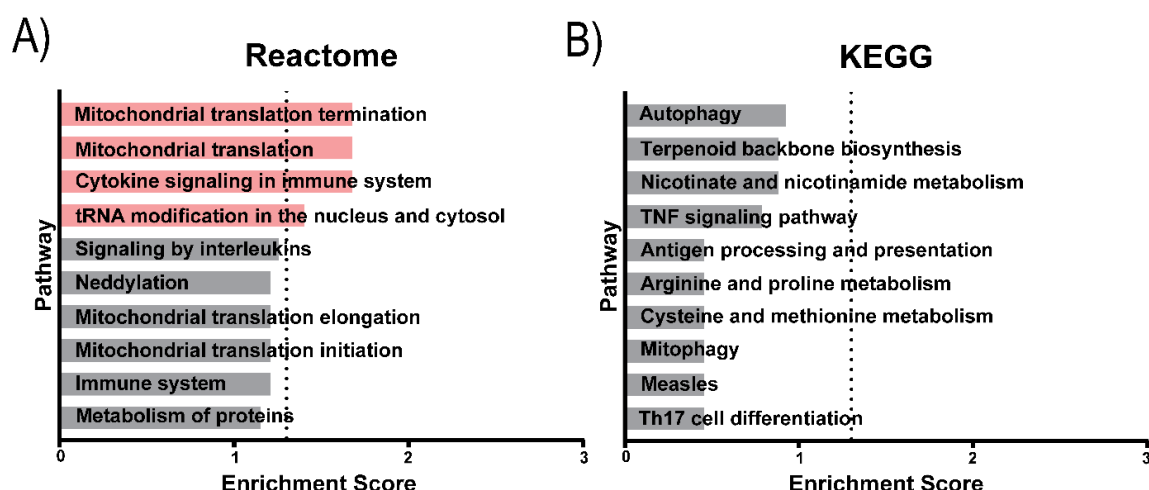


Figure 4.7. Reactome and KEGG pathway analysis of differentially expressed proteins in control NK cells after restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. **(A,B)** Quantitative proteomics was performed on Ctrl and CIML NK cells +/- IL-12/15, analysing enriched pathways. Significantly upregulated ($\text{Log}_2\text{FC} > 1$, $p\text{-value} < 0.05$) proteins in Ctrl + IL-12/15 NK cells were analysed for biological pathway enrichment using the **(A)** Reactome and **(B)** KEGG databases. Significantly enriched pathways ($-\text{Log}_{10}\text{Adj.pvalue} > 1.3$) are shown in red and non-significant pathways ($-\text{Log}_{10}\text{Adj.pvalue} < 1.3$) are shown in grey. Enrichment score = $-\text{Log}_{10}\text{Adj.pvalue}$. Dotted line represents significance threshold ($-\text{Log}_{10}\text{Adj.pvalue} = 1.3 = \text{Adj.p} < 0.05$). Quantitative proteomics was performed on N=4 donors.

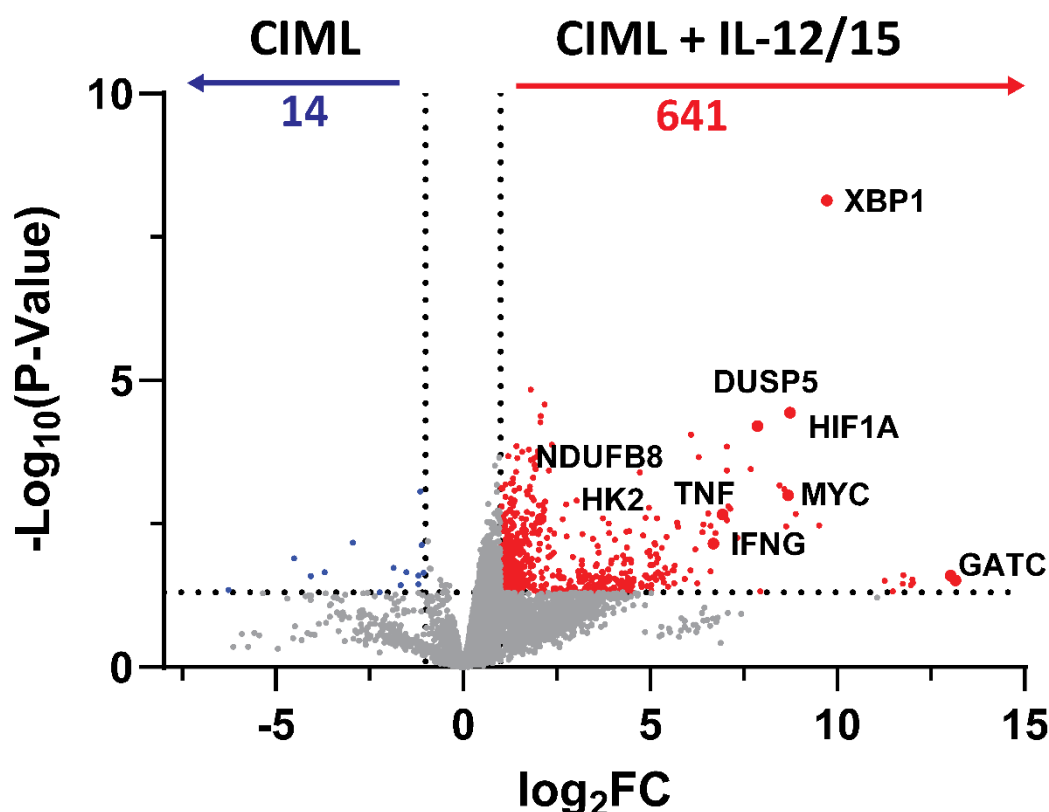


Figure 4.8. Differential expression analysis of resting CIML NK cells versus CIML NK cells + IL-12/15.

PBMC were isolated from fresh healthy human blood and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. Volcano plot of 8380 total identified proteins displaying differences in relative abundance between CIML and CIML + IL-12/15 NK cells. The plot represents the $-\log_{10}$ of the p-value versus $\log_2$ of the fold change of protein expression. Differentially expressed proteins (red and blue) have a $\log_2$ fold change >1 or <-1 and a $-\log_{10}(\text{p-value}) > 1.35$. Proteins highlighted in red are enriched in CIML NK cells + IL-12/15, and those highlighted in blue are enriched in resting CIML NK cells. Quantitative proteomics was performed on N=4 donors.

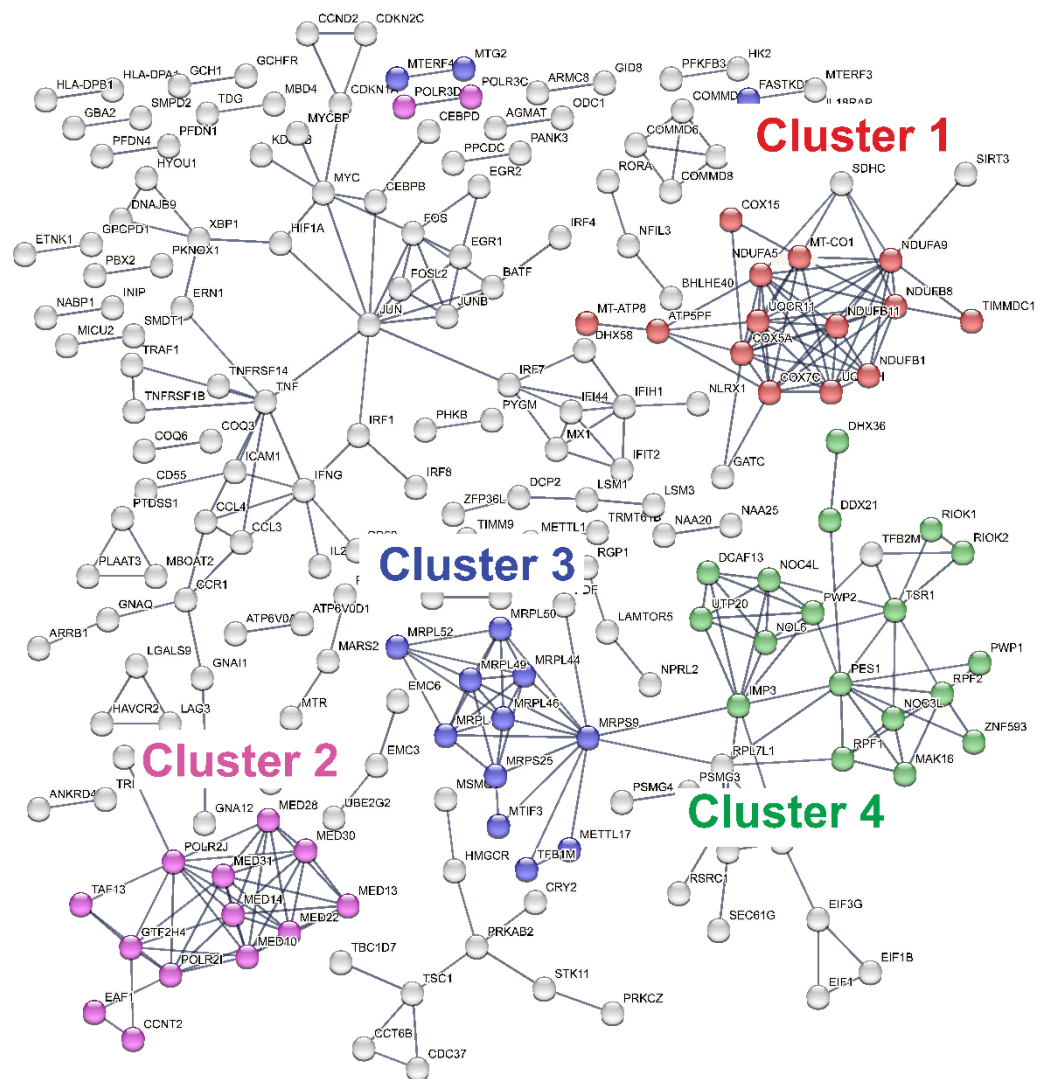


Figure 4.9. STRING analysis of differentially expressed proteins in CIML NK cells after restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. Protein-protein interaction network of the 641 differentially expressed proteins in CIML NK cells after restimulation, analysed using STRING. Full STRING network where network edges indicate both functional and physical protein associations with a minimum required interaction threshold score of the highest confidence (0.900). Local network clustering identified 4 main clusters of highly functionally and physically associated proteins. Disconnected nodes hidden for clarity.

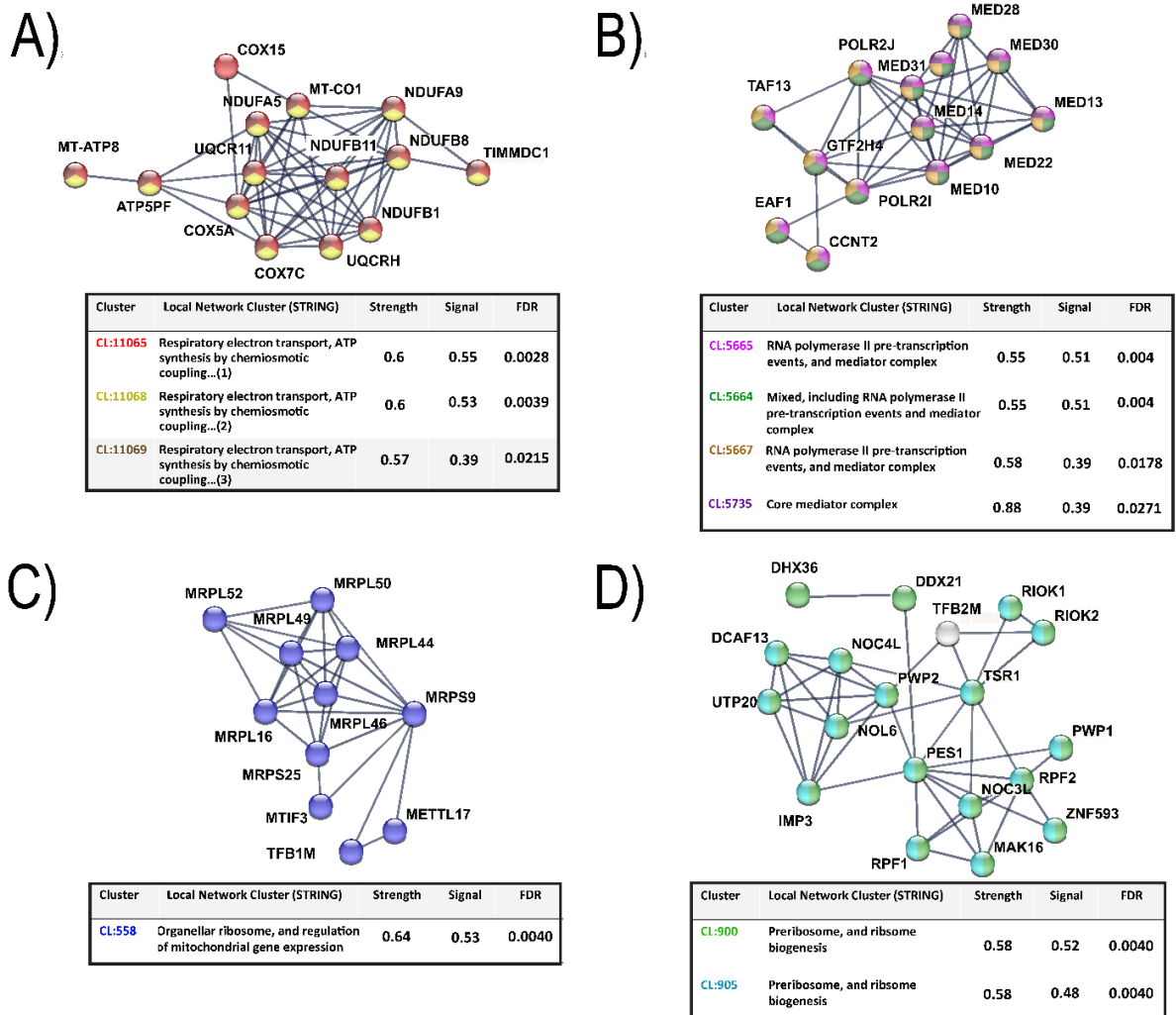


Figure 4.10. Clustering of highly associated proteins in CIML NK cells after restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. **(A-D)** Isolated STRING analysis clusters identified from the 641 differentially expressed proteins in CIML NK cell after restimulation: **(A)** Cluster 1 – electron transport and ATP synthesis (CL:11065, CL:11068, CL:11069), **(B)** Cluster 2 – RNA polymerase II and core mediator complex (CL:5665, CL:5664, CL:5667, CL:5735), **(C)** Cluster 3 – Mitochondrial ribosome and gene expression (CL:558) and **(D)** Cluster 4 – preribosome and ribosome biogenesis (CL:900, CL905). Isolated STRING networks with a minimum required interaction threshold score of the highest confidence (0.900). Disconnected nodes hidden for clarity.

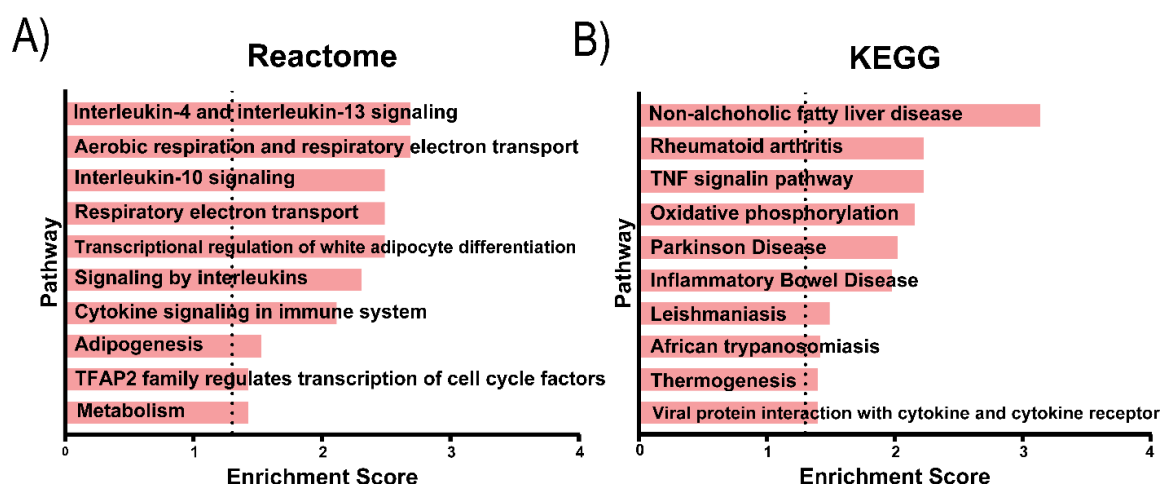


Figure 4.11. Reactome and KEGG pathway analysis of differentially expressed proteins in CIML NK cells after restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. **(A,B)** Quantitative proteomics was performed on Ctrl and CIML NK cells +/- IL-12/15, analysing enriched pathways. Significantly upregulated ($\text{Log}_2\text{FC} > 1$, $p\text{-value} < 0.05$) proteins in CIML + IL-12/15 NK cells were analysed for biological pathway enrichment using the **(A)** Reactome and **(B)** KEGG databases. Significantly enriched pathways ($-\text{Log}_{10}\text{Adj.pvalue} > 1.3$) are shown in red. Enrichment score = $-\text{Log}_{10}\text{Adj.pvalue}$. Dotted line represents significance threshold ($-\text{Log}_{10}\text{Adj.pvalue} = 1.3 = \text{Adj.p} < 0.05$). Quantitative proteomics was performed on N=4 donors.

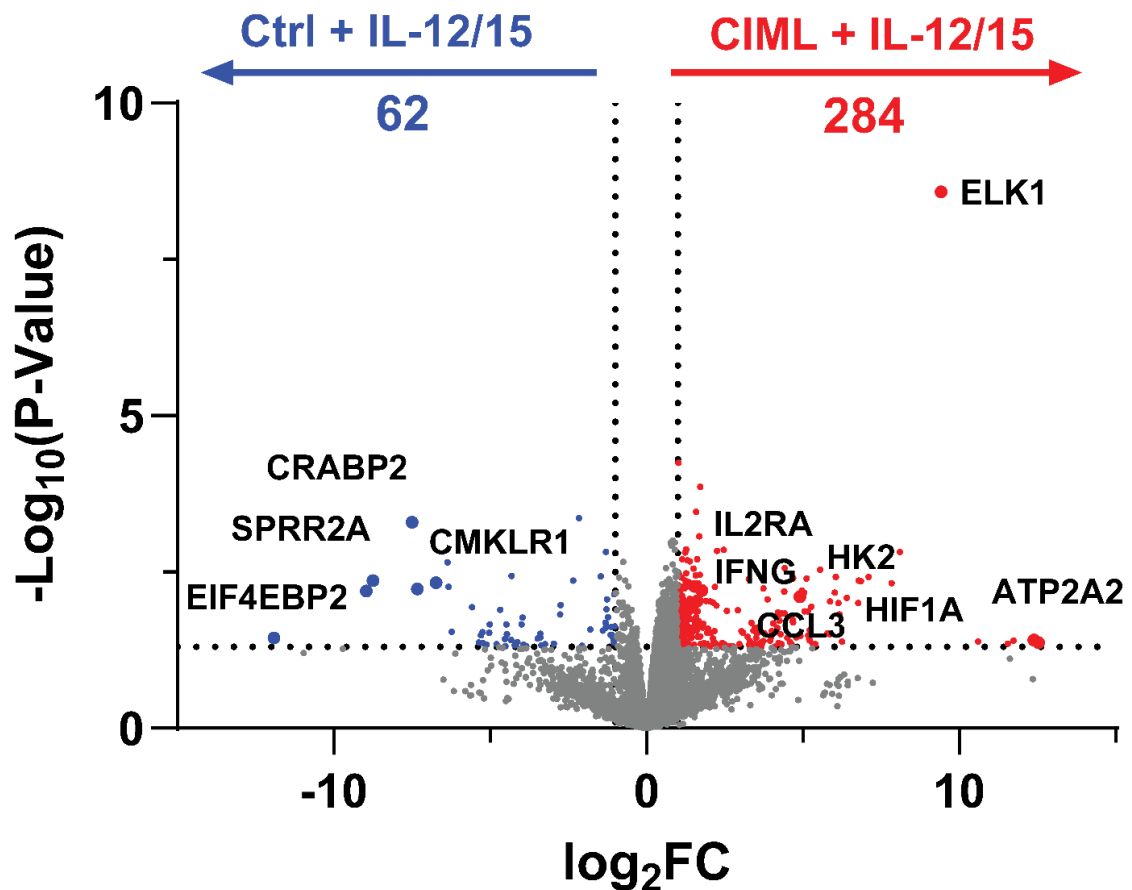


Figure 4.12. Differential expression analysis of control and CIML NK cells + IL-12/15.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days the Ctrl and CIML NK cells were restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. Volcano plot of 8260 total identified proteins displaying differences in relative abundance between Ctrl + IL-12/15 and CIML + IL-12/15 NK cells. The plot represents the $-\log_{10}$ of the p-value versus $\log_2$ of the fold change of protein expression. Differentially expressed proteins (red and blue) have a $\log_2$ fold change >1 or <-1 and a $-\log_{10}(\text{p-value}) > 1.35$. Proteins highlighted in red are enriched in CIML NK cells + IL-12/15, and those highlighted in blue are enriched in resting CIML NK cells. Quantitative proteomics was performed on N=4 donors.

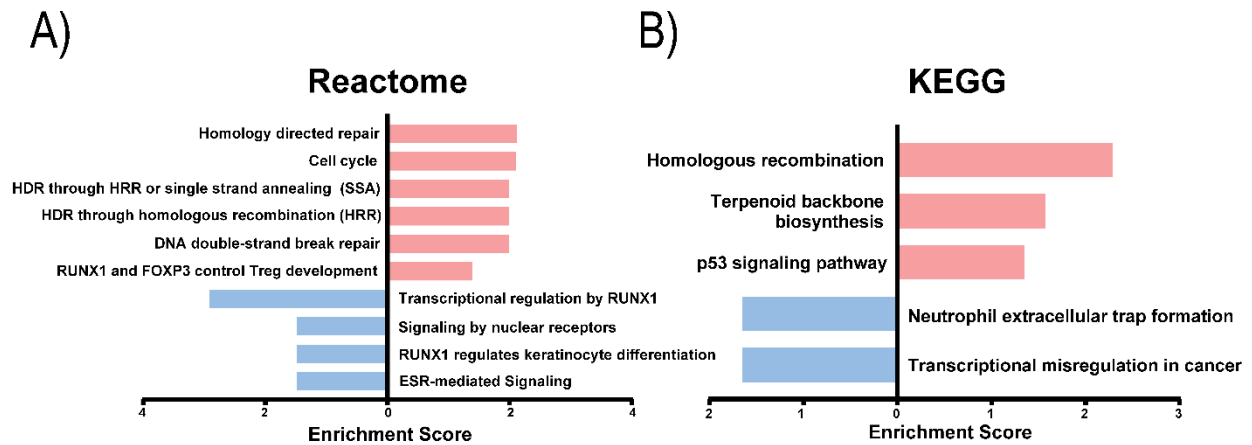


Figure 4.13. Reactome and KEGG pathway analysis of differentially expressed proteins in control and CIML NK cells after restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. **(A,B)** Quantitative proteomics was performed on Ctrl and CIML NK cells + IL-12/15, analysing enriched pathways. Significantly upregulated ($\text{Log}_2\text{FC} > 1$, $p\text{-value} < 0.05$) proteins in CIML + IL-12/15 NK cells were analysed for biological pathway enrichment using the **(A)** Reactome and **(B)** KEGG databases. Significantly enriched pathways ($-\text{Log}_{10}\text{Adj.pvalue} > 1.3$) in CIML + IL-12/15 NK cells are shown in red and enriched pathways in Ctrl + IL-12/15 in blue. Enrichment score = $-\text{Log}_{10}\text{Adj.pvalue}$. Dotted line represents significance threshold ($-\text{Log}_{10}\text{Adj.pvalue} = 1.3 = \text{Adj.p} < 0.05$). Quantitative proteomics was performed on N=4 donors.

A)

All 18 enriched pathways in Ctrl NK cells + IL-12/15

Rank	ID	Set size	NES	p.adjust
1	GOBP_PEPTIDE_CROSS_LINKING	15	3.051	7.14E-05
2	GOBP_AMINOGLYCAN_CATABOLIC_PROCESS	16	2.87	0.0002227
3	GOBP_GLYCOSAMINOGLYCAN_CATABOLIC_PROCESS	14	2.658	0.0022327
4	GOBP_ENDOTHELIAL_CELL_APOPTOTIC_PROCESS	30	2.47	0.0022327
5	GOBP_REGULATION_OF_MICROTUBULE_BASED_MOVEMENT	15	2.501	0.0060762
6	GOBP_POSITIVE_REGULATION_OF_CHEMOTAXIS	56	2.013	0.0078807
7	GOBP_OLIGOSACCHARIDE_CATABOLIC_PROCESS	10	2.401	0.0101404
8	GOBP_GLYCOPROTEIN_CATABOLIC_PROCESS	24	2.171	0.019736
9	GOBP_ANTIBACTERIAL_HUMORAL_RESPONSE	22	2.149	0.0222162
10	GOBP_KERATINIZATION	13	2.223	0.0245103
11	GOBP_KERATINOCYTE_DIFFERENTIATION	56	1.866	0.0249715
12	HALLMARK_COAGULATION	72	1.774	0.0249715
13	GOBP_SENSORY_PERCEPTION_OF_MECHANICAL_STIMULUS	50	1.971	0.0306585
14	HALLMARK_KRAS_SIGNALING_DN	43	2.046	0.0386924
15	GOBP_PROTEIN_ACTIVATION_CASCADE	13	2.083	0.0455363
16	GOBP_NEGATIVE_REGULATION_OF_EPITHELIAL_CELL_APOPTOSIS	28	1.995	0.0455363
17	GOBP_GANGLIOSIDE_METABOLIC_PROCESS	12	2.099	0.0459327
18	GOBP_CERAMIDE_CATABOLIC_PROCESS	12	2.088	0.0475313

B)

Top 20 enriched pathways in CIML NK cells + IL-12/15

Rank	ID	Set size	NES	p.adjust
1	GOBP_MITOCHONDRIAL_TRANSLATION	120	-2.25742	9.13E-08
2	GOBP_MITOCHONDRIAL_GENE_EXPRESSION	151	-2.19634	9.13E-08
3	GOBP_RIBOSOME_BIOGENESIS	290	-1.88965	9.13E-08
4	GOBP_RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS	419	-1.77792	9.13E-08
5	GOBP_RRNA_PROCESSING	205	-1.85185	3.27E-07
6	GOBP_RRNA_METABOLIC_PROCESS	235	-1.78525	8.67E-07
7	HALLMARK_MYC_TARGETS_V1	196	-1.79971	6.17E-06
8	GOBP_CARBOHYDRATE_DERIVATIVE_BIOSYNTHETIC_PROCESS	383	-1.59399	6.82E-06
9	HALLMARK_OXIDATIVE_PHOSPHORYLATION	181	-1.8228	2.36E-05
10	GOBP_RIBOSOMAL_SMALL_SUBUNIT_BIOGENESIS	101	-1.9711	2.59E-05
11	HALLMARK_MTORC1_SIGNALING	170	-1.75735	0.000121
12	GOBP_CELLULAR_RESPIRATION	182	-1.72486	0.000121
13	GOBP_MITOCHONDRIAL_TRANSMEMBRANE_TRANSPORT	71	-1.99352	0.000193
14	GOBP_MITOCHONDRIAL_TRANSPORT	127	-1.78833	0.000295
15	GOBP_AEROBIC_RESPIRATION	153	-1.73312	0.000432
16	GOBP_CYTOPLASMIC_TRANSLATION	145	-1.72405	0.000555
17	GOBP_PROTON_MOTIVE_FORCE_DRIVEN_ATP_SYNTHESIS	64	-1.96544	0.001248
18	GOBP_NUCLEOSIDE_TRIPHOSPHATE_BIOSYNTHETIC_PROCESS	96	-1.81115	0.0022
19	GOBP_MITOCHONDRIAL_TRANSPORT	127	-1.78833	0.000295
20	GOBP_AEROBIC_RESPIRATION	153	-1.73312	0.000432

Table 4.1. The most enriched GSEA pathways in control vs CIML NK cells after restimulation.

(A,B) GSEA was performed on the entire proteomic datasets to identify pathways enriched across the full range of protein expression, independent of differential expression thresholds. Normalised enrichment scores (NES) are shown, with negative values indicating pathways enriched in CIML NK cells + IL-12/15. (A) All enriched pathways in Ctrl NK cells + IL-12/15 and (B) only the top 20 enriched pathways in CIML NK cells + IL-12/15. Significant Pathways ranked by adjusted p-value. Set size indicates proteins upregulated within the biological process gene set ontology (GO:BP) or HALLMARK.

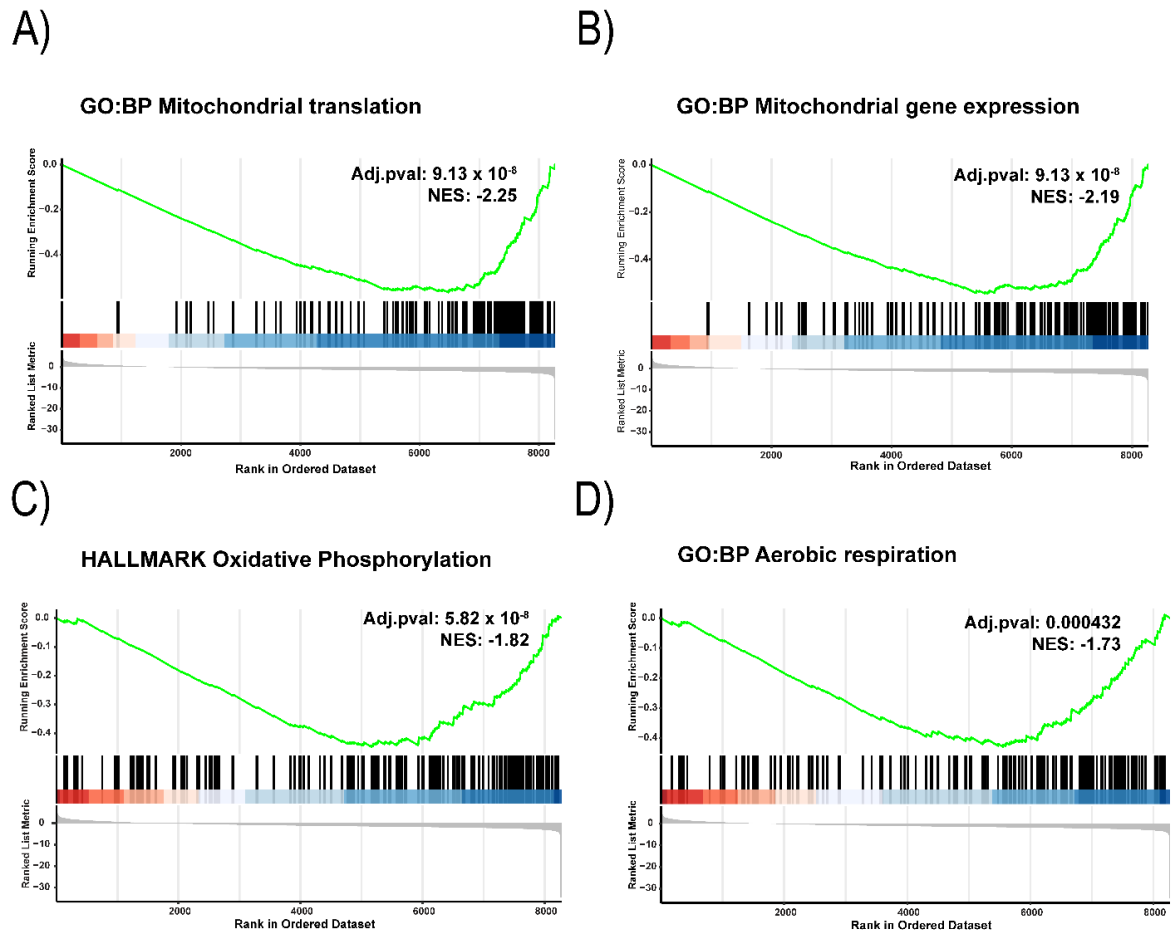


Figure 4.14. GSEA analysis reveals increased mitochondrial protein translation and OXPHOS enrichment in CIML NK cells after restimulation compared to control NK cells.

(A-D) GSEA was performed on the entire proteomic datasets to identify pathways enriched across the full range of protein expression, independent of differential expression thresholds. Proteins were ranked by expression differences between Ctrl and CIML NK cells after restimulation, and enrichment was assessed using predefined pathway gene sets. Normalised enrichment scores (NES) are shown, with negative values indicating pathways enriched in CIML NK cells. Enriched CIML NK cell pathways included **(A)** GO:BP Mitochondrial Translation (M11131), **(B)** GO:BP Mitochondrial Gene Expression (M24915), **(C)** HALLMARK Oxidative phosphorylation (M5936), and **(D)** GO:BP Aerobic respiration (M11785). Pathways with an adjusted p-value of <0.05 were considered statistically significant.

4.5 CIML NK cell mitochondrial dynamics and features after restimulation.

Mitochondria are complex organelles that exist in numerous states that impact NK cell energy metabolism and general function. In order to determine the CIML NK cell mitochondrial phenotype post-stimulation, proteomic and flow cytometric data were integrated to investigate mitochondrial dynamics, size, and bioenergetic features. First, CIML NK cells mitochondrial properties were investigated in terms of protein compartmentalisation after restimulation. Although there was no significant increase in total mitochondrial content, a trending increase was observed in outer ($p=0.0970$) (GO:0005741) and inner ($p=0.0802$) (GO:0005743) mitochondrial membrane proteins (Figure 4.15A-C) in restimulated CIML NK cells compared to stimulated control NK cells. A trending increase was also observed in total CIML NK cell mitoribosomal proteins ($p=0.0940$) (GO:0005762) after restimulation compared to stimulated control NK cells (Figure 4.15D). Analysis of protein content of mitochondrial bioenergetic compartments revealed trending increases in OXPHOS ($p=0.0941$) (GO:0006119), ETC ($p=0.0636$) (GO:0022900), and ATP synthesis ($p=0.0544$) (GO:0042773) associated proteins and, importantly, a significant increase in mitochondrial DNA (mtDNA) encoded proteins in CIML NK cells after restimulation (Figure 4.15E-H). There are 13 known mtDNA structural genes in humans, and all 13 encode for ETC proteins, 3 cytochrome c oxidase subunits, cytochrome b, 7 NADH dehydrogenase subunits, and 2 ATP synthase subunits [314]. Regarding mitochondrial dynamics, mitochondrial fission or fragmentation is associated with poor NK cell function and NK cells in disease states, whereas mitochondrial fusion is generally associated with mitochondrial fitness and respiratory efficiency [97, 107]. CIML NK cells had no change in the mitochondrial fusion-associated proteins, mitofusin 2 (MFN2) or the mitochondrial elongation factor 1 (MIEF1) but did show a trend towards increased mitofusin 1 (MFN1) ($p=0.0564$) expression post-restimulation compared to stimulated control NK cells (Figure 4.16A). Furthermore, no change was observed in the mitochondrial fission protein 1 (FIS1) (Figure 4.16B).

To investigate mitochondrial features further, flow cytometric analysis of mitochondrial membrane potential generation and mitochondrial ROS generation after CIML NK cell restimulation was performed. Control and CIML NK cells were

treated and stained with NAO, TMRM, and mitoSOX to measure mitochondrial mass, membrane potential, and ROS production, respectively, as described in section 2.2.3.1. In Chapter 3, it was demonstrated that CIML NK cells significantly increase mitochondrial mass compared to control NK cells at rest. Interestingly, although CIML NK cells increase mitochondrial translation of mitochondrial proteins upon restimulation, there appears to be a trend towards a decrease ($p=0.0588$) in their overall mitochondrial mass compared to resting CIML NK cells (Figure 4.17A,B). However, although their overall mitochondrial mass decreases, restimulation of CIML NK cells resulted in significantly increased overall membrane potential compared to stimulated control NK cells (Figure 4.18A,B), further supporting their rapid induction of OXPHOS compared to control NK cells after stimulation. It was also demonstrated also defined in Chapter 3 that CIML NK cells have significantly increased mitochondrial ROS at rest compared to control NK cells. In contrast, CIML NK cells exhibited no increases in mitochondrial ROS upon restimulation (Figure 4.19A,B), suggesting efficient OXPHOS and potentially enhanced redox management in the mitochondria. Furthermore, it was found that CIML NK cells have a trend towards an increase ($p=0.0653$) in mitochondrial membrane potential relative to mitochondrial mass upon restimulation compared to control NK cells (Figure 4.20A,B). It was also observed that CIML NK cells have no change in mitochondrial ROS production relative to mitochondrial mass after restimulation (Figure 4.21A,B).

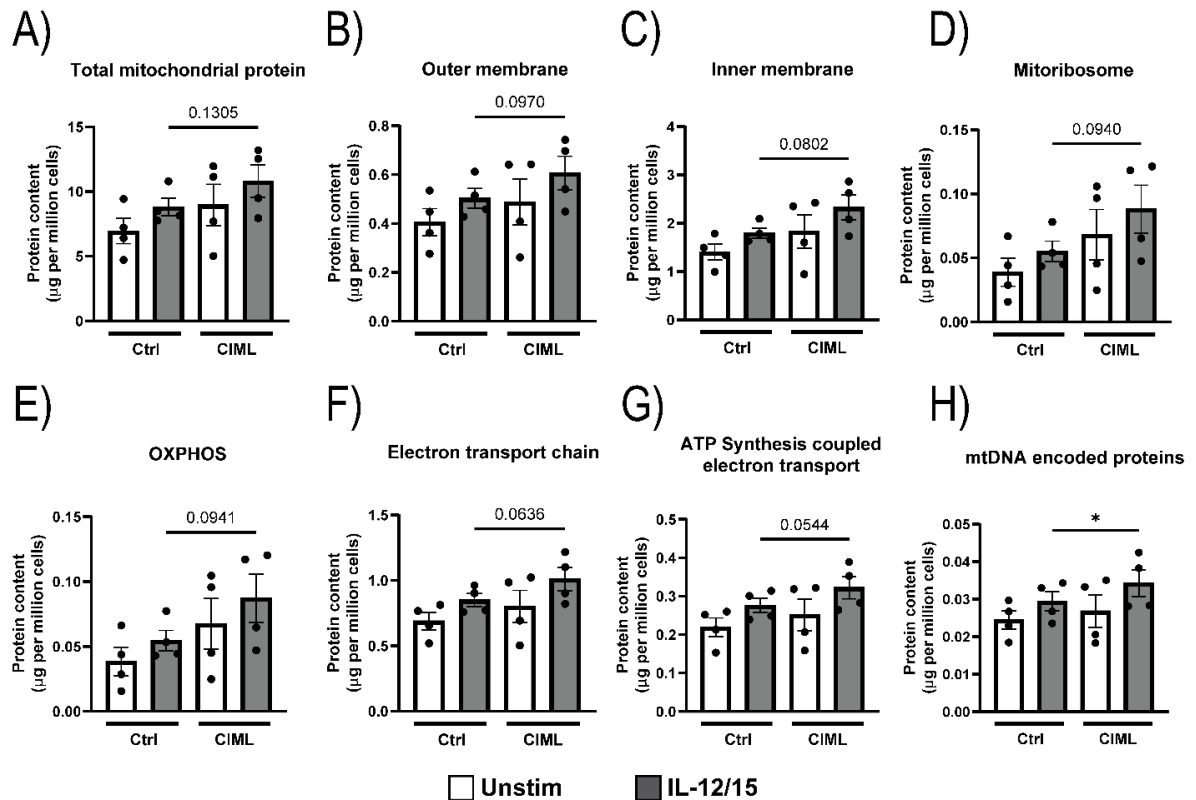


Figure 4.15. Total protein content analysis of mitochondrial protein compartments in control versus CIML NK cells after stimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. **(A-H)** Quantitative proteomics was performed on restimulated Ctrl and CIML NK cells, analysing the average distribution of proteins into mitochondrial compartments. The protein content of **(A)** total mitochondria (GO:0005739), **(B)** mitochondrial outer (GO:0005741) and **(C)** inner (GO:0005743) membranes, **(D)** mitoribosome (GO:0005762), **(E)** OXPHOS (GO:0006119), **(F)** ETC (GO:0022900), **(G)** ATP synthesis coupled ETC (GO:0042773) and **(H)** mitochondrial DNA (mtDNA) encoded proteins in micrograms per million cells before and after restimulation in Ctrl and CIML NK cells. Total protein content per million cells in micrograms was calculated as $\Sigma(((CN \times MW)/N) \times 10^{12})$ where CN = Protein copy number, MW = Protein molecular weight, and N = Avogadro's constant. Data is mean $\pm$ SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (*p<0.05)

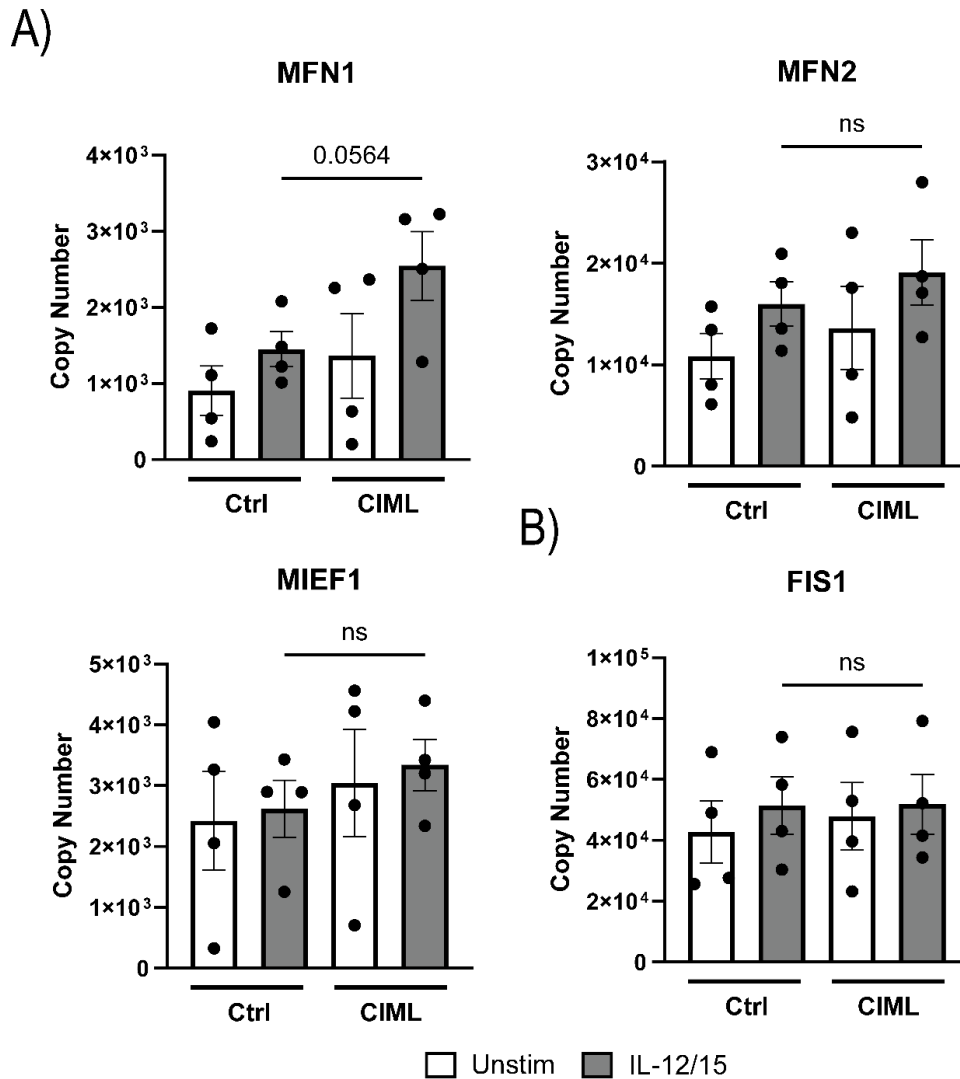


Figure 4.16. CIML NK cells may rapidly induce mitochondrial fusion after restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. **(A,B)** Quantitative proteomics was performed on Ctrl and CIML NK cells +/- IL-12/15, analysing paired copy numbers per cell of proteins involved in mitochondrial **(A)** fusion and **(B)** fission. Data is mean +/- SEM of N=4 donors. Statistical analysis was performed using a paired t-test.

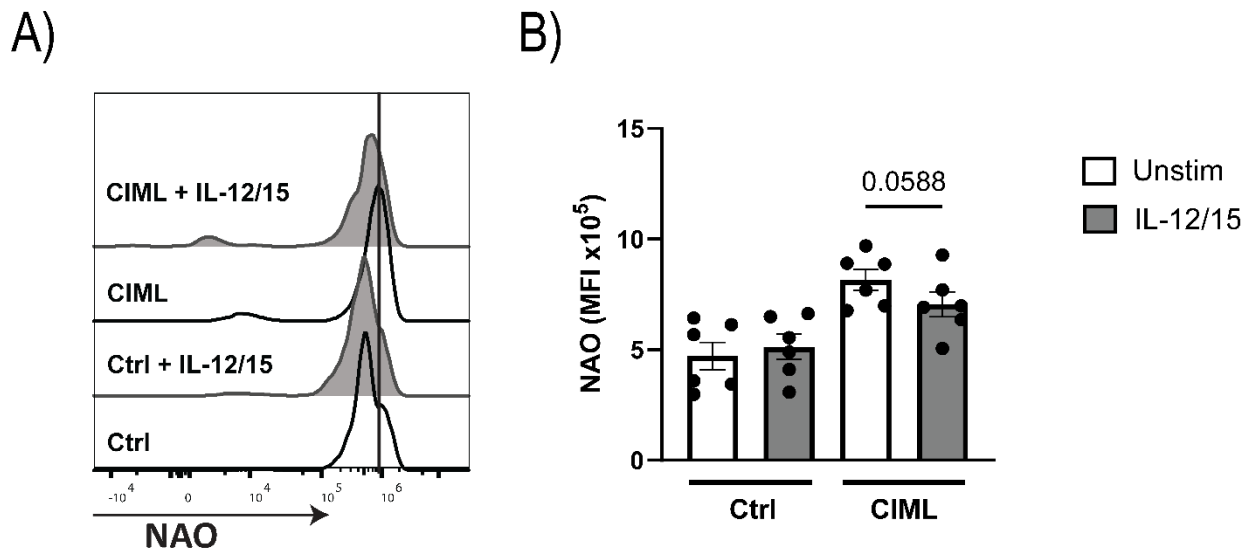


Figure 4.17. Mitochondrial mass in control versus CIML NK cells post-restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. Mitochondrial mass was determined by staining with the nonyl acridine orange (NAO) probe. Cells were incubated with 50 nM NAO for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A,B)** Flow cytometry analysis of NAO staining on Ctrl vs CIML NK cells +/- IL-12/15. **(A)** Representative histogram showing NAO staining signal and **(B)** pooled MFI values of NAO signal. Data are **(A)** representative or **(B)** mean +/- SEM of N=6 donors. Statistical analysis was performed using a paired t-test.

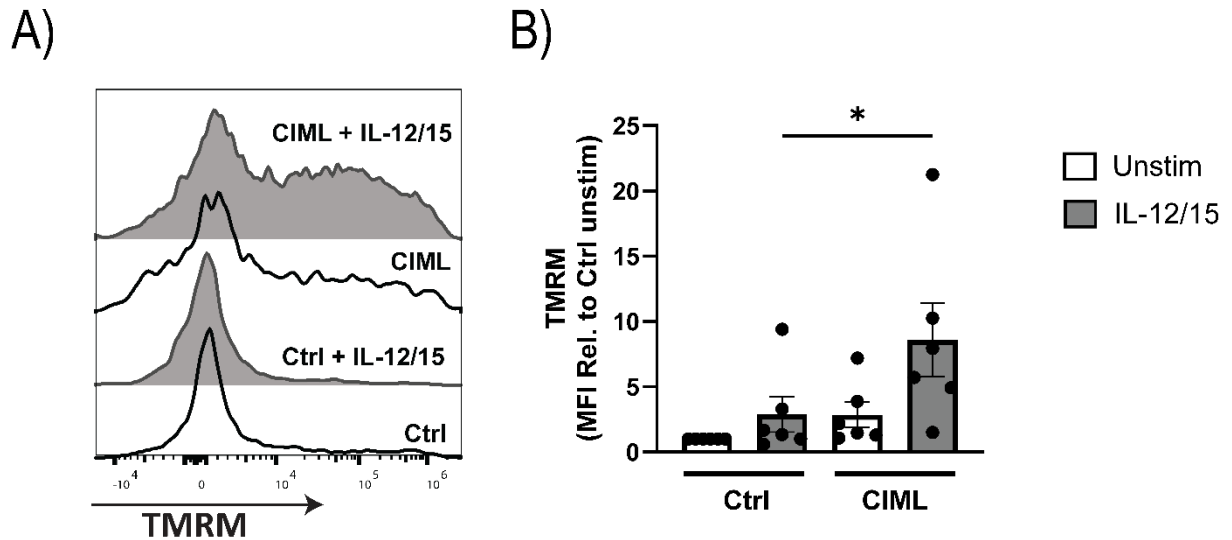


Figure 4.18. CIML NK cells rapidly generate mitochondrial membrane potential after restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. Membrane potential was determined by staining with tetramethylrhodamine methyl ester (TMRM) dye. Cells were incubated with 3 nM TMRM for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A,B)** Flow cytometry analysis of TMRM staining on Ctrl vs CIML NK cells +/- IL-12/15. **(A)** Representative histogram showing TMRM staining signal **(B)** pooled MFI values of TMRM signal relative to Ctrl unstim. Data are **(A)** representative or **(B)** mean +/- SEM of N=6 donors. Statistical analysis was performed using a paired t-test. (* $p < 0.05$).

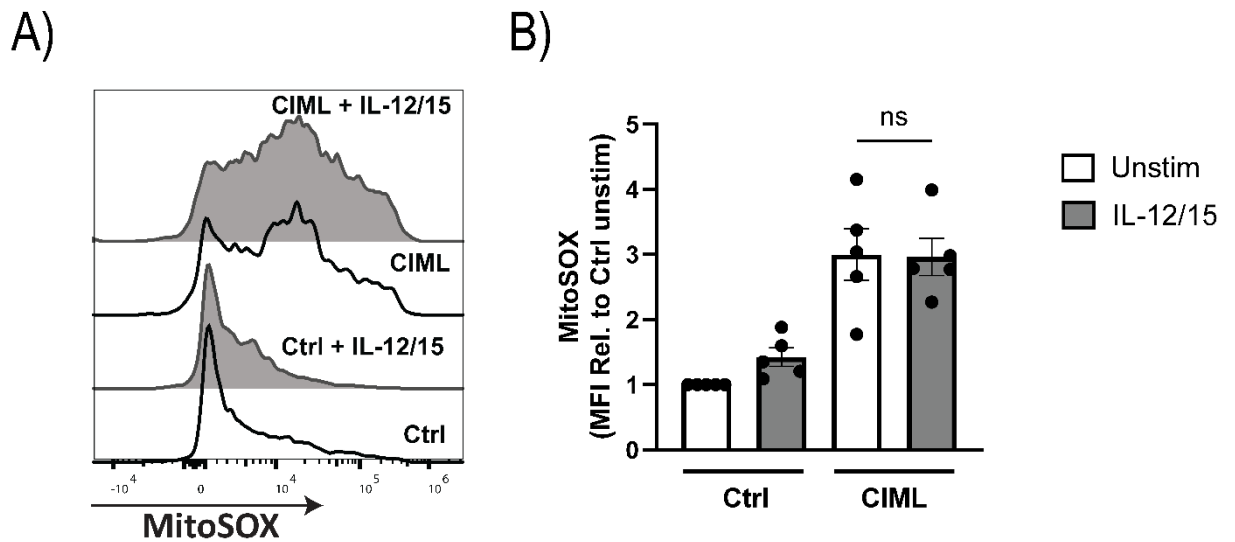


Figure 4.19. CIML NK cells effectively manage mitochondrial ROS post-restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml) and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18-hours. After 7 days the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6-hours and then analysed. Mitochondrial ROS was determined by staining with mitoSOX dye. Cells were incubated with 2 μ M mitoSOX red dye for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A,B)** Flow cytometry analysis of mitoSOX staining on Ctrl vs CIML NK cells +/- IL-12/15. **(A)** Representative histogram showing mitoSOX staining signal and **(B)** pooled MFI values of mitoSOX signal relative to Ctrl unstim. Data are **(A)** representative or **(B)** mean +/- SEM of N=5 donors. Statistical analysis was performed using a paired t-test.

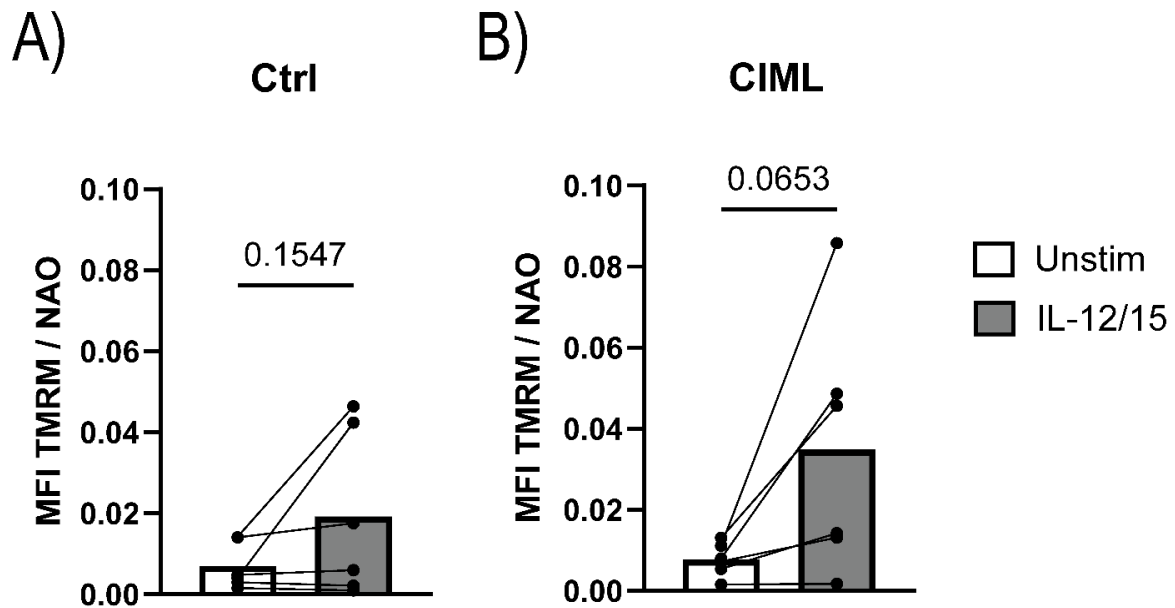


Figure 4.20. CIML NK cells have a potential increase in mitochondrial membrane potential relative to mass, compared to control NK cells after restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. Cells were incubated with 3 nM TMRM and 50 nM NAO for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A,B)** Flow cytometry analysis of TMRM relative to NAO staining on Ctrl vs CIML NK cells +/- IL12/15. **(A,B)** Pooled data of TMRM:NAO MFI ratio values in **(A)** Ctrl and **(B)** CIML NK cells +/- IL12/15. Data are mean +/- SEM of N=6 donors. Statistical analysis was performed using a paired t-test.

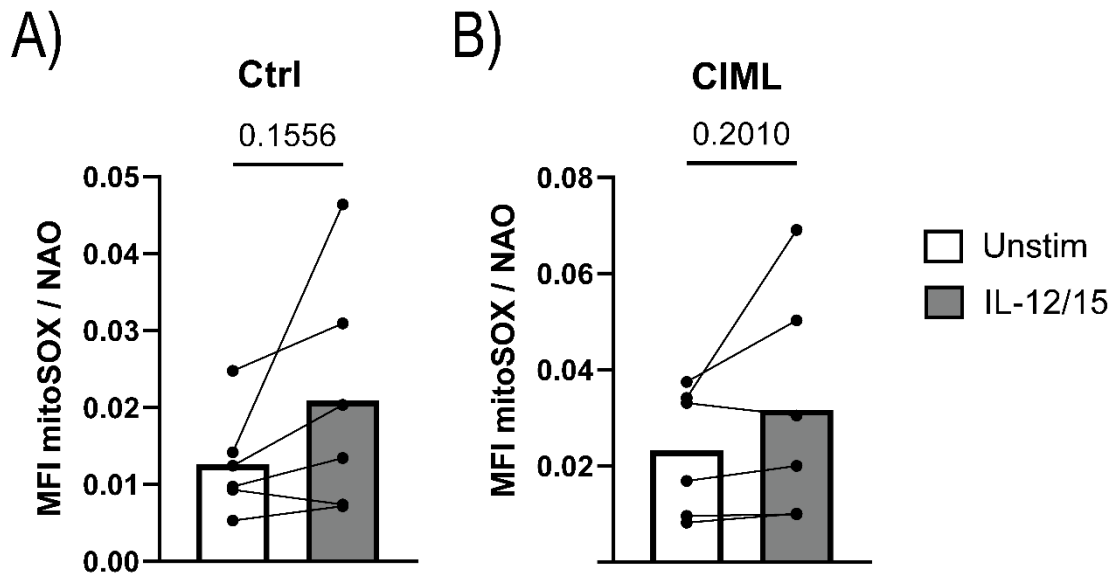


Figure 4.21. CIML NK cells have no increase in mitochondrial ROS relative to mass after restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. Cells were incubated with 2 μ M mitoSOX red and 50 nM NAO for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A,B)** Flow cytometry analysis of mitoSOX relative to NAO staining on Ctrl vs CIML NK cells +/- IL12/15. **(A,B)** Pooled data of mitoSOX:NAO MFI ratio values in **(A)** Ctrl and **(B)** CIML NK cells +/- IL12/15. Data are mean +/- SEM of N=6 donors. Statistical analysis was performed using a paired t-test.

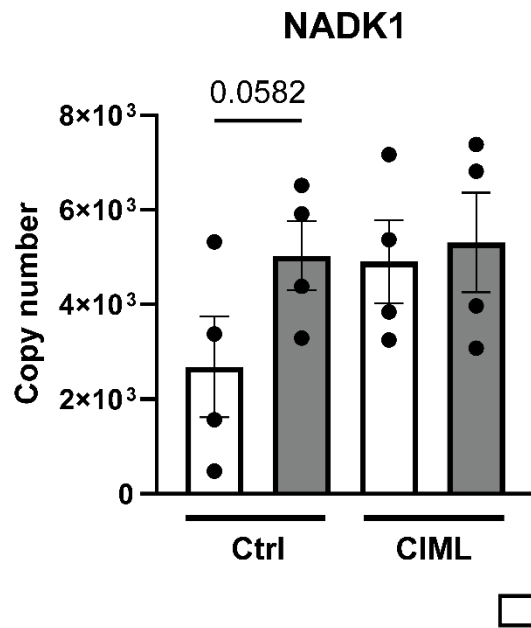
4.6 CIML NK cells may require the pentose phosphate pathway for fully effective mitochondria during restimulation.

In Chapter 3, it was demonstrated that CIML NK cells had upregulated expression of antioxidant enzymes, increased PPP activity, and cytosolic NAD kinase expression, indicating an enhanced potential for redox management. Results presented thus far in this chapter suggest that CIML NK cells demonstrate a better ability to manage mitochondrial ROS production after restimulation. To investigate this further, quantitative proteomic and flow cytometric analyses were integrated as referred to above. It was found that control NK cells have a trend towards increased ($p=0.0582$) expression of NADK1 after stimulation ($p=0.0582$), whereas CIML NK cells did not, indicating that CIML NK cells have a constitutively high expression of cytosolic NADK that does not change upon restimulation (Figure 4.22A). Control NK cells significantly upregulated NADK2 after stimulation, while there was a trend towards increased expression in CIML NK cells post restimulation ($p=0.0983$), suggesting both control and CIML NK cells may upregulate mitochondrial production of NADP^+ after stimulation (Figure 4.22B). Glutathione, as discussed earlier, is a major source of reductive capacity in immune cells and can be transported into the mitochondria from the cytosol to exert its reductive effects. SLC25A39 has been identified as the primary mitochondrial importer of glutathione, and has been shown to regulate ROS production and act as a crucial link between iron homeostasis and OXPHOS [143, 315, 316]. Although the SLC25A39 protein was not in the proteomic datasets, SLC25A40, another glutathione transporter, was significantly upregulated in CIML NK cells after restimulation compared to control NK cells after stimulation, consistent with increased mitochondrial glutathione uptake (Figure 4.23).

As the PPP is an important source of cytosolic reductive potential, which can be important in sustaining mitochondrial redox balance, the role of the PPP in mitochondrial activity in CIML NK cells during restimulation was investigated. To assess this, mitochondrial parameters in CIML NK cells after restimulation were analysed by flow cytometry as referred to above, in the presence or absence of the PPP inhibitor 6-aminonicotinamide (6-AN). CIML NK cells had a trend towards decreased mitochondrial mass upon restimulation; however, inhibition of

the PPP resulted in no change in mitochondrial mass after restimulation in CIML NK cells (Figure 4.24A,B). Interestingly, inhibition of the PPP resulted in a significant decrease in mitochondrial membrane potential during restimulation of CIML NK cells compared to control NK cells (Figure 4.25A,B), suggesting a role for the PPP in mitochondrial bioenergetic capacity. Finally, inhibition of the PPP resulted in no change in mitochondrial ROS accumulation in restimulated CIML NK cells (Figure 4.26A,B).

A)



B)

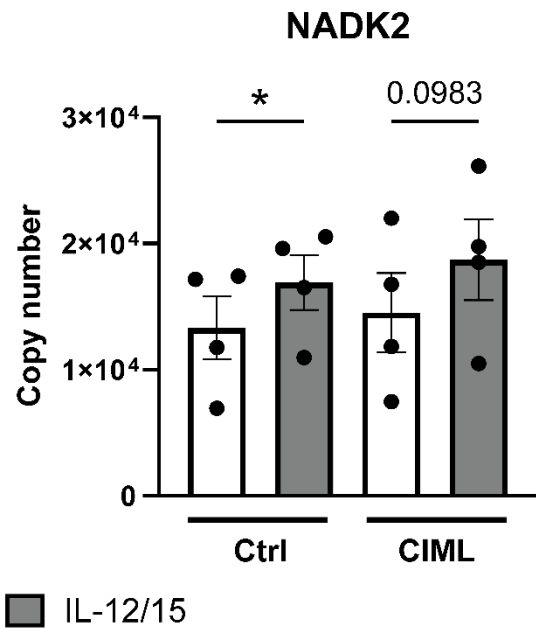


Figure 4.22. CIML NK cells have constitutively high expression of NADK1 and do not upregulate NADK2 compared to controls after restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. **(A,B)** Quantitative proteomics was performed on Ctrl and CIML NK cells +/- IL-12/15, analysing paired copy numbers per cell of **(A)** cytosolic and **(B)** mitochondrial NAD kinase (NADK). Data is mean +/- SEM of N=4 donors. Statistical analysis was performed using a paired t-test and a one-way ANOVA. (* $p < 0.05$).

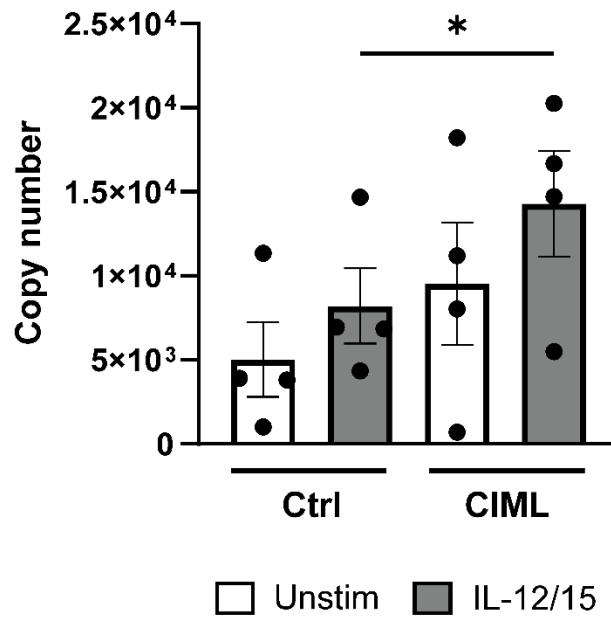
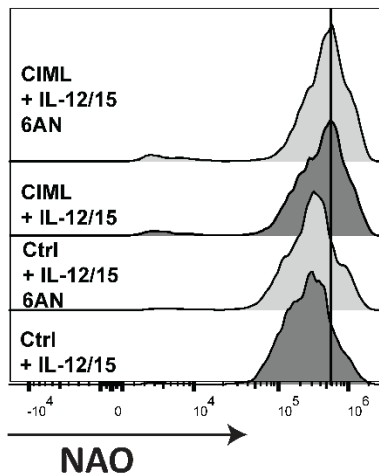


Figure 4.23. CIML NK cells significantly increase expression of the mitochondrial glutathione transporter SLC25A40 after restimulation compared to control NK cells.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. Quantitative proteomics was performed on Ctrl and CIML NK cells +/- IL-12/15, analysing paired copy numbers per cell of the hypothesised mitochondrial glutathione transporter SLC25A40. Data is mean +/- SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (* $p < 0.05$).

A)



B)

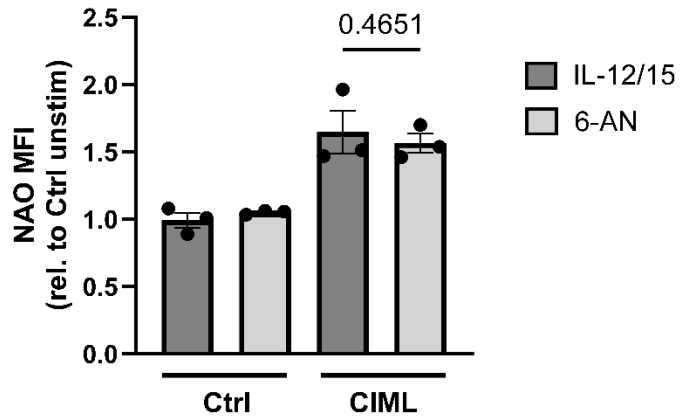


Figure 4.24. Inhibition of the pentose phosphate pathway does not affect mitochondrial mass after CIML NK cell restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) +/- 50 μ M of the PPP inhibitor 6-AN for 6 hours and then analysed. Mitochondrial mass was determined by staining nonyl acridine orange (NAO) probe. Cells were incubated with 50 nM NAO for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A,B)** Flow cytometry analysis of NAO staining on Ctrl and CIML NK cells after restimulation +/- 6-AN. **(A)** Representative histogram showing NAO staining signal **(B)** pooled raw MFI values of NAO signal relative to Ctrl unstim. Data are **(A)** representative or **(B)** mean +/- SEM of N=3 donors. Statistical analysis was performed using a paired t-test.

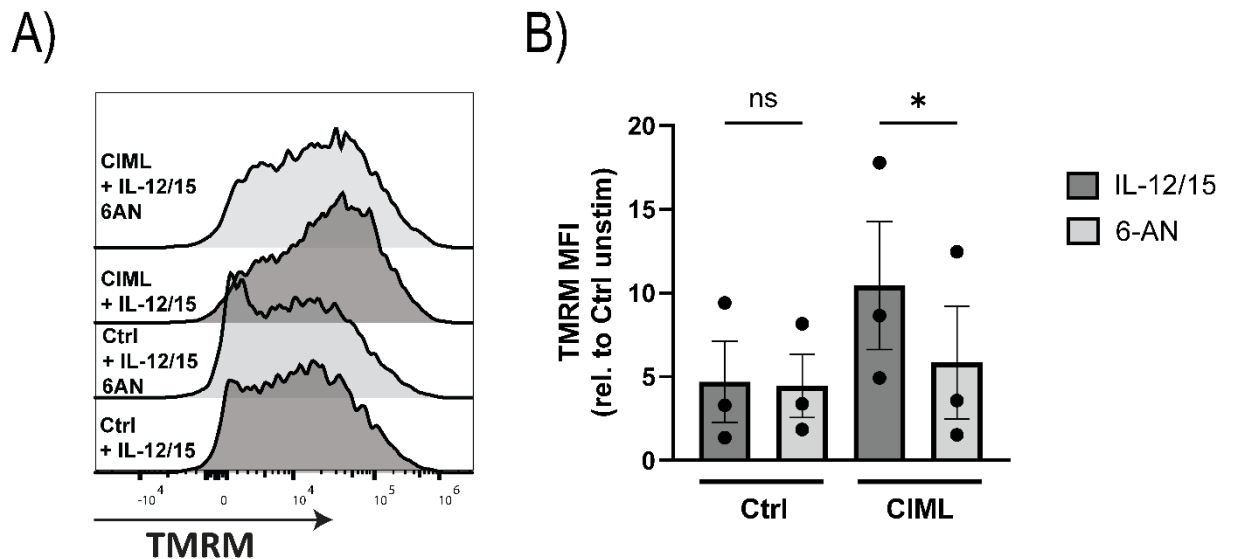


Figure 4.25. The pentose phosphate pathway may be important for CIML NK cell mitochondrial membrane potential generation post-restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) +/- 50 μ M of the PPP inhibitor 6-AN for 6 hours and then analysed. Mitochondrial membrane potential was determined by staining with tetramethylrhodamine methyl ester (TMRM) dye. Cells were incubated with 3 nM TMRM for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A,B)** Flow cytometry analysis of TMRM staining on Ctrl and CIML NK cells after restimulation +/- 6-AN. **(A)** Representative histogram showing TMRM staining signal **(B)** pooled raw MFI values of TMRM signal relative to Ctrl + IL-12/15. Data are **(A)** representative or **(B)** mean +/- SEM of N=3 donors. Statistical analysis was performed using a one-way ANOVA. (*p<0.05).

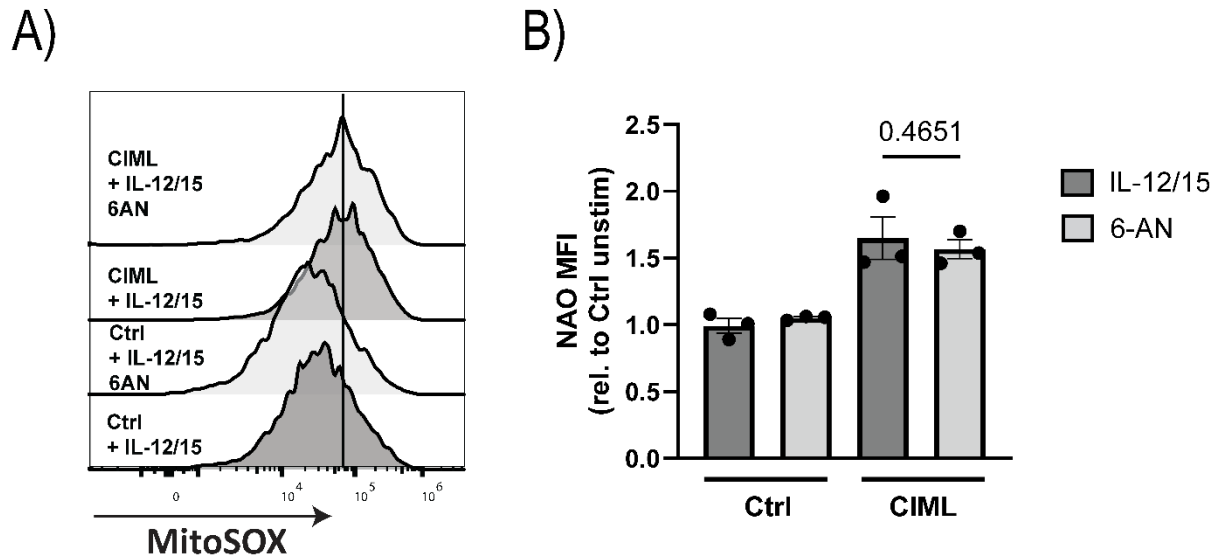


Figure 4.26. Inhibition of the pentose phosphate pathway does not affect mitochondrial ROS after CIML NK cell restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) +/- 50 μ M of the PPP inhibitor 6-AN for 6 hours and then analysed. Mitochondrial membrane potential was determined by staining with tetramethylrhodamine methyl ester (TMRM) dye. Cells were incubated with 3 nM TMRM for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A,B)** Flow cytometry analysis of TMRM staining on Ctrl and CIML NK cells after restimulation +/- 6-AN. **(A)** Representative histogram showing TMRM staining signal **(B)** pooled raw MFI values of TMRM signal relative to Ctrl + IL-12/15. Data are **(A)** representative or **(B)** mean +/- SEM of N=3 donors. Statistical analysis was performed using a paired t-test.

4.7 Mitochondrial metabolism and the pentose phosphate pathway may be important for CIML NK cell-enhanced secondary responses.

It is clear that CIML NK cells rapidly induce mitochondrial OXPHOS after restimulation and have increased mitochondrial membrane potential compared to stimulated control NK cells. In order to generate this membrane potential, cells must take up and break down nutrients, which are oxidised by the ETC, resulting in a proton gradient in the mitochondrial inner membrane, which is harnessed to drive ATP synthesis [39] [86]. The main fuels for this process include pyruvate derived from glucose, fatty acids, and amino acids such as glutamine, which feed the TCA cycle via FAO and glutaminolysis, respectively [39]. Earlier in this chapter, no changes were observed in amino acid metabolism in CIML NK cells after restimulation compared to stimulated control NK cells, but there was an observed trend towards an increase in FAO-associated proteins (Figure 4.3), indicating that fatty acids may be important fuels for the rapid burst of CIML NK cell mitochondrial activity. Another important mitochondrial fuel is pyruvate, which is formed from glycolysis and converted into acetyl coenzyme A (acetyl-CoA), which feeds the TCA cycle, generating NADH and FADH₂ for electron transport. The conversion of pyruvate to acetyl-CoA is carried out by pyruvate dehydrogenase (PDH), an enzyme within the pyruvate dehydrogenase complex (PDC). In order to determine if mitochondrial pyruvate metabolism was important for CIML NK cell metabolism post-restimulation, quantitative proteomic analysis was performed as referred to above. Quantitative proteomics revealed a trend towards an increase ($p=0.0705$) in the total protein content of the PDC in CIML NK cells after restimulation (Figure 4.27A) and a trend towards increased ($p=0.0744$) expression of proteins that make up the PDC, such as the PDH E1 alpha subunit (Figure 4.27B).

The rapid induction of OXPHOS protein translation and membrane potential generation suggests more fuels are being burned in the mitochondria, which may underpin energetic demands for enhanced CIML NK cell secondary functions. The pentose phosphate pathway was also shown earlier to potentially have a role in maintaining mitochondrial membrane potential upon restimulation of CIML NK cells (Figure 4.25A,B). To investigate these observations further, mitochondrial

ATP synthesis, PDH activity, and the PPP were inhibited during stimulation of control and CIML NK cells. Inhibition of the ATP synthase with a low dose concentration of oligomycin (40nM) resulted in a trend towards decreased IFN γ production in CIML NK cells after restimulation, while having no effect on stimulated control NK cells (Figure 4.28 A,B). Similarly, CIML NK cells make significantly more granzyme B at rest and after restimulation compared to control NK cells, but inhibition of ATP synthase resulted in a return to resting levels of granzyme B expression after restimulation in CIML NK cells (Figure 4.29A,B). Inhibition of PDH using CPI-613, preventing pyruvate-derived acetyl-CoA from feeding the TCA cycle, resulted in a trend towards decreased IFN γ (Figure 4.30A,B) and granzyme B (Figure 4.31A,B) production in CIML NK cells after restimulation and again showed a modest effect on control NK cell IFN γ and granzyme B expression after stimulation. Finally, inhibition of the PPP resulted in a more detrimental effect on IFN γ (Figure 3.32A,B) and granzyme B (Figure 3.33A,B) expression in CIML NK cells after restimulation compared to stimulated control NK cells. However, it is important to note that data for the control NK cells in this experiment were limited to n=2, and therefore these results must be interpreted with caution. Additional experiments are required to fully elucidate the role of these pathways in control and CIML NK cell metabolism upon restimulation.

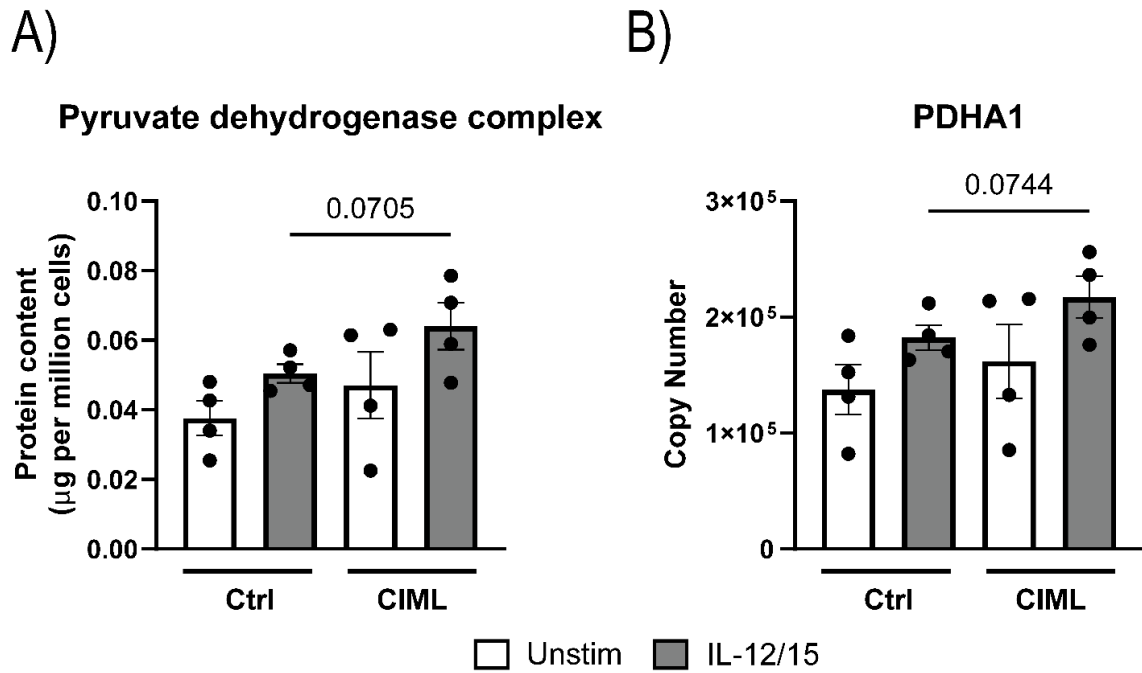


Figure 4.27. Pyruvate dehydrogenase complex and PDHA1.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. Quantitative proteomics was performed on Ctrl and CIML NK cells +/- IL-12/15, analysing paired total protein of **(A)** the pyruvate dehydrogenase (PDH) complex (PDC) (GO:0045354) and **(B)** paired copy numbers per cell of the PDH E1 subunit alpha 1 (PDHA1). Total protein content per million cells in micrograms was calculated as $\Sigma(((CN \times MW)/N) \times 10^{12})$ where CN = Protein copy number, MW = Protein molecular weight, and N = Avogadro's constant. Data is mean +/- SEM of N=4. donors **(A)**. Statistical analysis was performed using a paired t-test.

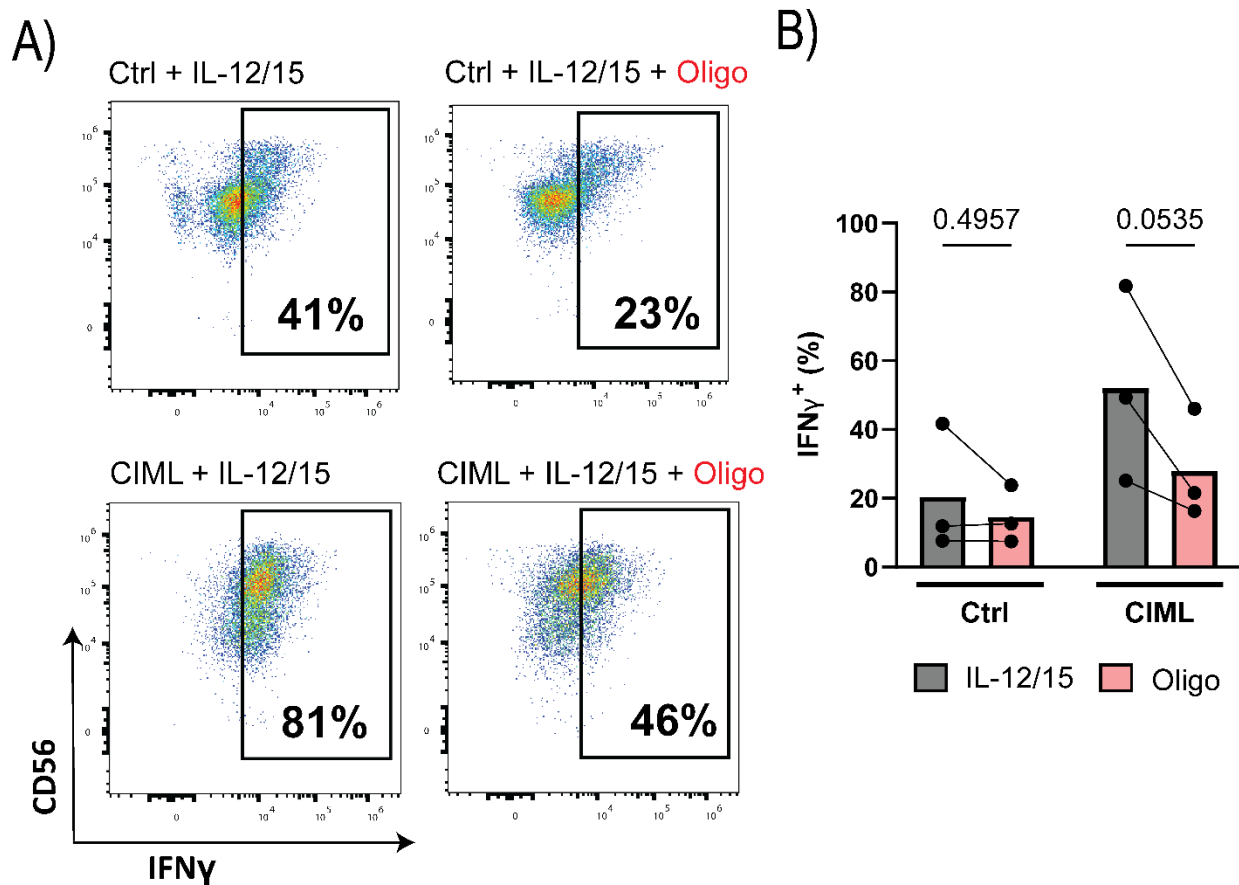


Figure 4.28. Mitochondrial ATP synthesis may be necessary for enhanced IFN γ production in CIML NK cells upon restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) +/- 40 nM of the ATP synthase inhibitor oligomycin (oligo) for 6 hours. Cells were subsequently stained for surface markers, followed by intracellular staining for granzyme B (GZMB) and acquired on Cytex Aurora. **(A,B)** Flow cytometry analysis of GZMB staining on Ctrl and CIML NK cells before and after stimulation +/- oligo. **(A)** Representative histogram showing IFN γ staining signal **(B)**, pooled percentage positive values of IFN γ . Data are **(A)** representative or **(B)** mean +/- SEM of N=3 donors. Statistical analysis was performed using a two-way ANOVA with a Tukey's multiple comparisons test.

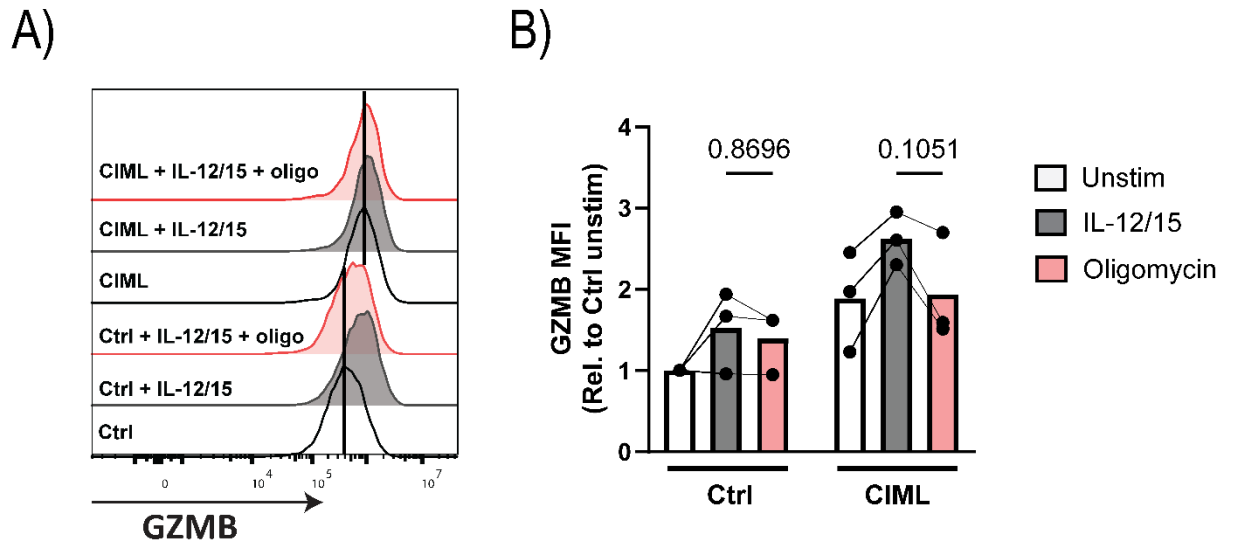


Figure 4.29. Mitochondrial ATP synthesis may be necessary for enhanced granzyme B production in CIML NK cells upon restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) +/- 40 nM of the ATP synthase inhibitor oligomycin (oligo) for 6 hours. Cells were subsequently stained for surface markers, followed by intracellular staining for granzyme B (GZMB) and acquired on Cytex Aurora. **(A,B)** Flow cytometry analysis of GZMB staining on Ctrl and CIML NK cells before and after stimulation +/- oligomycin. **(A)** Representative histogram showing GZMB staining signal **(B)** pooled MFI values of GZMB signal relative to Ctrl unstim. Data are **(A)** representative or **(B)** mean +/- SEM of N=3 donors. Statistical analysis was performed using a two-way ANOVA with a Tukey's multiple comparisons test.

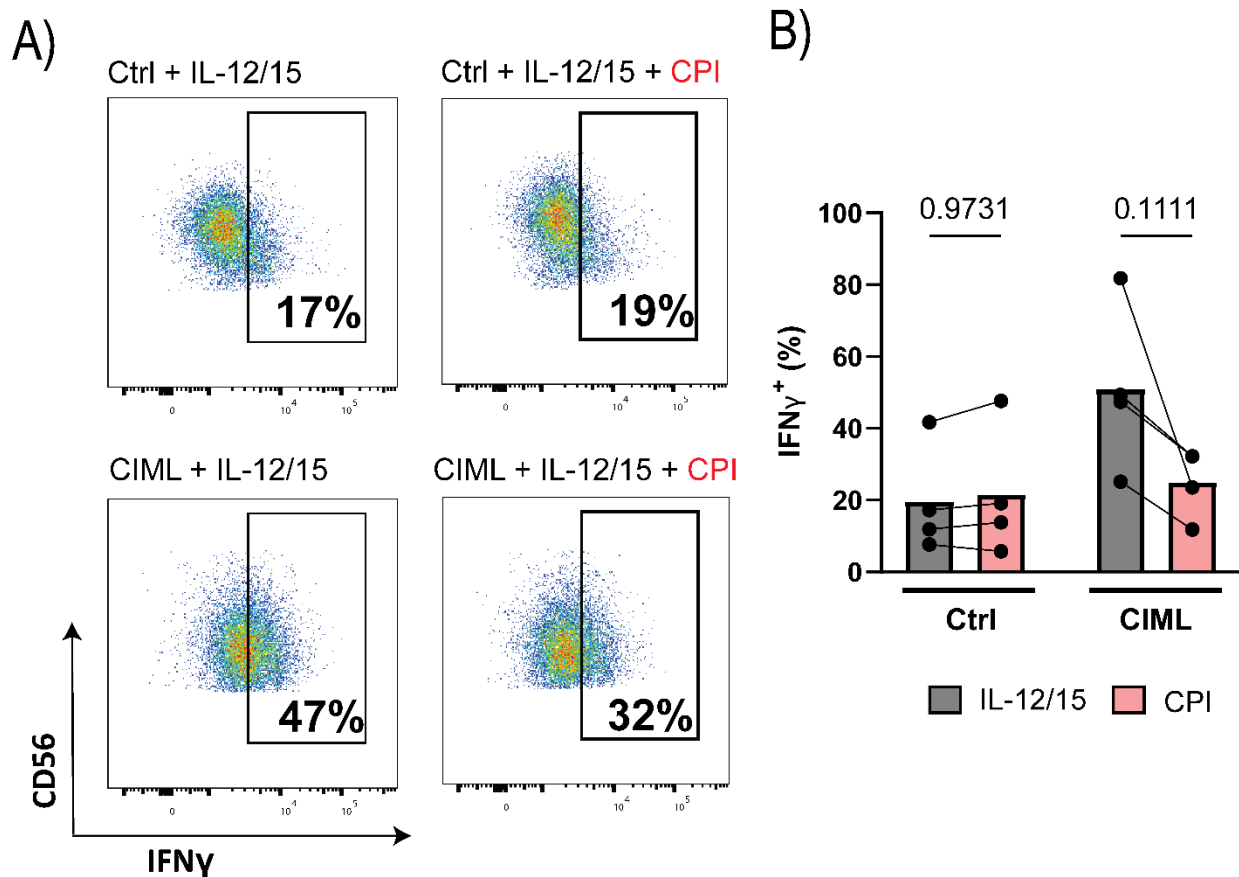


Figure 4.30. Mitochondrial pyruvate metabolism may be necessary for enhanced IFN γ production in CIML NK cells upon restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) +/- 50 μ M of the PDH inhibitor CPI-613 (CPI) for 6 hours. Cells were subsequently stained for surface markers, followed by intracellular staining for IFN γ and acquired on Cytex Aurora. **(A,B)** Flow cytometry analysis of IFN γ staining on Ctrl and CIML NK cells before and after stimulation +/- oligomycin. **(A)** Representative histogram showing IFN γ staining signal **(B)**, pooled percentage positive values of IFN γ . Data are **(A)** representative or **(B)** mean +/- SEM of N=4 donors. Statistical analysis was performed using a two-way ANOVA with a Tukey's multiple comparisons test.

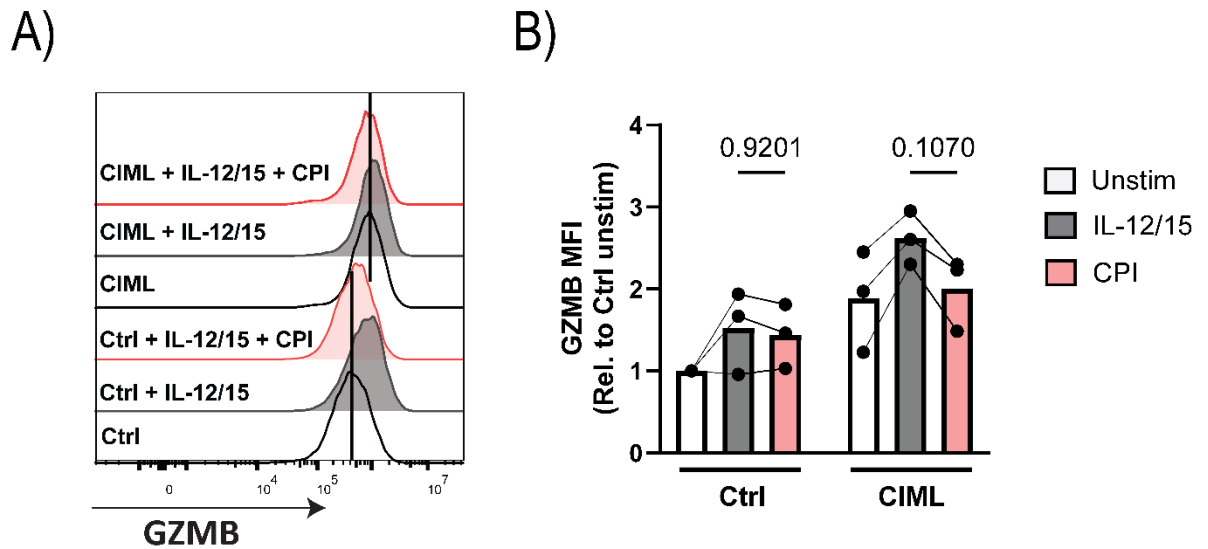


Figure 4.31. Mitochondrial pyruvate metabolism may be necessary for enhanced granzyme B production in CIML NK cells upon restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or stimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) +/- 50 μ M of the PDH inhibitor CPI-613 (CPI) for 6 hours. Cells were subsequently stained for surface markers, followed by intracellular staining for granzyme B (GZMB) and acquired on Cytex Aurora. **(A,B)** Flow cytometry analysis of GZMB staining on Ctrl and CIML NK cells before and after stimulation +/- CPI. **(A)** Representative histogram showing GZMB staining signal **(B)**, pooled MFI values of GZMB signal relative to Ctrl unstim. Data are **(A)** representative or **(B)** mean +/- SEM of N=3 donors. Statistical analysis was performed using a two-way ANOVA with a Tukey's multiple comparisons test. (* $p < 0.05$).

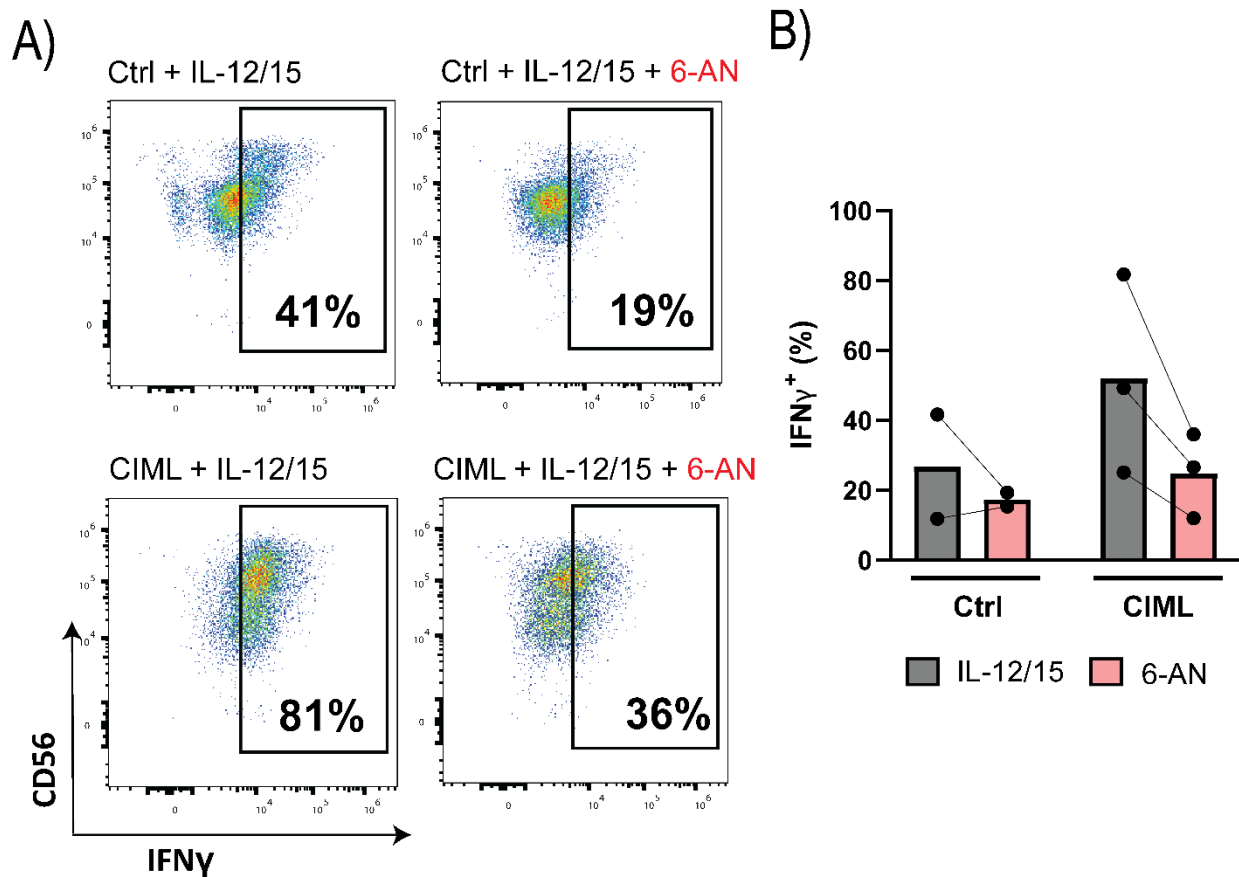


Figure 4.32. The pentose phosphate pathway may be necessary for enhanced granzyme B production in CIML NK cells upon restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) +/- 10 μ M of the PPP inhibitor 6-aminonicotinamide (6-AN) for 6 hours. Cells were subsequently stained for surface markers, followed by intracellular staining for IFN γ and acquired on Cytex Aurora. **(A,B)** Flow cytometry analysis of IFN γ staining on Ctrl and CIML NK cells before and after stimulation +/- oligomycin. **(A)** Representative histogram showing IFN γ staining signal **(B)** pooled percentage positive values of IFN γ . Data are **(A)** representative or **(B)** mean +/- SEM of N=2-3 donors. Statistical analysis was performed using a two-way ANOVA with a Tukey's multiple comparisons test.

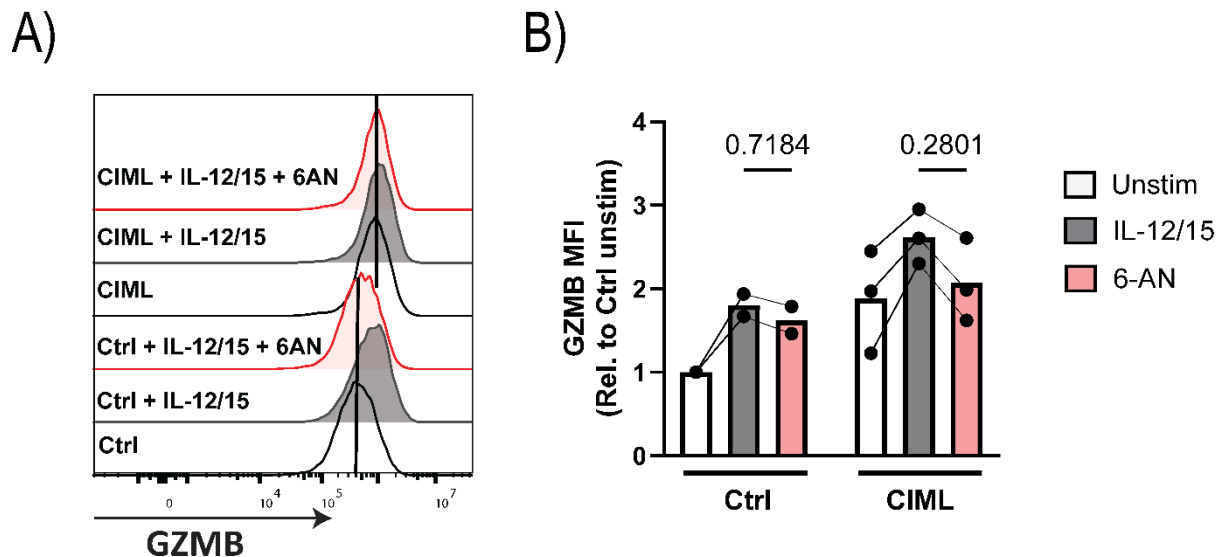


Figure 4.33. The pentose phosphate pathway may be necessary for enhanced granzyme B production in CIML NK cells upon restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or stimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) +/- 10 μ M of the PPP inhibitor 6-aminonicotinamide (6-AN) for 6 hours. Cells were subsequently stained for surface markers, followed by intracellular staining for granzyme B (GZMB) and acquired on Cytex Aurora. **(A,B)** Flow cytometry analysis of GZMB staining on Ctrl and CIML NK cells before and after stimulation +/- 6-AN. **(A)** Representative histogram showing GZMB staining signal **(B)**, pooled MFI values of GZMB signal relative to Ctrl unstim. Data are **(A)** representative or **(B)** mean +/- SEM of N=2-3 donors. Statistical analysis was performed using a two-way ANOVA with a Tukey's multiple comparisons test.

4.8 Discussion

Understanding how CIML NK cells effectively mount enhanced effector functions will ultimately shape their advancement as an immunotherapy. Although metabolic manipulation in the generation and engineering of immunotherapies was not particularly targeted in the past, there is increasing interest in exploring metabolic alterations for next-generation cell therapies [317-319]. Here, it is demonstrated that CIML NK cells rapidly induce translation of OXPHOS-associated proteins and mitochondrial membrane potential, which may be the bioenergetic foundation of enhanced CIML NK cell secondary functions. The research presented in this chapter provides novel mechanistic insights as to how CIML NK cells alter their metabolism to mount such efficient and potent effector functions, which will help with rationale design.

In chapter 3, it was demonstrated that there is an accumulation of mitochondria in CIML NK cells. Results in this chapter demonstrate that CIML NK cells may rapidly mobilise this increase in mitochondrial mass for energy production. Multidimensional analysis of proteomic data using quantitative, STRING, and pathway analyses all suggest that CIML NK cells undergo rapid upregulation of OXPHOS, ETC, and mitochondrial ATP generation-associated proteins. This observation was supported by flow cytometric data that CIML NK cells rapidly induce mitochondrial membrane potential compared to control NK cells after restimulation. This suggests that CIML NK cells have preestablished mitochondrial mass poised for rapid energy generation upon restimulation and the potential to prioritise effector molecule production. A similar phenotype has been identified in adaptive immune cells. For example, CD8⁺ memory T cells have greater mitochondrial mass and greater spare respiratory capacity compared to CD8⁺ effector T cells, indicating a potential readiness for rapid OXPHOS induction [320]. The data also suggest that CIML NK cells may undergo mitochondrial fusion after restimulation, which was also shown to drive CD4⁺ memory T cell formation and improve immunotherapeutic adoptive transfer outcomes in mice [321]. This indicates that CIML NK cells use similar mechanisms to classical memory cells for function, memory development, and recall responses. Furthermore, in adaptive NK cells that arise in murine and human CMV infection, there is significantly increased mitochondrial ROS

production 7 days after infection, but no change in mitochondrial mass, and significantly decreased mitochondrial potential. In the previous chapter, CIML NK cells displayed a significant increase in mitochondrial mass; this mass had no change in membrane potential but did have significantly increased ROS generation. Also worth noting is a study demonstrating that the ability to remove dysfunctional mitochondria is essential for NK cell memory formation [300]. Again, this demonstrates a link between mitochondrial regulation and memory formation, resulting in better recall responses. It is possible that the mitochondria, through an intricate balance of mass accumulation and adequate ROS management, contribute towards driving memory formation in CIML NK cells while facilitating enhanced effector functions through rapid OXPHOS induction.

Results in this chapter also demonstrated that CIML NK cells do not increase mitochondrial ROS after restimulation, which was observed after stimulation of conventional NK cells overnight with IL-12/IL-15 and IL-18, as shown by other studies [223]. NADK enzymes catalyse the phosphorylation of NAD^+ to NADP^+ , which can subsequently be reduced to NADPH. While NADK is primarily localised in the cytosol, its mitochondrial isomer, NADK2, carries out this function within the mitochondria. Interestingly, it is shown in this study that CIML NK cells had constitutively high expression of NADK that did not change with stimulation and showed no significant change in NADK2 expression compared to control stimulated NK cells. Although CIML NK cells had the ability to manage mitochondrial ROS production after stimulation, their intrinsic mitochondrial ability to produce NADP^+ was no different from control NK cells. NADK2 has been shown to be important for fatty acid synthesis, lipoylation of PDH that is essential for mitochondrial pyruvate oxidation, and importantly, ETC assembly and translation of mitochondrial proteins [322]. Furthermore, methotrexate, a common cancer treatment, has been shown to inhibit NADK2 activity in murine hepatocytes, but overexpression of NADK2 using an adenovirus ameliorated this effect through rescuing mitochondrial NADPH levels [323]. CIML NK cells do not appear to upregulate mitochondrial $\text{NADP}^+/\text{NADPH}$ generating mechanisms, such as increased expression of NADK2, relative to control NK cells upon restimulation. As a result, CIML NK cells may be less reliant on intrinsic mitochondrial mechanisms for regulating mitochondrial ROS upon restimulation.

Instead, CIML NK cells may depend on the import of cytosol-derived reducing factors into the mitochondria via transporters such as SLC25A39 and SLC25A40, which are associated with sustaining mitochondrial integrity and effective energy metabolism [143, 315]. Although this may be adequate for enhanced functions *in vitro*, immune cells from patients with cancer and other diseases associated with oxidative stress are known to harbour cells with dysfunctional mitochondria, driven by the disease state, which may affect CIML NK clinical efficacy [97, 324, 325]. Overexpression of proteins involved in intrinsic mechanisms of mitochondrial ROS management, such as NADK2, may improve NK cell resistance to oxidative stress following adoptive transfer into cancer patients, thereby supporting more robust functions in the periphery or TME.

Glucose has been appreciated as an important fuel for CIML NK cells, which rely on its metabolism for both their generation, sustainment, and effective secondary responses [220, 222, 284]. In the previous chapter, and as shown in other studies, it is suggested that CIML NK cells have accumulated mitochondria that are not bioenergetically more active than controls [220, 221]. It was also demonstrated that there was more pyruvate in the supernatants from CIML NK cells, indicating that it may not be an important fuel for CIML NK cells, pointing towards a more energy-dormant mitochondrial profile. However, in this chapter, it is demonstrated that, upon restimulation and inhibition of PDH or ATP synthase, CIML NK cells may lose their characteristic enhanced IFN γ and granzyme B production. This indicates that CIML NK cells are functionally reliant on OXPHOS and that OXPHOS feeding metabolic pathways may redirect pyruvate into the mitochondria for aerobic respiration upon restimulation. Similar observations have been made in IL-4/IL-13 trained bone marrow-derived murine macrophages, where effective secondary responses against *Mycobacterium tuberculosis* were abrogated with oligomycin treatment. Interestingly, glucose depletion in this study enhanced IL-4/IL-13 trained macrophage IL-6 and TNF α production during rechallenge, whereas inhibition of glutaminolysis decreased responses, indicating preferential mitochondrial metabolism of amino acids rather than glucose products [326]. Similarly, *in vivo* lipopolysaccharide (LPS) trained alveolar murine macrophages showed increased glycolysis and OXPHOS 16 hours post *ex vivo* rechallenge with *Streptococcus pneumoniae*

[327]. Indeed, CIML NK cells show some metabolic similarities with canonical trained immune cells after restimulation.

The role of the PPP is ill-defined in human NK cell function and metabolism. Studies in murine cells demonstrated that upon stimulation with IL-2/IL-12, NK cells may upregulate PPP metabolism, with potential increases in *G6pd* mRNA. Similarly, PKM2, a key glycolytic enzyme, was shown to be essential for maintaining R5P pools in IL-2/12 stimulated murine NK cells [94, 328]. However, the PPP has been studied in more detail in other cell types, with a more recent study showing that small hairpin RNA-mediated deletion of PKM2 elevated PPP metabolism in CD8⁺ T cells and that PPP agonism resulted in an improved CD8⁺ T cell phenotype with enhanced tumour killing in mice [329]. Cytokine-induced killer (CIK) cells, which feature a mixed NK and T cell phenotype, were shown to upregulate glucose and PPP metabolism and had increased intracellular NADPH in prolonged culture environments [330]. Regulatory T cells also rely on PPP metabolism, particularly TKT activity, for glycolysis, oxidative stress regulation, and restriction of DNA hypermethylation, acting as a bridge between regulatory T cell metabolism and epigenetic control [74]. In this chapter, it was shown that the PPP is important for secondary responses, and its inhibition results in a collapse in mitochondrial membrane potential after restimulation, indicating it has a role in efficient mitochondrial ATP production. Although the PPP contributes to ROS management, and inhibition of the PPP elevates mitochondrial ROS in resting CIML NK cells, no changes were observed in mitochondrial ROS production after inhibition of the PPP during restimulation. The PPP also produces R5P for nucleotide synthesis and the subsequent generation of, molecules involved in cell bioenergetics [66]. In Chapter 3, it was demonstrated that R5P from PPP metabolism feeds nucleotide synthesis, and it is well established that mitochondria demand a constant nucleotide supply in order to function [331]. It was also demonstrated that there is a significant upregulation in mtDNA-encoded proteins in the mitochondria after restimulation of CIML NK cells, suggesting they would need an increased supply of nucleotides for transcription of mtDNA-derived proteins. Similarly, nucleotide pools influence AMP/ADP supply. Uptake of these nucleotides is stimulated by membrane potential generation in the mitochondria, and they can be subsequently

phosphorylated by ATP synthase to generate ATP [122, 332, 333]. Thus, it is clear that the PPP has an important role in efficient mitochondrial energy metabolism in restimulated CIML NK cells; however, future studies must investigate which aspect of PPP metabolism is supporting mitochondrial membrane potential and enhanced effector function.

Overall, this chapter uses proteomic and flow cytometric analyses to identify novel mechanistic insights into CIML NK cell secondary responses. This study has demonstrated that CIML NK cells undergo a rapid metabolic shift towards OXPHOS using preestablished mitochondrial mass. This enhanced OXPHOS is supported by fuels such as glucose-derived pyruvate and pathways like the PPP, which, through an unclear mechanism, has an important role in the generation of mitochondrial membrane potential. Together, these pathways are likely to coordinate to support enhanced effector function in a mechanism unique to CIML NK cells that is not observed in control NK cells. The data suggests FAO may also be important in this system, and future studies should investigate the role of FAO during CIML NK cell restimulation, as it is a major mitochondrial metabolic pathway of energy generation, important for NK cell responses against infection and cancer [172, 173]. Ultimately, this demonstrates that CIML NK cells not only mimic functional characteristics of classic memory cells and canonical innate immune-trained cells but also undergo similar aspects of metabolic rewiring used for facilitating enhanced function. This could be potentially harnessed in next-generation CIML NK cell immunotherapies.

Chapter 5. Functional and metabolic heterogeneity of CIML NK cell subsets.

5.1 Introduction.

It is well established that human NK cells are made up of a range of subsets. The best characterised are separated based on their expression of the surface marker CD56. The CD56^{dim} subset is associated with cytotoxicity, displaying high basal expression of granzyme B and CD16, which binds antibodies that are opsonising target cells, thus triggering ADCC and target cell lysis. The CD56^{bright} subset is more proinflammatory, producing large amounts of IFN γ and TNF α upon activation, but must upregulate cytotoxic machinery, unlike CD56^{dim} NK cells. These properties are supported by differences in metabolism, with CD56^{bright} NK cells upregulating nutrient uptake transporters like CD71, GLUT1, SLC7A5, and SLC3A2 for enhanced glucose, iron, and amino acid metabolism to meet these bioenergetic and biosynthetic demands [15, 334, 335]. Other, more unique and epigenetically distinct NK cell subsets exist in humans, such as an adaptive-like NK cell phenotype that arises post-HCMV infection, and have been associated with enhanced immune potential against cancer [336, 337]. Thus, it is not surprising that heterogeneity has recently been observed within CIML NK cells as outlined below.

CIML NK cells are induced *in vitro* and are derived from conventional CD56^{dim} and CD56^{bright} NK cells. Unsurprisingly, due to the natural heterogeneity of conventional NK cells, CIML NK cells have recently been shown to comprise various subsets with transcriptional and epigenetic differences. The epigenetic landscape of CIML NK cells was untangled by Foltz et al., who describe how the bulk CIML NK cell population is comprised of long-lived CD56^{bright}s and CD56^{dim} NK cells, a primed or “effector conventional” NK (effcNK) and an “enriched memory-like” (eML) NK cell phenotype. These effcNK cells show a high degree of epigenetic similarity to control NK cells (low dose IL-15), thus do not meet one of the main criteria of innate immune training, which is epigenetic remodeling [194]. On the other hand, eMLs exhibit vast epigenetic and transcriptional differences between both control NK cells and effcNK cells, and are also epigenetically unique to adaptive NK cells, classifying them as a bona fide innate

immune-trained cell. In summary, CIML NK cells are made up of long-lived conventional NK cells, a primed NK cell phenotype (effcNKs), and an enriched trained subpopulation (eMLs), which exhibit enhanced functional responses [219]. In this chapter, a publicly available single-cell RNA-sequencing (scRNA-seq) dataset generated by Foltz et al. was used to search for metabolic and additional functional differences between effcNKs and eML NK cells.

This chapter tests the hypothesis that eML NK cells represent a functionally and metabolically distinct trained subset, supported by coordinated metabolic reprogramming. This reprogramming is expected to align bioenergetic and biosynthetic pathways with eML NK cell demands, enabling sustained and amplified functionality. Furthermore, it is hypothesised that the emergence of the eML NK cell subset may account for the functional and metabolic differences observed in the total CIML NK cell population, as described in the previous chapters.

5.2 Aims.

- To identify effcNK cells and eML NK cells at the RNA level and *in vitro*.
- To characterise functional and phenotypic differences between effcNK cells and eML NK cells.
- To investigate metabolic differences between effcNK cells and eML NK cells.

5.3 Identification of effcNK and eML NK cells.

Foltz et al. describe how eMLs have markedly reduced expression of *KIT* (CD117) and *B3GAT1* (CD57) but have significantly increased gene expression of *KLRC1* (NKG2A) and *ENTPD1* (CD39) [219]. Prior to carrying out more substantial analysis of functional/metabolic characteristics in effcNKs versus eMLs, it was first necessary to ensure that the two subsets could be identified based on RNA expression of markers specific to eMLs. UMAP visualisation of gene expression revealed that effcNK and eML NK cells do indeed have reduced expression of the conventional-like NK cell markers, CD57 (Figure 5.1A) and CD117 (Figure 5.1B). In terms of eML markers, both effcNKs and eMLs had high expression of NKG2A (Figure 5.1C), but eMLs had a notable enrichment of CD39 gene expression (Figure 5.1D). Visualisation of these markers using violin plots further validated CD117 and CD57 negativity (Figure 5.2A), with double positivity for NKG2A and CD39 in eML NK cells (Figure 5.2B). Furthermore, eML NK cells were shown to have epigenetic and transcriptional differences distinct from HCMV adaptive NK cells, indicating a unique memory profile. HCMV adaptive NK cells are positive for *KLRC2* (NKG2C) and negative for *FCER1G* (FcεR1γ), and both effcNK and eMLs had low expression of NKG2C (Figure 5.3B) and high expression of FcεR1γ (Figure 5.3B), suggesting that there is a transcriptional distinction between cytokine-induced eML NK cells and CMV adaptive NK cells [338]. Moreover, unlike adaptive NK cells, eMLs express NKG2A and lack CD57. Foltz et al. also reported that effcNKs and eMLs can be identified by flow cytometry using these markers, and that eMLs make up approximately 27% of the bulk CIML NK cell population. Indeed, flow cytometric analysis revealed the emergence of an eML population in CIML NK cells (Figure 5.4A,B), and consistent with previous findings, it was found that eMLs NK made up less than 1% of all circulating NK cells analysed *ex vivo* (Figure 5.5A), less than 5% of bulk control NK cells and were on average $29.8 \pm 2.6\%$ of the total bulk CIML NK cell population (Figure 5.5B).

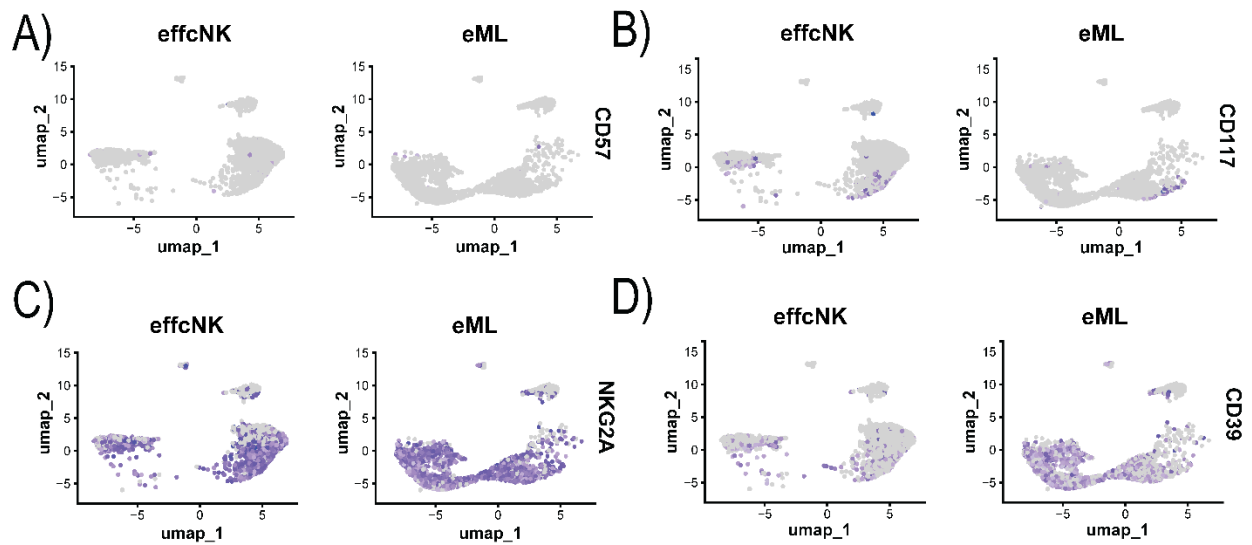


Figure 5.1. UMAP visualisation of gene markers associated with effcNKs and eMLs.

(A-D) Foltz et al. isolated single cells from CIML NK cells by flow cytometry based on expression of CD117, CD57, NKG2A, and CD39 markers. Primed “effcNK” cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. NK cells were then barcoded and sequenced by 10X Genomics scRNA-seq. Single cell populations of effector conventional NK (effcNK) cells and enriched memory-like (eML) NK cells were isolated from purified healthy human CIML NK cells. UMAPs display expression of genes encoding for (A) CD57, (B) CD117, (C) NKG2A, and (D) CD39. N=1 donor. Raw data available at [DOI: 10.1126/sciimmunol.adk4893](https://doi.org/10.1126/sciimmunol.adk4893).

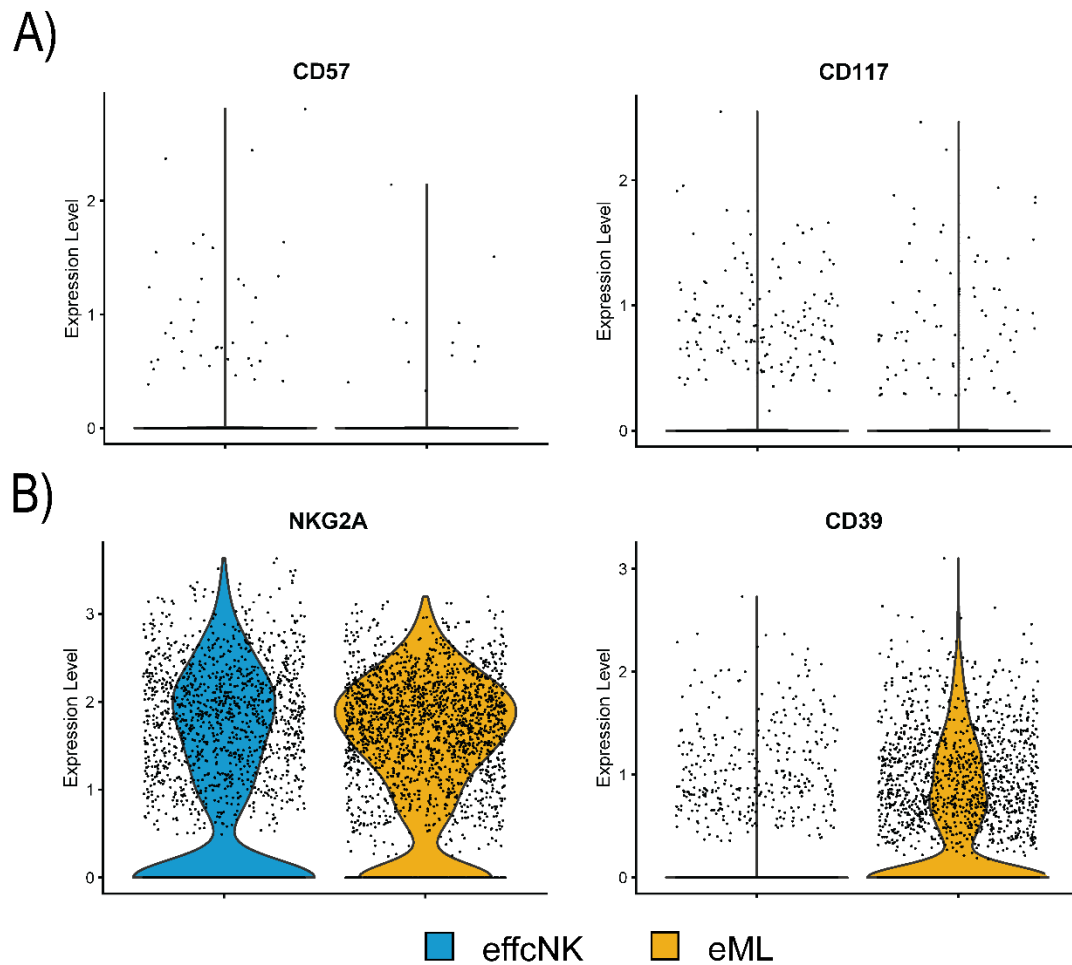


Figure 5.2. Violin plot of gene markers associated with effcNKs and eMLs.

(A,B) Foltz et al. isolated single cells from CIML NK cells by flow cytometry based on expression of CD117, CD57, NKG2A, and CD39 markers. Primed “effcNK” cells purified as the CD117⁻CD57⁻NKG2A⁻CD39⁻ population and trained “eML” NK cells purified as the CD117⁻CD57⁻NKG2A⁺CD39⁺ population. NK cells were then barcoded and sequenced by 10X Genomics scRNA-seq. Single cell populations of effector conventional NK (effcNK) cells and enriched memory-like (eML) NK cells were isolated from purified healthy human CIML NK cells. Violin plots display expression of genes encoding for **(A)** CD57, CD117, **(B)** NKG2A, and CD39. N=1 donor. Raw data available at [DOI: 10.1126/sciimmunol.adk4893](https://doi.org/10.1126/sciimmunol.adk4893).

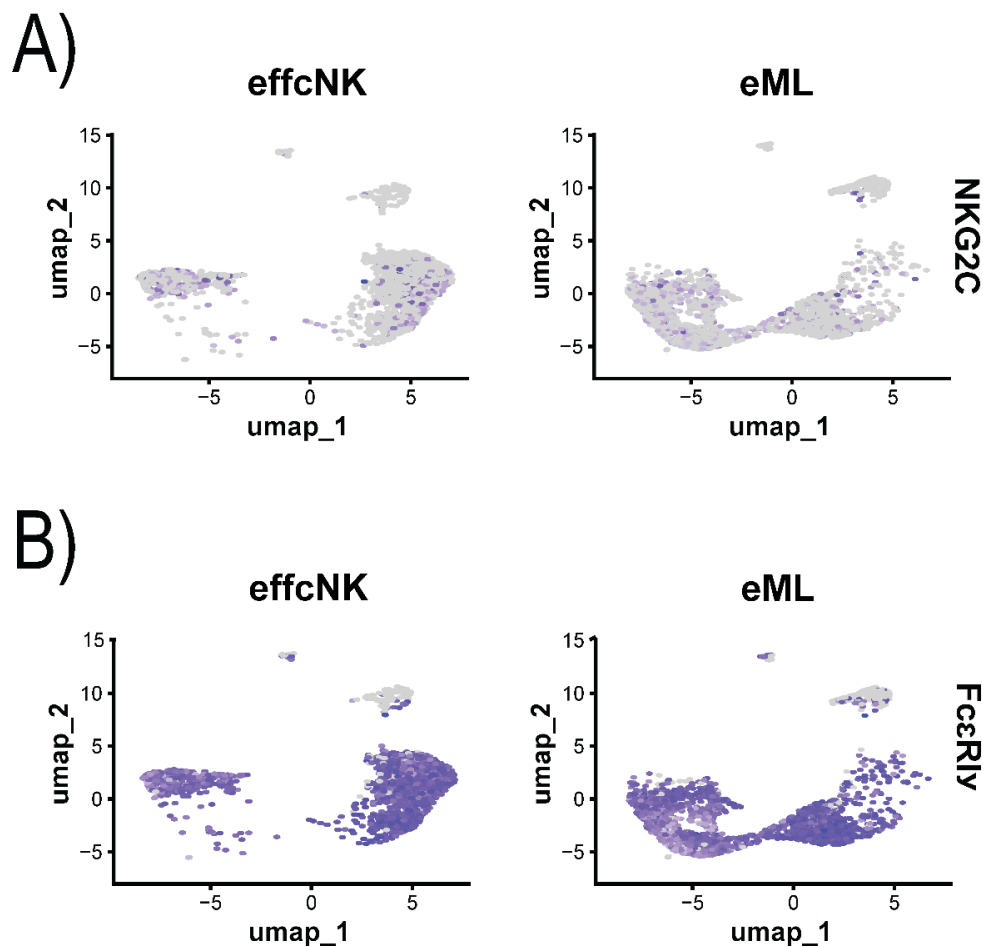


Figure 5.3. UMAP visualisation of adaptive NK cell markers.

(A,B) Foltz et al. isolated single cells from CIML NK cells by flow cytometry based on expression of CD117, CD57, NKG2A, and CD39 markers. Primed “effcNK” cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. NK cells were then barcoded and sequenced by 10X Genomics scRNA-seq. Single cell populations of effector conventional NK (effcNK) cells and enriched memory-like (eML) NK cells were isolated from purified healthy human CIML NK cells. UMAPs display expression of genes encoding for the HCMV-associated markers **(A)** NKG2C and **(B)** FcεRIγ. N=1 donor. Raw data available at [DOI: 10.1126/sciimmunol.adk4893](https://doi.org/10.1126/sciimmunol.adk4893).

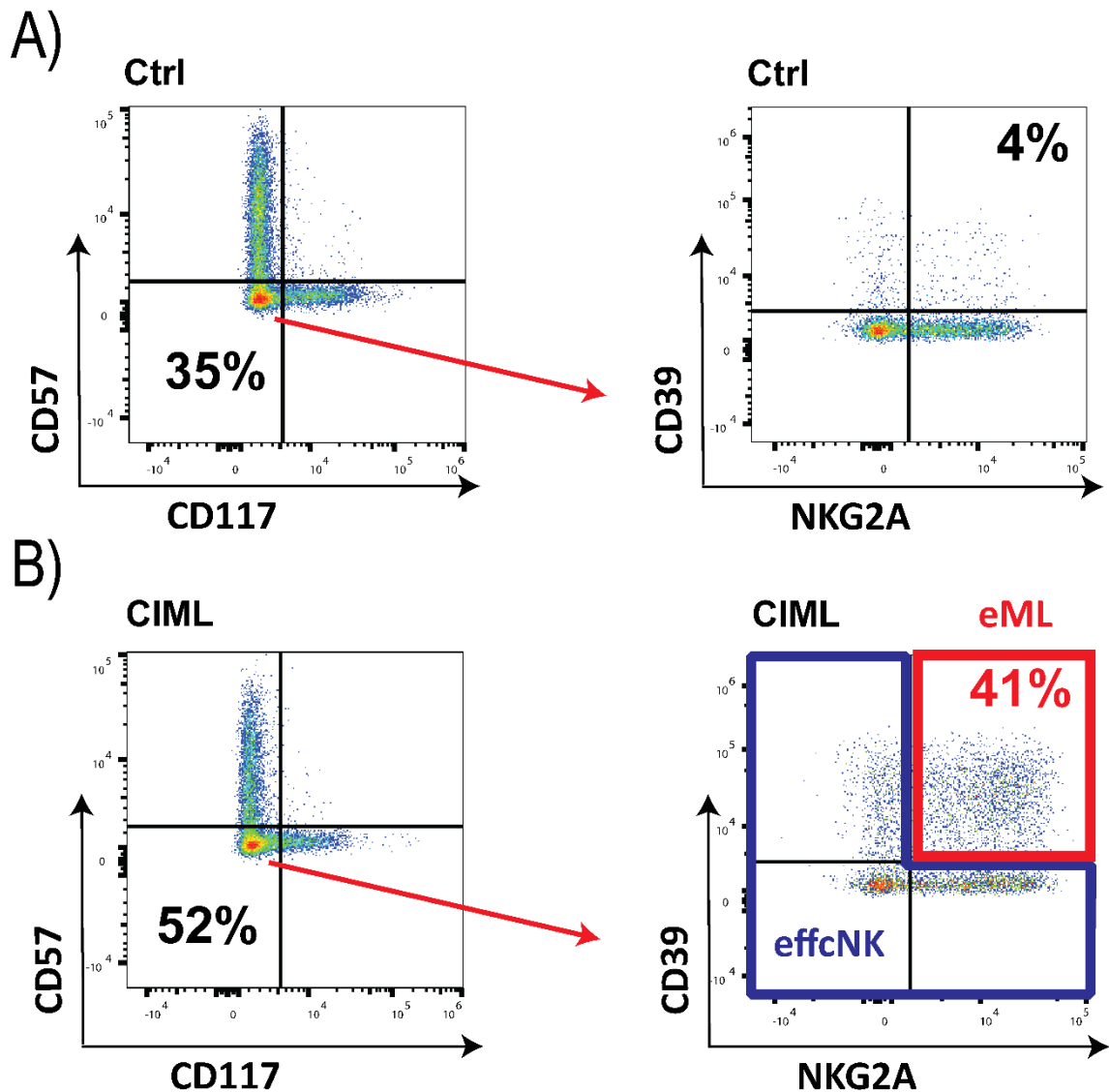


Figure 5.4. Gating strategy used to identify effcNKs and eMLs.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. NK cell subpopulations were analysed by flow cytometry *ex vivo*, or control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. CD56^{bright} NK cells were defined as CD117⁺ cells, and mature CD56^{dim} NK cells were defined as CD57⁺ cells. **(A,B)** NK cells negative for CD57 and CD117 and positive for NKG2A and CD39 were denoted as eML NK cells (red box). NK cells negative for CD57 and CD117 but also negative for eML markers were denoted as effcNK cells (blue box). Representative dot plots showing expression of CD57, CD117, NKG2A, and CD39 in **(A)** Ctrl NK cells and **(B)** CIML NK cells.

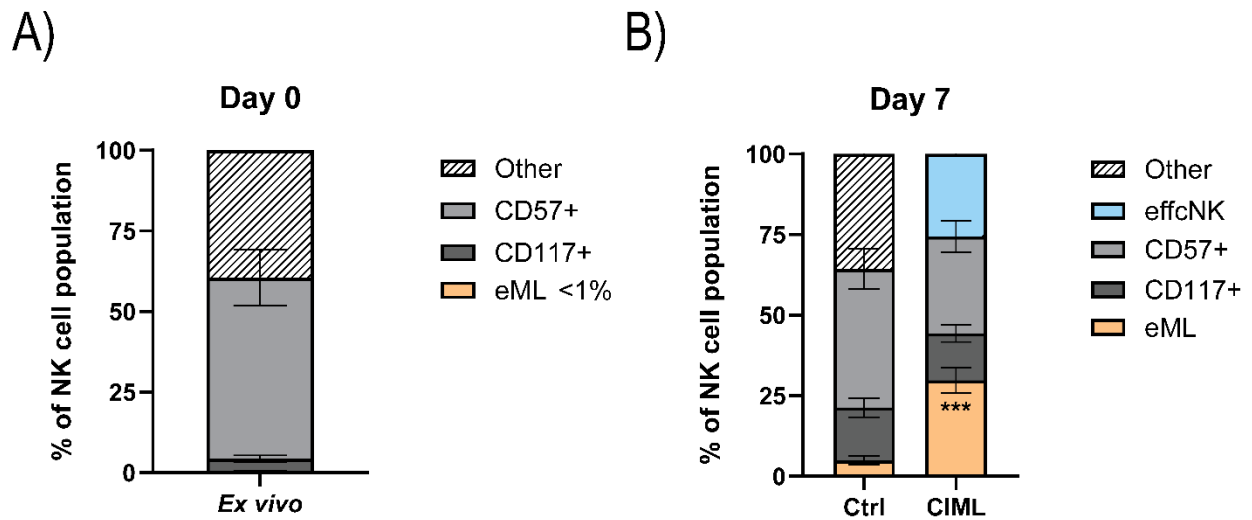


Figure 5.5. eML expansion within the bulk CIML NK cell population.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. NK cell subpopulations were analysed by flow cytometry *ex vivo*, or control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. CD56^{bright} NK cells were defined as CD117⁺ cells, and mature CD56^{dim} NK cells were defined as CD57⁺ cells. NK cells negative for CD57 and CD117 and positive for NKG2A and CD39 were denoted as eML NK cells. NK cells negative for CD57 and CD117 but also negative for eML markers were denoted as effcNK cells. **(A,B)** Distributions of NK cell subpopulations **(A)** *ex vivo* (day 0) and in **(B)** Ctrl and CIML NK cells (day 7). Flow cytometry analysis of Ctrl and CIML NK cells proliferation by Ki-67 expression. Data is mean $\pm$ SEM of N=3-8 donors. Data was analysed using a paired t-test (** $p < 0.001$).

5.4 eML NK cells have increased markers of proliferation.

To investigate genes with significantly different expression levels between effcNKs and eMLs, differential expression analysis was performed on RNA expression levels. Differentially expressed genes were identified as genes with a log2 fold change of greater than 1 or less than -1 and that showed statistical significance (adj.p<0.05). This identified 781 differentially expressed genes, 291 of which were upregulated in effcNKs and 490 of which were upregulated in eMLs (Figure 5.6A). Foltz et al. used scRNA-seq to compare eMLs, CD56^{bright}, CD56^{dim}, and NKG2C⁺ (HCMV adaptive) and found that RNA expression of *IFNG*, *FTH1*, *GNLY*, *PKM*, *GAPDH*, *PGD*, and glutathione s-transferase pi 1 (*GSTP1*) was upregulated in eML NK cells [219]. This study did not, however, compare the most differentially expressed genes between effcNK and eML NK cells. To investigate this, the top 30 most differentially expressed genes in effcNK and eML NK cells were ranked by adjusted p-value and found *GAPDH*, *FTH1*, *PGD*, and *GSTP1* were again amongst the most differentially expressed genes in eML NK cells (Table 5.1A). Interestingly, three of the five main PPP enzymes, *PGD*, *TALDO1*, and *TKT*, were amongst the most differentially expressed genes, along with glycolytic genes, iron metabolism genes, and redox balance genes (Table 5.1A). The most differentially expressed genes in effcNKs included *HLA-E*, *CD69*, granzyme M (*GZMM*), and a mix of activating and inhibitory receptors such as *KLRD1*, *KLRB1*, and *KLRF1* together with those listed in Table 5.1B. To investigate functional pathway enrichment, the Reactome online software was used to identify enriched cellular activities with a threshold of adjusted p-value <0.05. Reactome analysis of differentially expressed genes in effcNKs revealed enriched pathways such as immunoregulatory interactions between lymphoid and non-lymphoid cells, signal transduction, chemokine receptor binding, signaling by tyrosine kinases, and integrin cell surface interactions (Figure 5.7A). Reactome pathway analysis of differentially expressed genes in eMLs revealed highly significant enrichment of proliferative pathways such as activation of the pre-replicative complex, mitotic G1 phase and G1 S transition, synthesis of DNA and DNA replication (Figure 5.7B). Furthermore, GSEA of all significantly increased genes, not just differentially expressed ones, revealed significant enrichment of genes involved in proliferative pathways, particularly the E2F targets (Figure 5.8A,B), such as the minichromosome maintenance 5 (*MCM5*)

and the Myb-related protein B (*MYBL2*) genes (Figure 5.8C). Finally, a significant increase in expression of the proliferation marker Ki-67 was observed in eMLs compared to effcNKs and control NK cells, as assessed using flow cytometry (Figure 3.15A-C). Taken together, this data suggests that eMLs have a more proliferative phenotype than effcNKs.

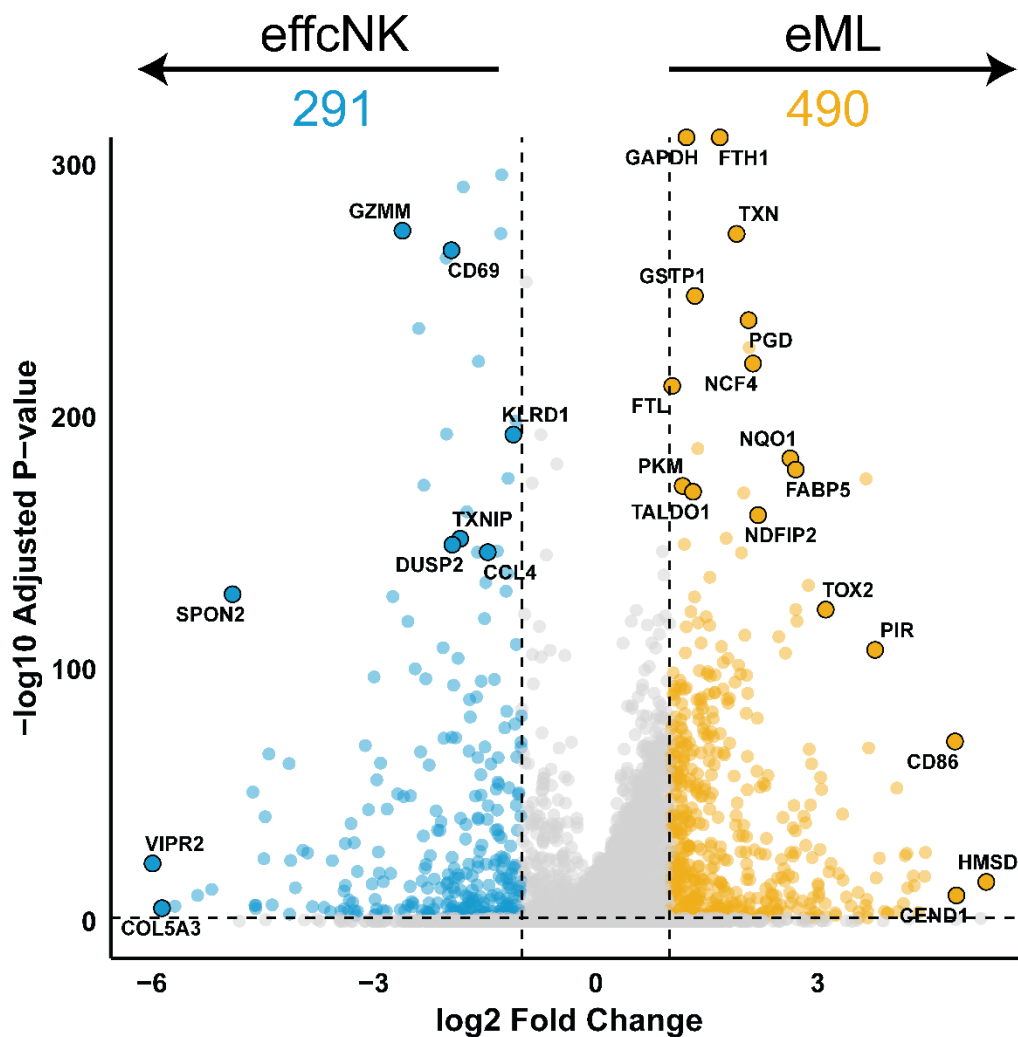


Figure 5.6. Differential expression analysis of effcNKs and eMLs.

Foltz et al. isolated single cells from CIML NK cells by flow cytometry based on expression of CD117, CD57, NKG2A, and CD39 markers. Primed “effcNK” cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. NK cells were then barcoded and sequenced by 10X Genomics scRNA-seq. Volcano plot of 10,815 total identified genes displaying differences in relative abundance between effcNK (blue) and eML (yellow) NK cells. The plot represents the -log₁₀ of the p-value versus log₂ of the fold change of gene expression. Differentially expressed genes (blue and yellow) have a log₂ fold change >1<-1 and a -log₁₀(adj.p-value) > 1.35.

A)

Top 30 most significant differentially expressed genes in eML NK cells

Rank	Gene name	Description	log2FC	p_val_adj
1	FTH1	Ferritin Heavy Chain 1	1.655765	0
2	GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	1.223924	1.93E-307
3	TXN	Thioredoxin	1.904135	2.16E-273
4	GSTP1	Glutathione S-Transferase Pi 1	1.34021	8.85E-249
5	PGD	Phosphogluconate Dehydrogenase	2.065657	3.56E-239
6	COTL1	Coactosin Like F-Actin Binding Protein 1	2.073123	2.92E-228
7	NCF4	Neutrophil Cytosolic Factor 4	2.125616	6.05E-222
8	FTL	Ferritin Light Chain	1.033534	4.54E-213
9	LGALS1	Galectin 1	1.378771	3.10E-188
10	NQO1	NAD(P)H Quinone Dehydrogenase 1	2.629011	2.66E-184
11	FABP5	Fatty Acid Binding Protein 5	2.702763	7.01E-180
12	MT1E	Metallothionein 1E	3.654306	3.42E-176
13	PKM	Pyruvate Kinase M1/2	1.175498	2.16E-173
14	TALDO1	Transaldolase 1	1.315416	4.34E-171
15	ANXA5	Annexin A5	2.000908	1.32E-170
16	NDFIP2	Nedd4 Family Interacting Protein 2	2.193608	7.98E-162
17	MMP25	Matrix Metalloproteinase 25	1.763259	1.34E-152
18	NME2	NME/NM23 Nucleoside Diphosphate Kinase 2	1.198862	3.12E-150
19	HLA-DRB1	Major Histocompatibility Complex, Class II, DR Beta 1	1.969553	7.38E-147
20	PPA1	Inorganic Pyrophosphatase 1	1.542952	3.75E-137
21	HLA-DRB5	Major Histocompatibility Complex, Class II, DR Beta 5	2.875199	5.38E-134
22	TESC	Tescalcin	1.335647	2.34E-129
23	DPP4	Dipeptidyl Peptidase 4	2.704041	2.41E-124
24	TOX2	TOX High Mobility Group Box Family Member 2	3.108388	2.44E-124
25	TKT	Transketolase	1.286933	1.65E-123
26	CHCHD10	Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 10	1.490839	1.81E-121
27	DUSP4	Dual Specificity Phosphatase 4	2.719473	1.03E-119
28	IL2RA	Interleukin 2 Receptor Subunit Alpha	1.384481	3.86E-119
29	ENTPD1	Ectonucleoside Triphosphate Diphosphohydrolase 1	1.539593	3.86E-118
30	NME1	NME/NM23 Nucleoside Diphosphate Kinase 1	2.015121	3.48E-114

B)

Top 30 most significant differentially expressed genes in effcNK cells

Rank	Gene name	Description	log2FC	p_val_adj
1	HLA-E	Major Histocompatibility Complex, Class I, E	-1.2709	0
2	BTG1	BTG Anti-Proliferation Factor 1	-1.7892	1.93E-307
3	GZMM	Granzyme M	-2.612	2.16E-273
4	MALAT1	Metastasis Associated Lung Adenocarcinoma Transcript 1	-1.2826	8.85E-249
5	CD69	CD69 Molecule	-1.9497	3.56E-239
6	PNRC1	Proline Rich Nuclear Receptor Coactivator 1	-2.0201	2.92E-228
7	KLRB1	Killer Cell Lectin Like Receptor B1	-2.3917	6.05E-222
8	CMC1	C-X9-C Motif Containing 1	-1.5845	4.54E-213
9	CST7	Cystatin F	-1.0758	3.10E-188
10	LITAF	Lipopolysaccharide Induced TNF Factor	-2.0158	2.66E-184
11	KLRD1	Killer Cell Lectin Like Receptor D1	-1.1134	7.01E-180
12	SARAF	Store-Operated Calcium Entry Associated Regulatory Factor	-1.1851	3.42E-176
13	FGFBP2	Fibroblast Growth Factor Binding Protein 2	-2.322	2.16E-173
14	PLAC8	Placenta Associated 8	-1.7418	4.34E-171
15	TXNIP	Thioredoxin Interacting Protein	-1.8312	1.32E-170
16	DUSP2	Dual Specificity Phosphatase 2	-1.9373	7.98E-162
17	ZFP36L1	ZFP36 Ring Finger Protein Like 1	-1.3294	1.34E-152
18	CCL4	C-C Motif Chemokine Ligand 4	-1.4593	3.12E-150
19	XCL2	X-C Motif Chemokine Ligand 2	-1.5969	7.38E-147
20	IER2	Immediate Early Response 2	-1.1894	3.75E-137
21	JUNB	JunB Proto-Oncogene, AP-1 Transcription Factor Subunit	-1.486	5.38E-134
22	CD247	CD247 Molecule	-1.2113	2.34E-129
23	SPON2	Spondin 2	-4.9073	2.41E-124
24	LINC00861	Long Intergenic Non-Protein Coding RNA 861	-2.7414	2.44E-124
25	KLRF1	Killer Cell Lectin Like Receptor F1	-1.5029	1.65E-123
26	ICAM2	Intercellular Adhesion Molecule 2	-2.5385	1.81E-121
27	SH2D2A	SH2 Domain Containing 2A	-1.0735	1.03E-119
28	C1orf162	Chromosome 1 Open Reading Frame 162	-2.0633	3.86E-119
29	CCL4L2	C-C Motif Chemokine Ligand 4 Like 2	-1.8614	3.86E-118
30	CX3CR1	C-X3-C Motif Chemokine Receptor 1	-2.4409	7.61E-101

Table 5.1. Top differentially expressed genes in effcNK and eML NK cells.

Table ranking the top 20 most significantly differentially expressed genes by in (A) eML NK cells and (B) effcNK cells ranked by adjusted p-value.

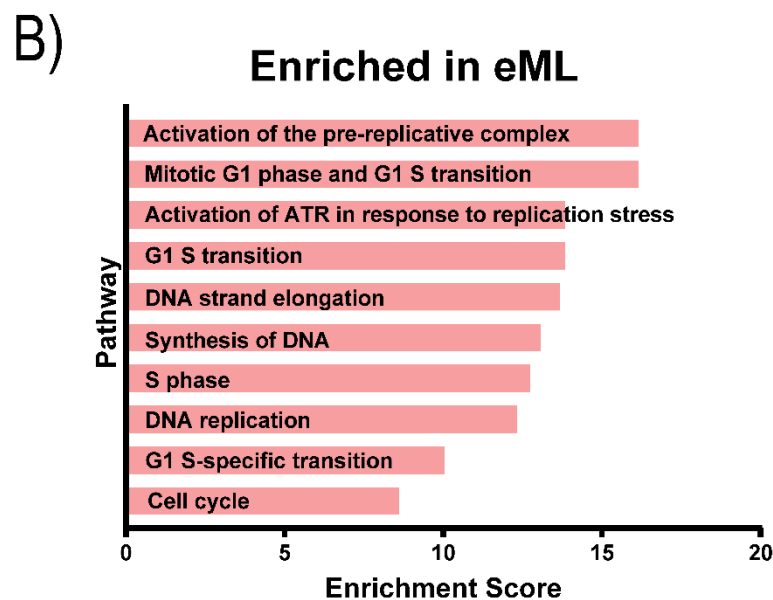
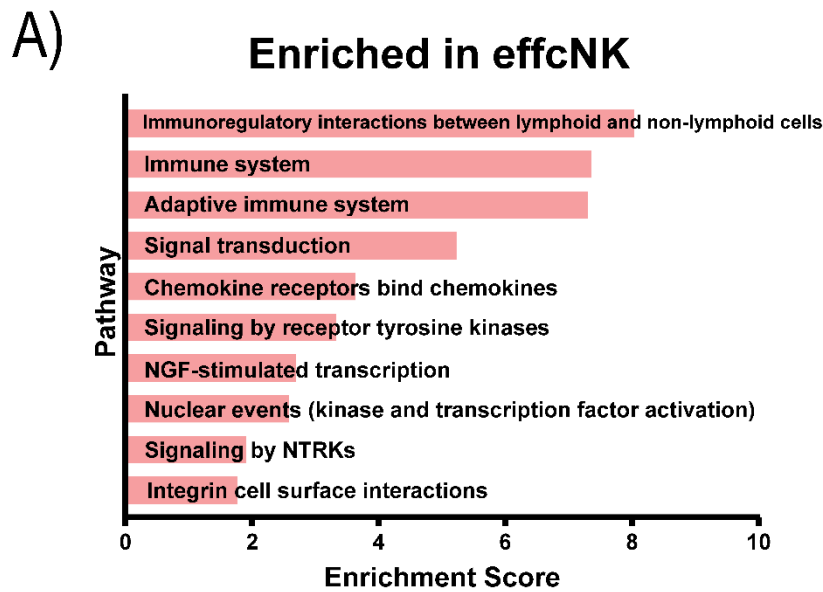


Figure 5.7. Pathway enrichment analysis of differentially expressed genes in effcNK cells and eML NK cells.

(A,B) Gene expression pathway analysis was performed on effcNK and eML NK cells, analysing enriched biological pathways. Significantly upregulated ($\text{Log}_2\text{FC} > 1$, $p\text{-value} < 0.05$) genes in effcNK and eML NK cells were analysed for functional enrichment using the Reactome database. Pathways highly associated with differentially expressed genes in **(A)** effcNK and **(B)** eML NK cells. Enrichment score = $-\text{Log}_{10}\text{Adj.pvalue}$.

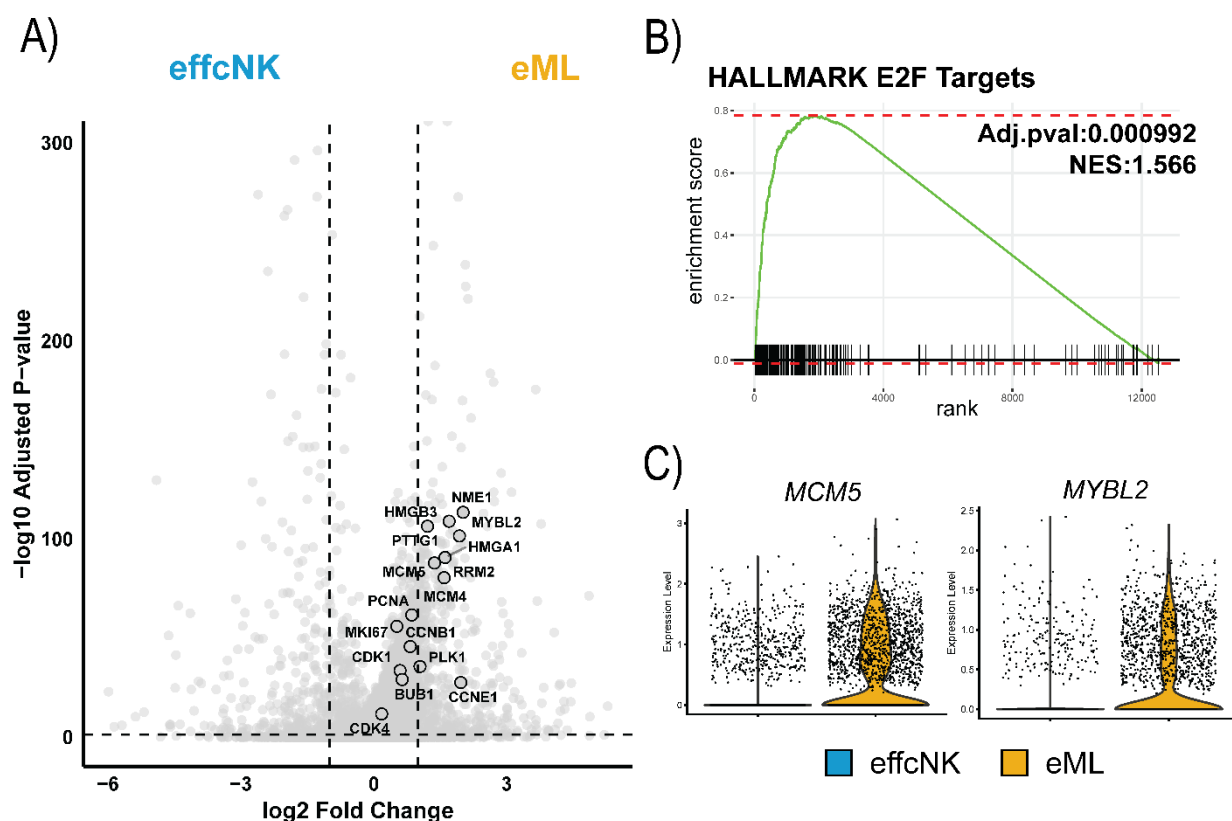


Figure 5.8. eMLs have increased expression of proliferation-associated genes.

(A-C) Foltz et al. isolated single cells from CIML NK cells by flow cytometry based on expression of CD117, CD57, NKG2A, and CD39 markers. Primed “effcNK” cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. NK cells were then barcoded and sequenced by 10X Genomics scRNA-seq. **(A-C)** Expression of genes involved in proliferation in effcNK cells and eML NK cells. **(A)** Volcano plot of differentially expressed genes in effcNK cells and eML NK cells, the plot represents the $-\log_{10}$ of the adjusted p-value versus $\log_2$ of the fold change of gene expression. Differentially expressed genes have a $\log_2$ fold change $>1<-1$ and a $-\log_{10}(\text{adj.p-value}) > 1.35$. Genes of interest are highlighted with a black border. **(B)** GSEA was performed on the entire gene expression datasets, independent of differential expression thresholds. Normalised enrichment scores (NES) are shown, with positive values indicating the HALLMARK E2F target pathway (M5925) is enriched in eML NK cells. **(C)** Violin plots visualising expression of highly differentially expressed proliferative genes *MCM5* and *MYBL2* in effcNK and eML NK cells. N=1 donor. Raw data available at [DOI: 10.1126/sciimmunol.adk4893](https://doi.org/10.1126/sciimmunol.adk4893).

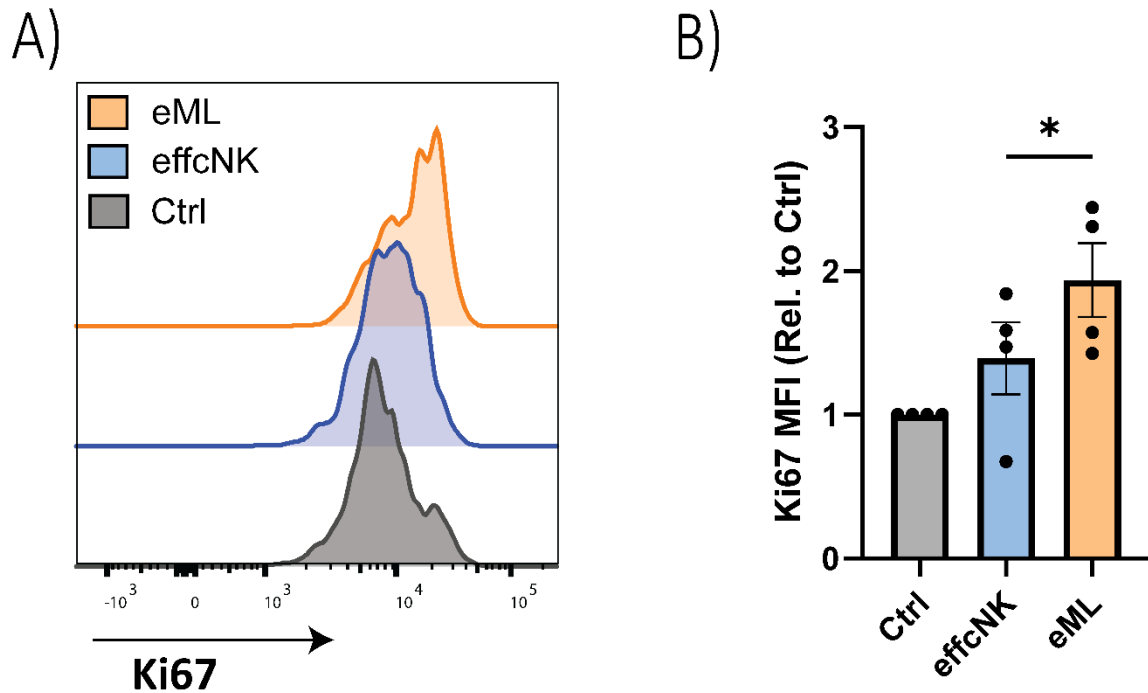


Figure 5.9. eMLs have increased expression of Ki67.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. **(A,B)** Flow cytometry analysis of Ki67 expression on Ctrl, effcNK cells, and eML NK cells. Primed “effcNK” cells identified as the CD117⁺CD57⁺NKG2A⁺CD39⁻ population and trained “eML” NK cells identified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. **(A)** Representative histogram showing the expression of Ki67 and **(B)** pooled MFI of Ki67 expression on effcNK cells and eML NK cells relative to Ctrl. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (* $p < 0.05$).

5.5 eML NK cells have increased cytotoxic potential.

Foltz et al. describe how eML NK cells exhibit increased IFN γ production after restimulation as well as enhanced killing of K562 cells [219]. Although their IFN γ production in response to restimulation with IL-12 and IL-15 and in response to K562 cocubation was significantly higher than effcNKs and control NK cells, their direct killing of K562s was comparable to that of effcNKs. In order to validate this, CIML NK cells were restimulated with IL-12 and IL-15 for 6 hours, and the subpopulations were assessed via flow cytometry. eMLs were found to have significantly increased IFN γ production compared to effcNKs and control NK cells after restimulation (Figure 5.10A-C). To investigate effcNK and eML cytotoxic potential further, the expression of cytotoxic genes in resting effcNKs and eMLs was analysed. Gene expression of cytolytic molecules was increased in eMLs, with increases observed in genes encoding for granzyme A, perforin, TRAIL, and slightly higher expression of granzyme B. Conversely, effcNKs exhibited increased gene expression of *GZMM* (Figure 5.11A-C). Furthermore, a significant increase in expression of granzyme B, was observed in resting eMLs compared to effcNKs and control NK cells, as assessed using flow cytometry (Figure 5.12A,B).

NK cells mediate cytotoxicity through recognising target cells via ligation of inhibitory and activating receptors, which can be manipulated by cancer for immune evasion. Given that pathway enrichment of differentially expressed genes in effcNKs had previously shown that there are enhanced immunoregulatory interactions between effcNKs and non-lymphoid cells (Figure 5.7A), analysis was next carried out to determine if these interactions are associated with the activation/inhibition of effcNKs. GSEA was carried out on all significantly increased genes and revealed enrichment of the “positive regulation of NK cell mediated immunity” pathway (GO:0002716) in effcNKs (Figure 5.13A,B), with increased expression of genes encoding for the activating receptors NKp46 and CD16 in these cells compared to eMLs (Figure 5.13C). However, when effcNKs and eMLs were assessed in terms of their inhibitory receptors, effcNKs also had significantly increased gene expression compared to eMLs (Figure 5.14A). GSEA revealed enrichment of the “negative regulation of NK cell mediated immunity” pathway (GO:0002716) in effcNKs (Figure

5.14A,B), characterised by increases in expression of genes encoding for potent inhibitory receptors such as TIGIT and the TGF β receptor TGFBR2 (Figure 5.14C). Taken together, these results suggest that eMLs have higher cytotoxic potential characterised by increased secondary responses, granzyme B reservoirs, and decreased expression of inhibitory receptors compared to effcNKs, suggesting they may be a better therapeutic option to target malignant cells.

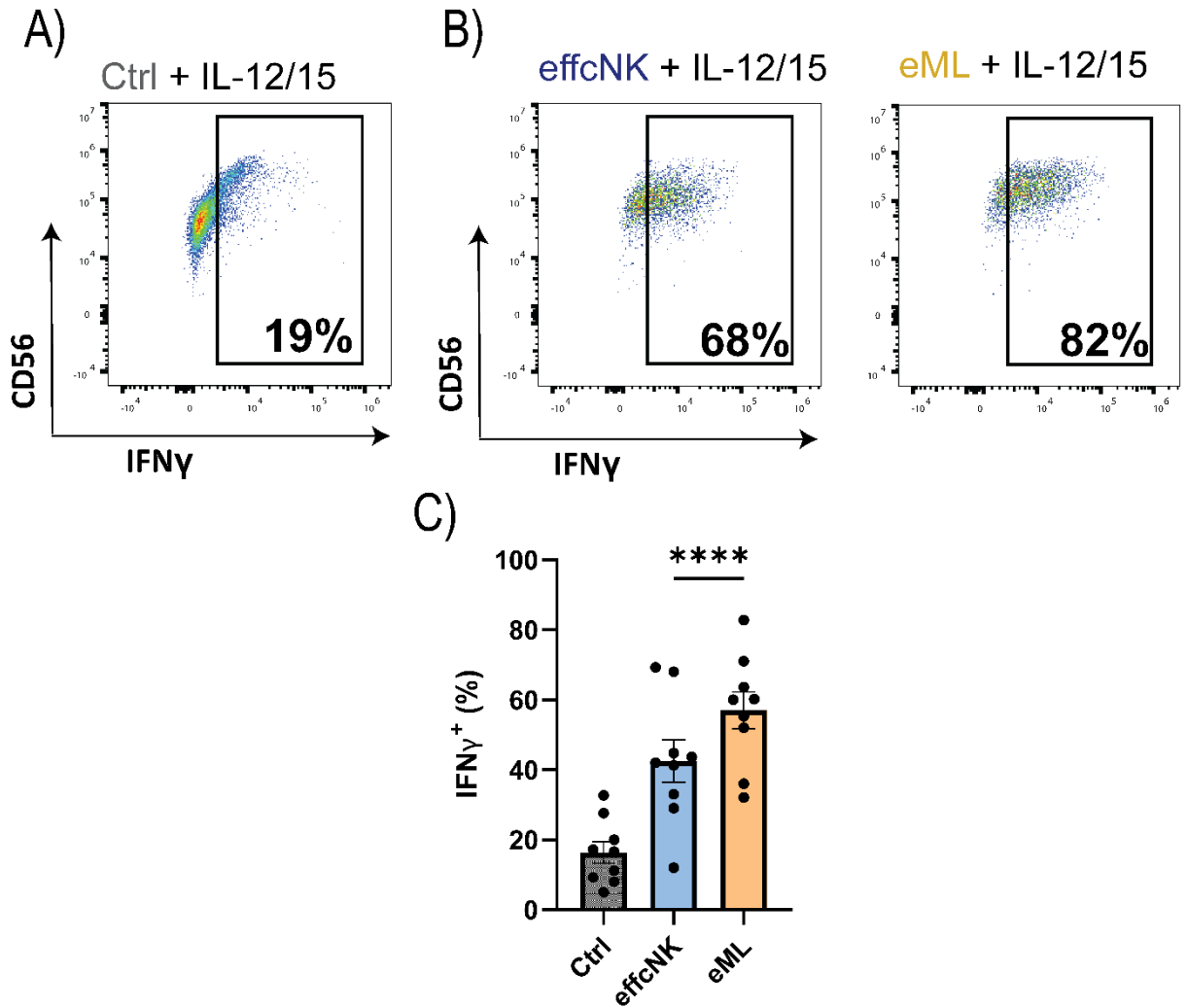


Figure 5.10. eMLs have increased IFN γ production after restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days the Ctrl and CIML NK cells were restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed (**A-C**). Flow cytometry analysis of IFN γ positivity on Ctrl, effcNK cells, and eML NK cells after restimulation. Primed “effcNK” cells identified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells identified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. (**A,B**) Representative dot plots showing IFN γ positivity in (**A**) Ctrl NK cells + IL-12/15, and (**B**) effcNK and eML NK cells + IL-12/15. (**C**) Pooled data indicating percentage positivity for IFN γ in Ctrl, effcNK, and eML NK cells + IL-12/15. Data are (**A,B**) representative or (**C**) mean $\pm$ SEM of N=9 donors. Statistical analysis was performed using a paired t-test. (****p<0.0001).

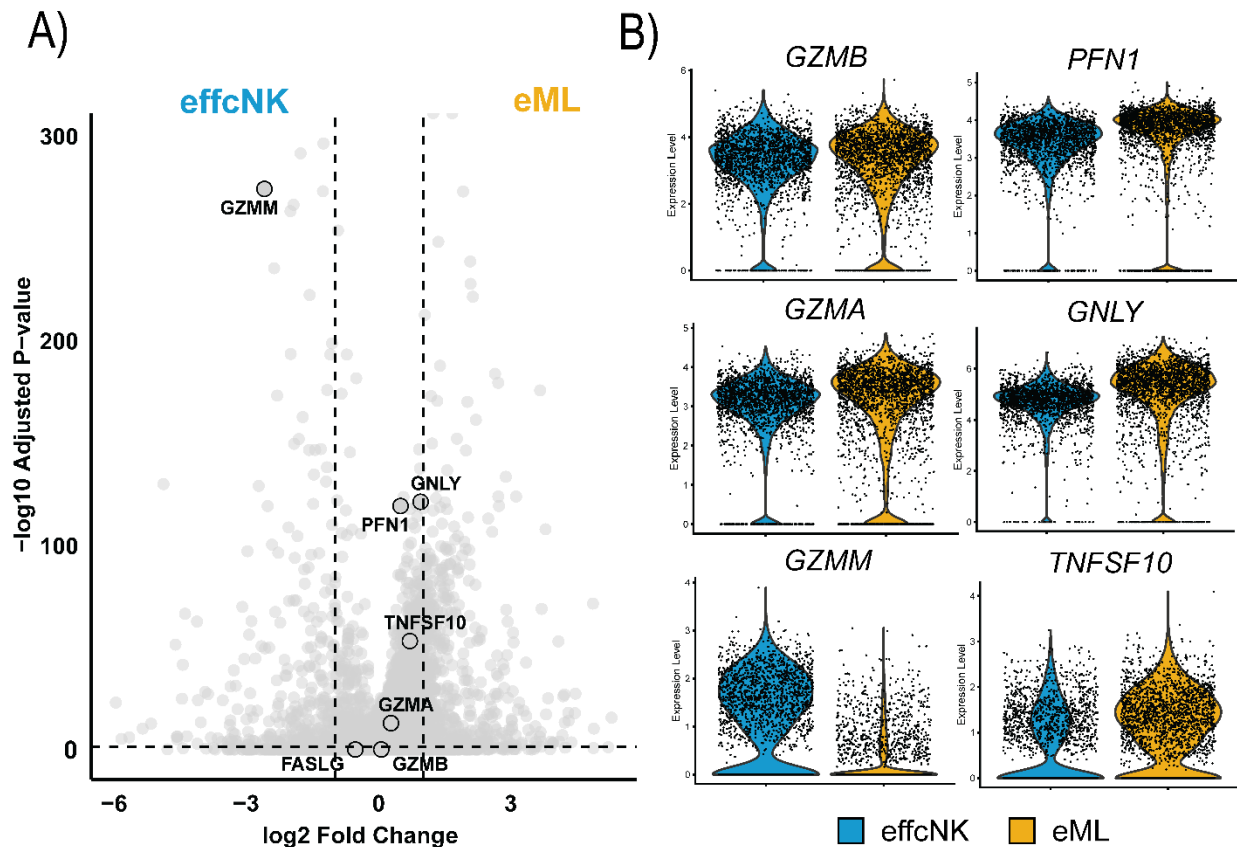


Figure 5.11. Cytotoxic gene expression in effcNK cells and eML NK cells.

(A,B) Foltz et al. isolated single cells from CIML NK cells by flow cytometry based on expression of CD117, CD57, NKG2A, and CD39 markers. Primed “effcNK” cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. NK cells were then barcoded and sequenced by 10X Genomics scRNA-seq. **(A)** Expression of cytotoxic genes in effcNK cells and eML NK cells. **(A)** Volcano plot of differentially expressed genes in effcNK cells and eML NK cells, the plot represents the $-\log_{10}$ of the adjusted p-value versus $\log_2$ of the fold change of gene expression. Differentially expressed genes have a $\log_2$ fold change >1 or <-1 and a $-\log_{10}(\text{adj. p-value}) > 1.35$. Genes of interest are highlighted with a black border. **(B)** Violin plots visualising expression of cytotoxic genes including granzyme A (GZMA), B (GZMB) and M (GZMM), perforin (PFN1), granulysin (GNLY) and TRAIL (TNFSF10) in effcNK and eML NK cells. N=1 donor. Raw data available at [DOI: 10.1126/sciimmunol.adk4893](https://doi.org/10.1126/sciimmunol.adk4893).

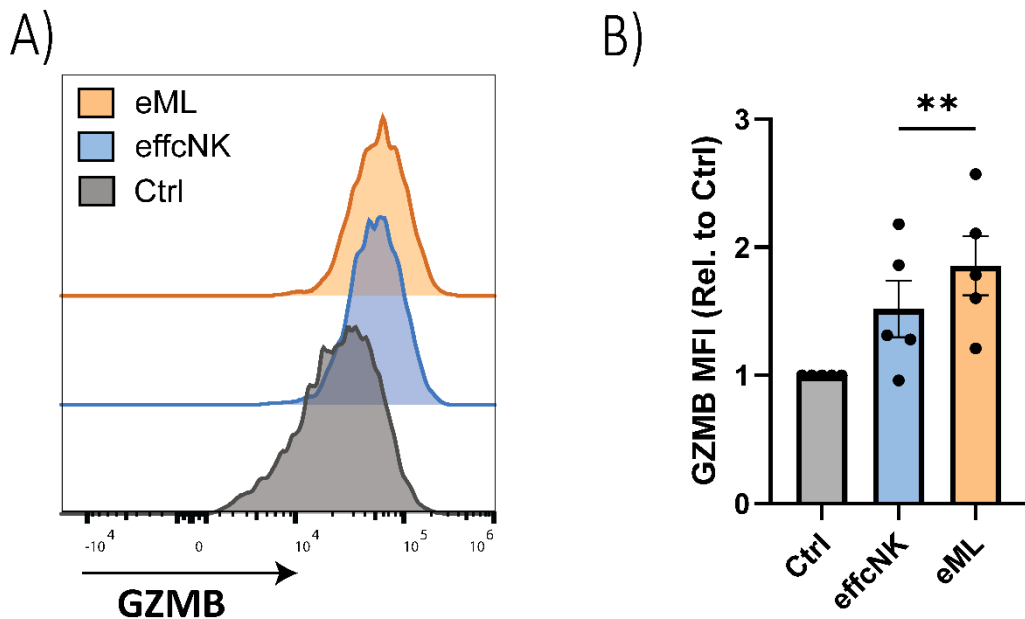


Figure 5.12. eMLs have increased expression of granzyme B.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. **(A,B)** Flow cytometry analysis of granzyme B (GZMB) expression on Ctrl, effcNK cells, and eML NK cells. Primed “effcNK” cells identified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells identified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. **(A)** Representative histogram showing the expression of GZMB and **(B)** pooled MFI of GZMB expression on effcNK cells and eML NK cells relative to Ctrl. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=5 donors. Statistical analysis was performed using a paired t-test. (**p<0.01).

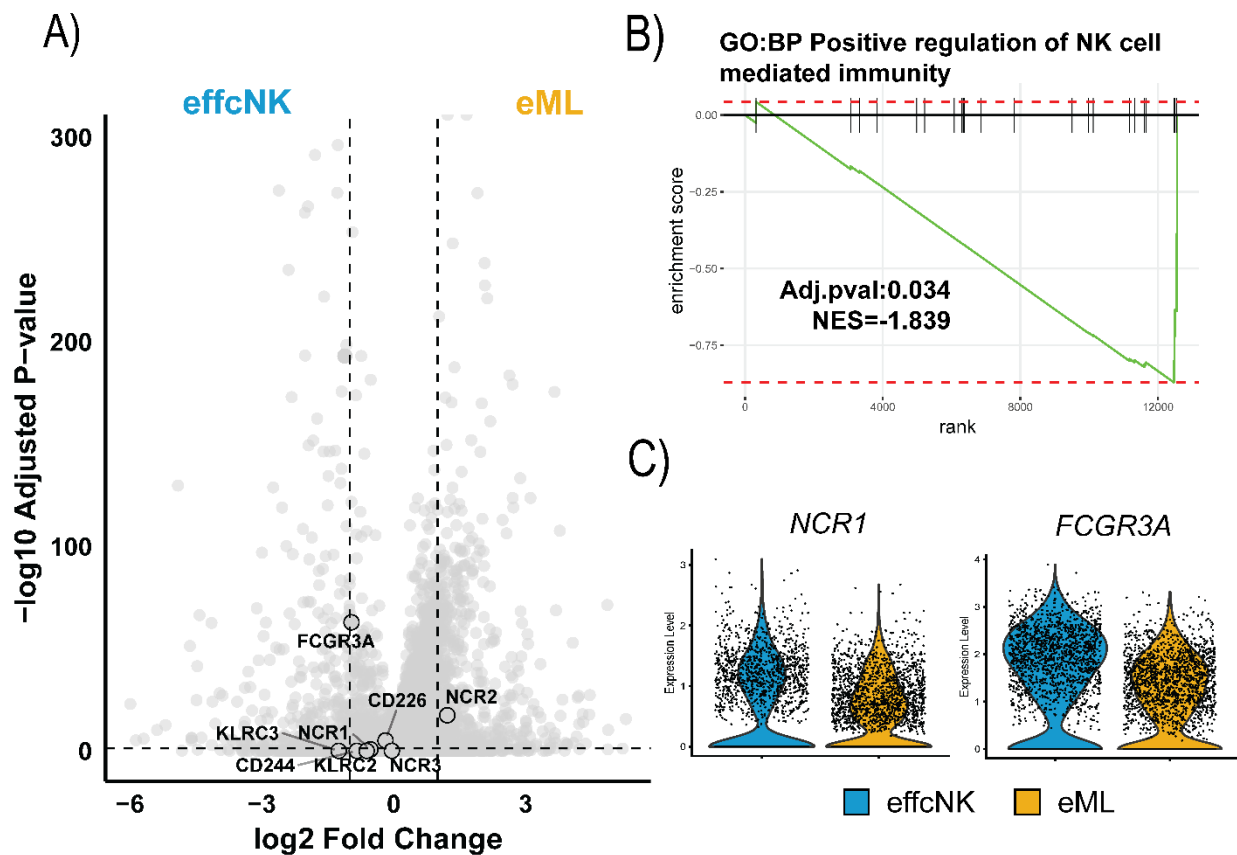


Figure 5.13. Gene expression of activating receptors in effcNK cells and eML NK cells.

(A-C) Foltz et al. isolated single cells from CIML NK cells by flow cytometry based on expression of CD117, CD57, NKG2A, and CD39 markers. Primed “effcNK” cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. NK cells were then barcoded and sequenced by 10X Genomics scRNA-seq. **(A-C)** Expression of activating receptor genes in effcNK cells and eML NK cells. **(A)** Volcano plot of differentially expressed genes in effcNK cells and eML NK cells, the plot represents the $-\log_{10}$ of the adjusted p-value versus $\log_2$ of the fold change of gene expression. Differentially expressed genes have a $\log_2$ fold change >1 and a $-\log_{10}(\text{adj.p-value}) > 1.35$. Genes of interest are highlighted with a black border. **(B)** GSEA was performed on the entire gene expression datasets, independent of differential expression thresholds. Normalised enrichment scores (NES) are shown, with negative values indicating enrichment of the GO:BP Positive regulation of NK cell mediated immunity (GO:0002717) pathway in effcNK NK cells. **(C)** Violin plots visualising expression of key activating receptor genes *NCR1* and *FCGR3A* in effcNK and eML NK cells. N=1 donor. Raw data available at [DOI: 10.1126/sciimmunol.adk4893](https://doi.org/10.1126/sciimmunol.adk4893).

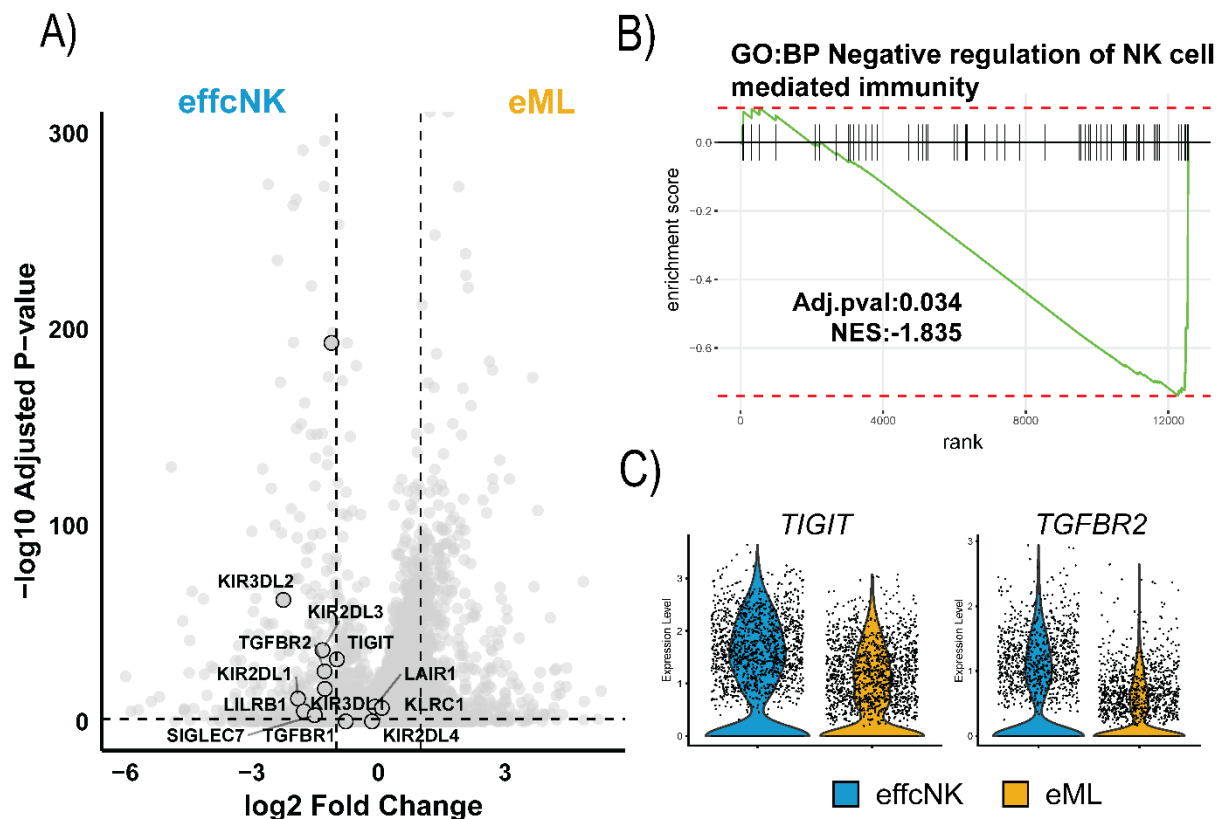


Figure 5.14. Gene expression of inhibitory receptors in effcNK cells and eML NK cells.

(A-C) Foltz et al. isolated single cells from CIML NK cells by flow cytometry based on expression of CD117, CD57, NKG2A, and CD39 markers. Primed “effcNK” cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. NK cells were then barcoded and sequenced by 10X Genomics scRNA-seq. **(A-C)** Expression of inhibitory receptor genes in effcNK cells and eML NK cells. **(A)** Volcano plot of differentially expressed genes in effcNK cells and eML NK cells, the plot represents the $-\log_{10}$ of the adjusted p-value versus $\log_2$ of the fold change of gene expression. Differentially expressed genes have a $\log_2$ fold change $>1<-1$ and a $-\log_{10}(\text{adj.p-value}) > 1.35$. Genes of interest are highlighted with a black border. **(B)** GSEA was performed on the entire gene expression datasets, independent of differential expression thresholds. Normalised enrichment scores (NES) are shown, with negative values indicating the GO:BP Negative regulation of NK cell mediated immunity (GO:0002716) pathway is enriched in effcNK NK cells. **(C)** Violin plots visualising expression of key inhibitory receptor genes *TIGIT* and *TGFBR2* in effcNK and eML NK cells. N=1 donor. Raw data available at [DOI: 10.1126/sciimmunol.adk4893](https://doi.org/10.1126/sciimmunol.adk4893).

5.6 effcNK and eML NK cells exhibit metabolic heterogeneity.

It is clear that effcNK cells and eMLs differ in terms of their proliferative and functional capabilities; thus, steps were taken to investigate the metabolic profiles of both effcNKs and eMLs. It was demonstrated in this chapter that glycolytic and PPP genes were among the most differentially expressed genes in eMLs (Table 5.1A). Consistent with this, GSEA of all significantly upregulated genes revealed significant upregulation of glycolysis, with all ten of the main glycolytic genes significantly increased in eMLs compared to effcNKs, particularly those encoding for GAPDH and PKM (Figure 5.15A,B). Furthermore, eMLs have significantly increased GLUT1 expression compared to effcNKs as assessed by flow cytometry (Figure 5.16A,B). PPP genes such as those encoding for TALDO1, TKT, and PGD were also differentially expressed and among the most significantly increased genes in eMLs (Figure 5.17A,B). In chapter 3, bulk CIML NK cell analysis revealed upregulated glycolysis, PPP, and purine synthesis intermediate metabolism. It is shown here that eMLs have enriched purine metabolism compared to effcNKs, with GSEA revealing significantly upregulated purine compound catabolic and metabolic processes (Figure 5.18A,B). Furthermore, eML NK cells had an increased expression of genes involved in iron metabolism (Figure 5.19A,B). The iron sequestering ferritin protein heavy and light chain genes were differentially expressed in eMLs, with *FTH1* being the most differentially expressed gene between effcNKs and eMLs (Table 5.1A, Figure 5.19A). Although there was low expression of *TFRC* (the gene encoding for CD71) in both effcNKs and eMLs, CD71 expression was increased in eMLs as assessed by flow cytometry (Figure 5.20A-C). Furthermore, eMLs had significantly increased transferrin uptake compared to control NK cells and effcNKs, again assessed by flow cytometry (Figure 5.21A-D). Finally, genes associated with redox management were enriched in eMLs, compared to effcNKs. Genes such as those encoding for TXN, NQO1, GSTP1, MT1E and PRDX1 were all differentially expressed in eML NK cells (Table 5.1A, Figure 5.22A-C). Taken together, this data suggests eMLs have increased glycolysis, PPP, purine synthesis, iron-associated metabolism and increased antioxidant capacity compared to effcNKs.

It was demonstrated in Chapters 3 and 4 that CIML NK cells had many mitochondrial differences compared to control NK cells. To investigate these further, mitochondrial features in resting eMLs and effcNKs were compared at the RNA level and by flow cytometry. GSEA analysis revealed significantly increased expression of OXPHOS-associated genes such as the mitochondrial ADP/ATP transporter, *SLC25A5*, and the cytochrome c oxidase subunit 5A, *COX5A*, in eMLs compared to effcNKs (Figure 5.23A-C). However, no differences were observed in mitochondrial mass (Figure 5.24A,B), and a decrease in mitochondrial membrane potential (Figure 5.25A,B) was observed in eMLs compared to effcNKs as assessed by flow cytometry. Furthermore, eML NK cells had decreased mitochondrial ROS production compared to effcNKs (Figure 5.26A,B). Although eMLs displayed increased OXPHOS-associated gene expression, effcNKs displayed increased mitochondrial membrane potential generation compared to eMLs at rest. Mitochondrial features were then investigated in effcNKs and eMLs after restimulation. Upon restimulation, no differences were observed between effcNKs and eMLs in terms of mitochondrial mass (Figure 5.27A,B). However, there was a trend towards increased mitochondrial membrane potential in effcNKs compared to eMLs, after restimulation (Figure 5.28A,B). Finally, no differences were observed in mitochondrial ROS production in either effcNKs or eMLs after restimulation (Figure 5.29A,B). Taken together, this data suggests that although eMLs had increased expression of OXPHOS-associated genes, effcNKs may actually have more mitochondrial activity at rest and after restimulation compared to eMLs.

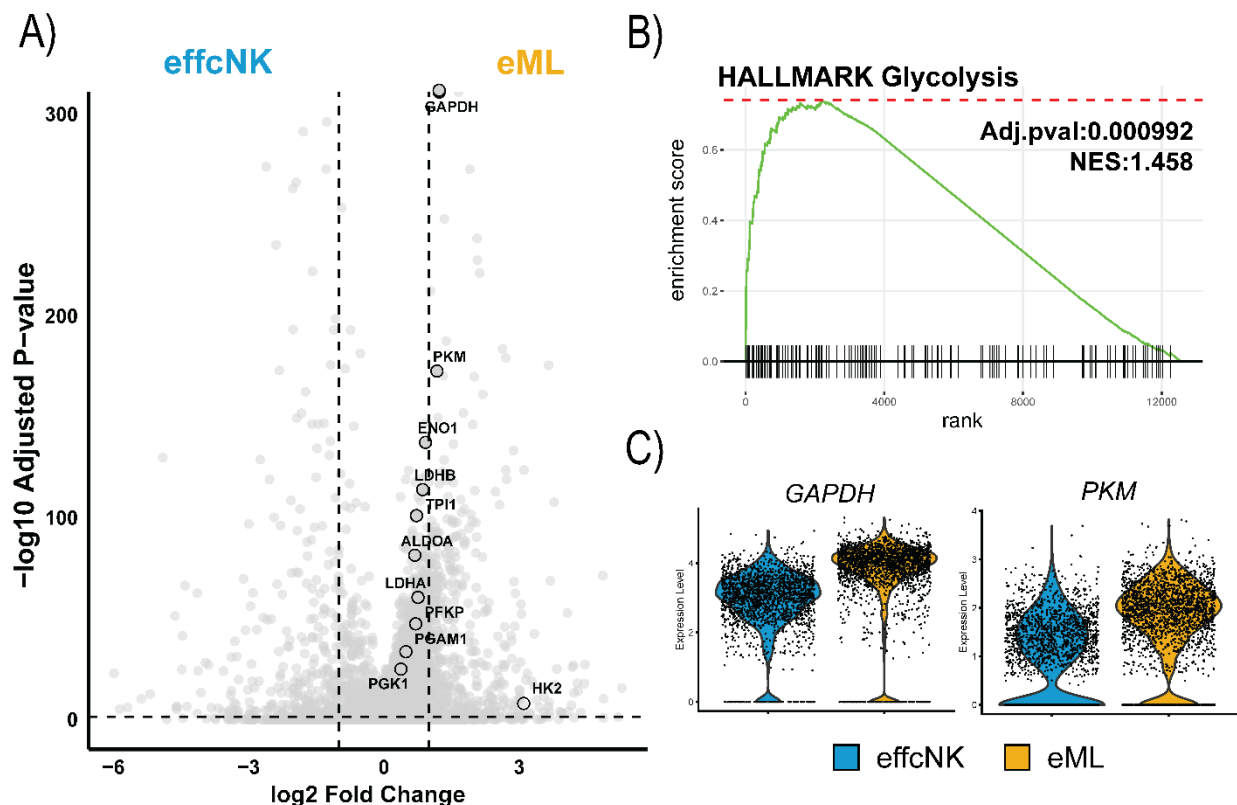


Figure 5.15. Glycolytic gene expression is increased in eMLs.

(A-C) Foltz et al. isolated single cells from CIML NK cells by flow cytometry based on expression of CD117, CD57, NKG2A, and CD39 markers. Primed “effcNK” cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. NK cells were then barcoded and sequenced by 10X Genomics scRNA-seq. **(A-C)** Expression of genes involved in glycolysis in effcNK cells and eML NK cells. **(A)** Volcano plot of differentially expressed genes in effcNK cells and eML NK cells, the plot represents the $-\log_{10}$ of the adjusted p-value versus $\log_2$ of the fold change of gene expression. Differentially expressed genes have a $\log_2$ fold change >1 and a $-\log_{10}(\text{adj.p-value}) > 1.35$. Genes of interest are highlighted with a black border. **(B)** GSEA was performed on the entire gene expression datasets, independent of differential expression thresholds. Normalised enrichment scores (NES) are shown, with positive values indicating the HALLMARK Glycolysis pathway (M5937) is enriched in eML NK cells. **(C)** Violin plots visualising expression of key glycolytic genes *GAPDH* and *PKM* in effcNK and eML NK cells. N=1 donor. Raw data available at [DOI: 10.1126/sciimmunol.adk4893](https://doi.org/10.1126/sciimmunol.adk4893).

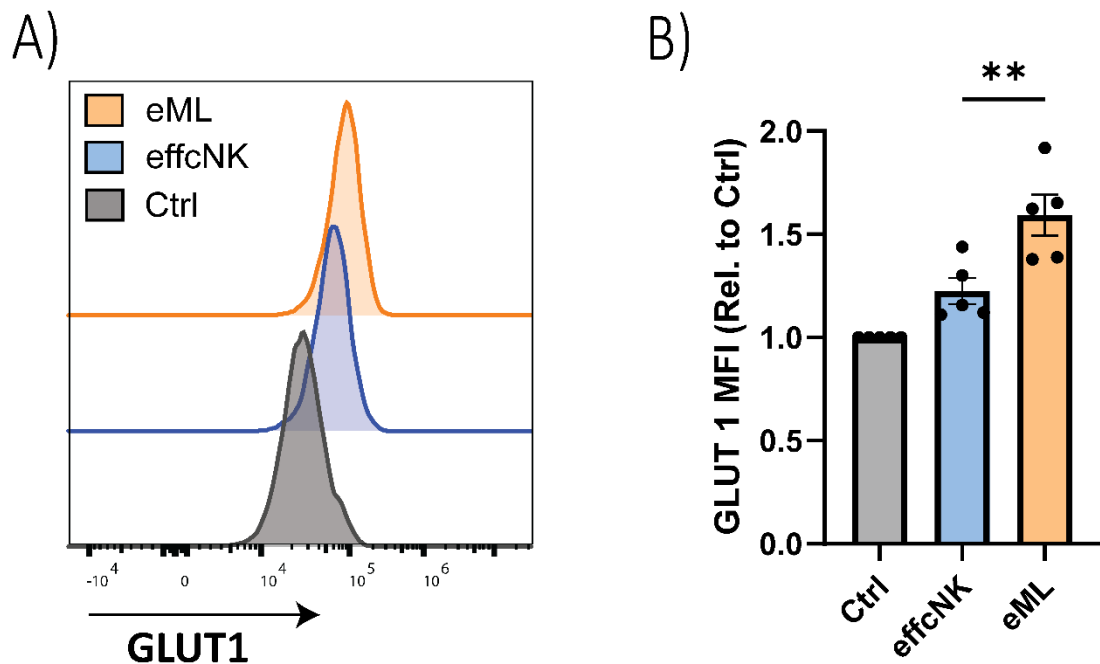


Figure 5.16. GLUT1 expression is increased on the surface of eMLs.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. **(A,B)** Flow cytometry analysis of Ki67 expression on Ctrl, effcNK cells, and eML NK cells. Primed “effcNK” cells identified as the CD117⁻CD57⁻NKG2A⁻CD39⁻ population and trained “eML” NK cells identified as the CD117⁻CD57⁻NKG2A⁺CD39⁺ population. **(A)** Representative histogram showing the expression of GLUT1 and **(B)** pooled MFI of GLUT1 expression on effcNK cells and eML NK cells relative to Ctrl. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=5 donors. Statistical analysis was performed using a paired t-test. (**p<0.01).

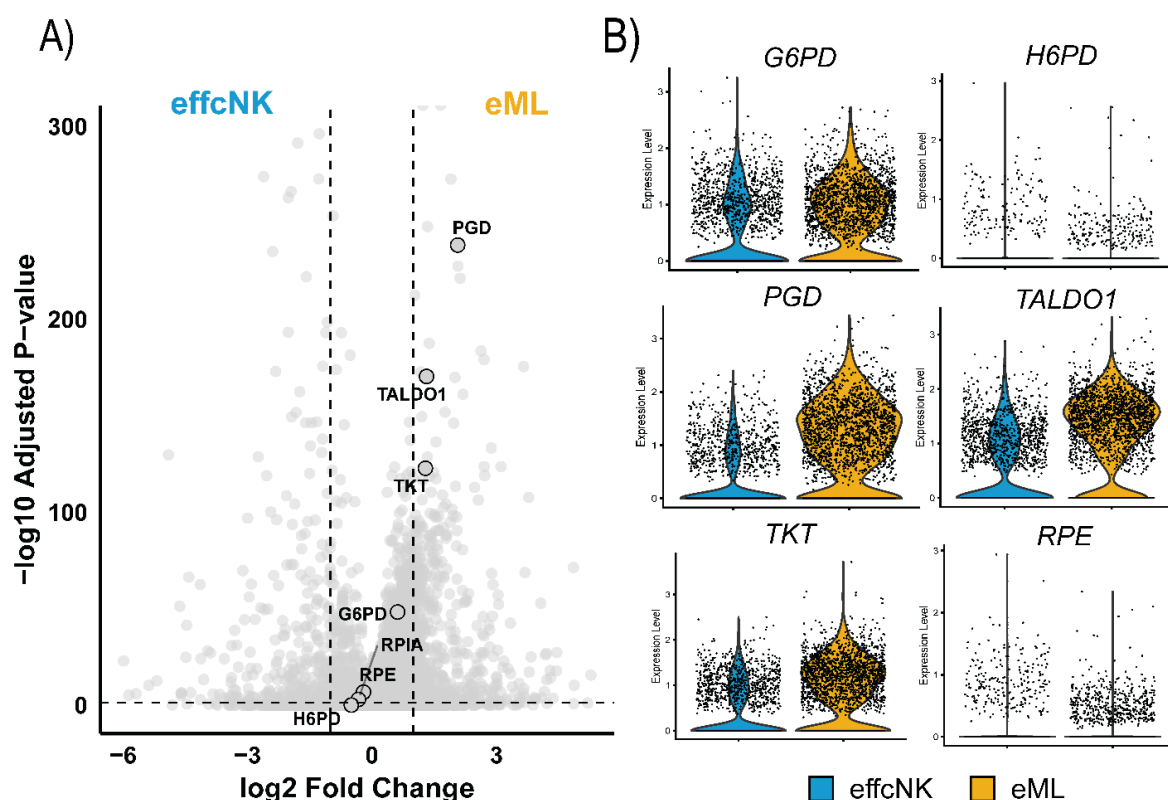


Figure 5.17. The pentose phosphate pathway gene expression is increased in eMLs.

(A,B) Foltz et al. isolated single cells from CIML NK cells by flow cytometry based on expression of CD117, CD57, NKG2A, and CD39 markers. Primed “effcNK” cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. NK cells were then barcoded and sequenced by 10X Genomics scRNA-seq. **(A,B)** Expression of genes involved in the PPP in effcNK cells and eML NK cells. **(A)** Volcano plot of differentially expressed genes in effcNK cells and eML NK cells, the plot represents the -log₁₀ of the adjusted p-value versus log₂ of the fold change of gene expression. Differentially expressed genes have a log₂ fold change >1<-1 and a -log₁₀(adj.p-value) > 1.35. Genes of interest are highlighted with a black border. **(B)** Violin plots visualising expression of PPP genes including *G6PD*, *H6PD*, *PGD*, *TALDO1*, *TKT*, and *RPE* in effcNK and eML NK cells. N=1 donor. Raw data available at [DOI: 10.1126/sciimmunol.adk4893](https://doi.org/10.1126/sciimmunol.adk4893).

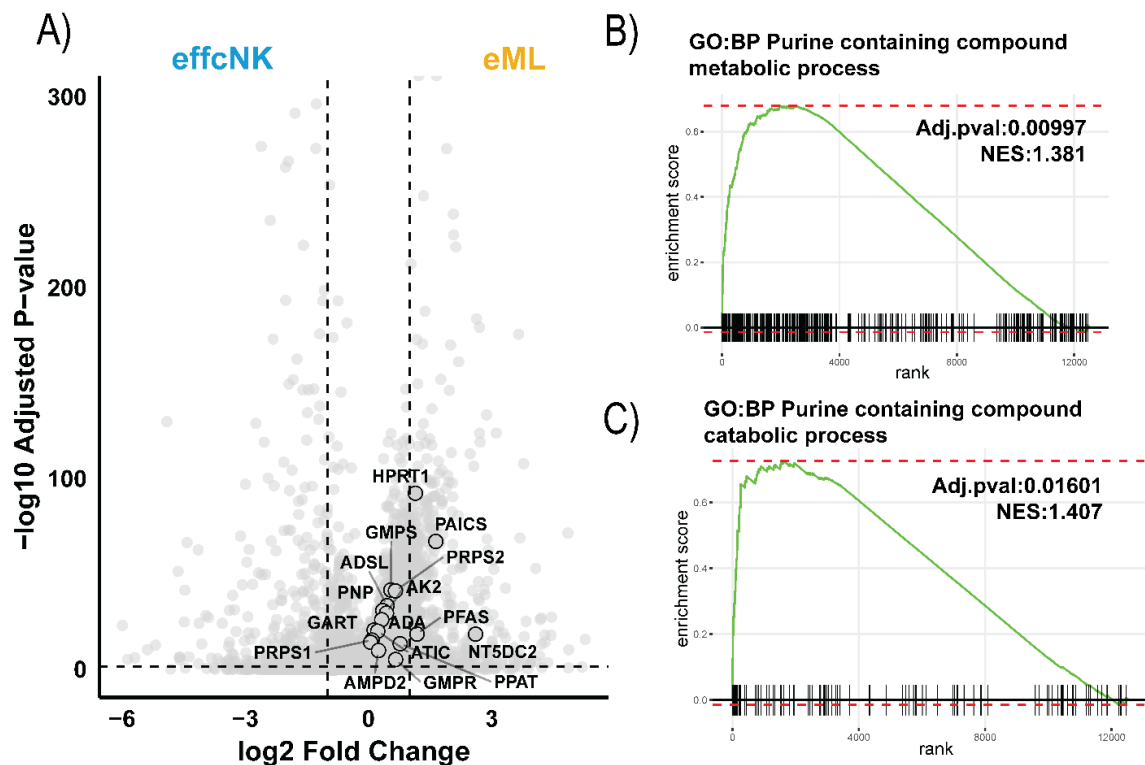


Figure 5.18. Purine metabolism gene expression is increased in eMLs.

(A,B) Foltz et al. isolated single cells from CIML NK cells by flow cytometry based on expression of CD117, CD57, NKG2A, and CD39 markers. Primed “effcNK” cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. NK cells were then barcoded and sequenced by 10X Genomics scRNA-seq. **(A,B)** Expression of genes involved in the PPP in effcNK cells and eML NK cells. **(A)** Volcano plot of differentially expressed genes in effcNK cells and eML NK cells, the plot represents the -log₁₀ of the adjusted p-value versus log₂ of the fold change of gene expression. Differentially expressed genes have a log₂ fold change >1<-1 and a -log₁₀(adj.p-value) > 1.35. Genes of interest are highlighted with a black border. **(B)** GSEA was performed on the entire gene expression datasets, independent of differential expression thresholds. Normalised enrichment scores (NES) are shown, with positive values indicating enrichment of the GO:BP Purine containing compound metabolic (GO:0072521) and catabolic (GO:0072523) pathways. N=1 donor. Raw data available at [DOI: 10.1126/sciimmunol.adk4893](https://doi.org/10.1126/sciimmunol.adk4893).

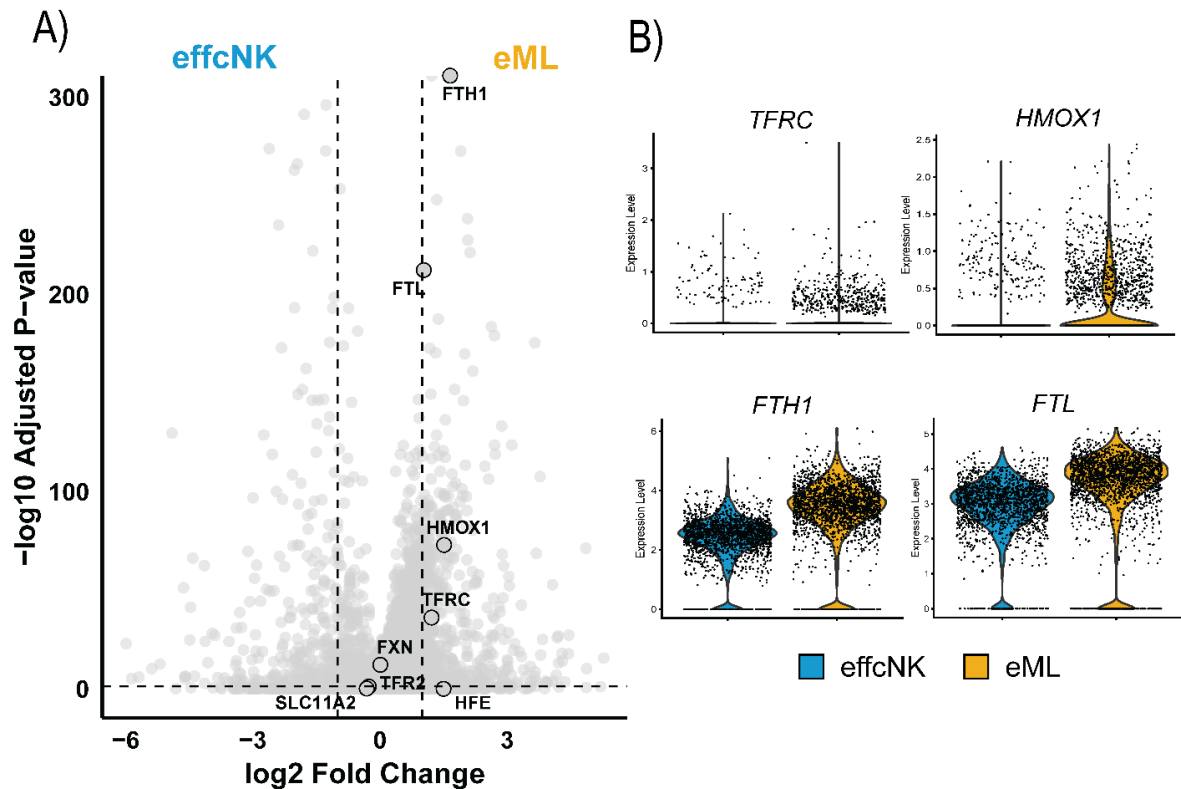


Figure 5.19. eMLs have increased expression of genes associated with iron metabolism.

(A,B) Foltz et al. isolated single cells from CIML NK cells by flow cytometry based on expression of CD117, CD57, NKG2A, and CD39 markers. Primed “effcNK” cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. NK cells were then barcoded and sequenced by 10X Genomics scRNA-seq. **(A,B)** Expression of genes involved in the iron metabolism in effcNK cells and eML NK cells. **(A)** Volcano plot of differentially expressed genes in effcNK cells and eML NK cells, the plot represents the $-\log_{10}$ of the adjusted p-value versus $\log_2$ of the fold change of gene expression. Differentially expressed genes have a $\log_2$ fold change >1 or <-1 and a $-\log_{10}(\text{adj.p-value}) > 1.35$. Genes of interest are highlighted with a black border. **(B)** Violin plots visualising expression of iron metabolism genes, including CD71 (*TFRC*), *HMOX1*, *FTH*, and *FTL* in effcNK and eML NK cells. N=1 donor. Raw data available at [DOI: 10.1126/sciimmunol.adk4893](https://doi.org/10.1126/sciimmunol.adk4893).

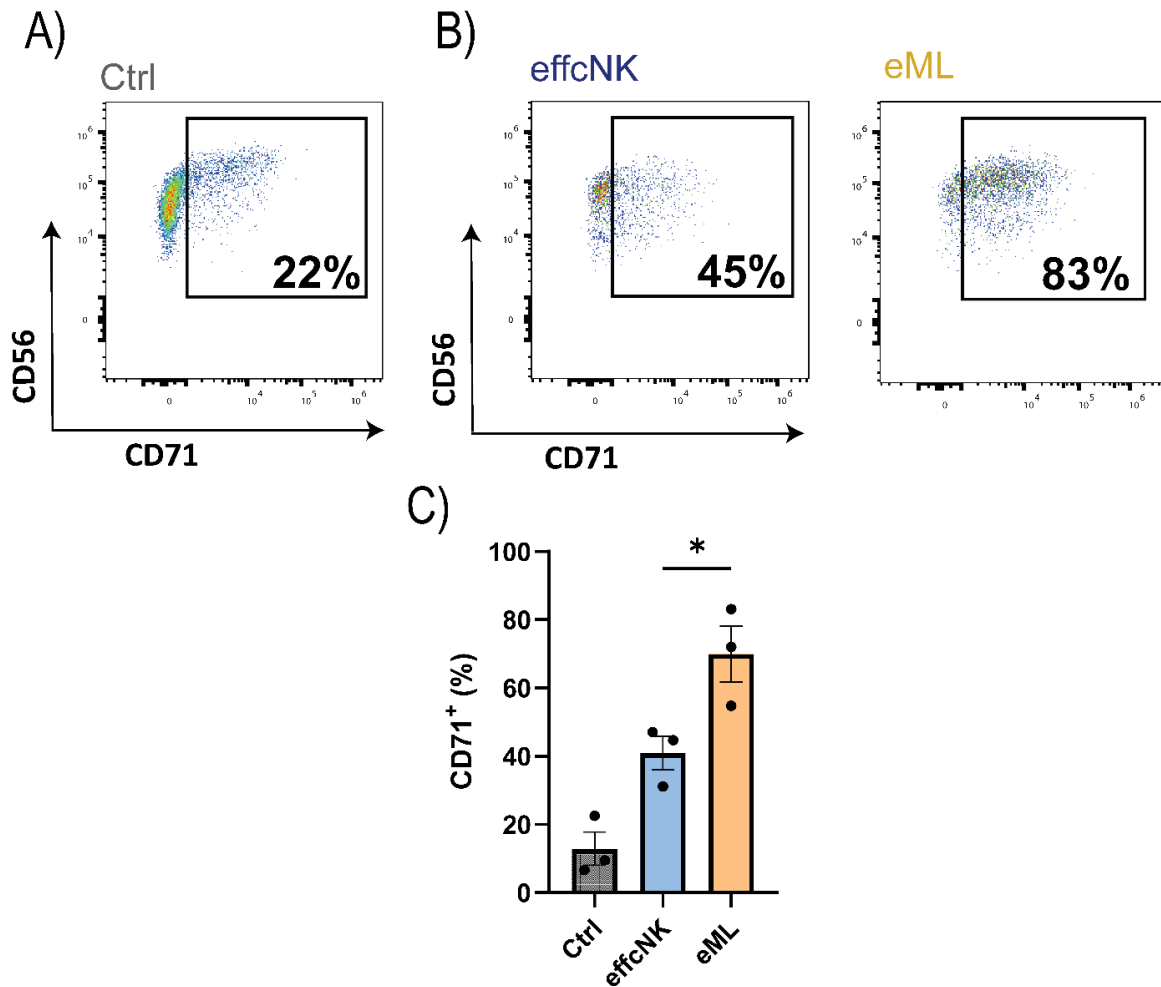


Figure 5.20. CD71 expression is increased on the surface of eMLs.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed (**A-C**). Flow cytometry analysis of CD71 positivity on Ctrl, effcNK cells, and eML NK cells. Primed “effcNK” cells identified as the CD117⁻CD57⁻NKG2A⁻CD39⁻ population and trained “eML” NK cells identified as the CD117⁻CD57⁻NKG2A⁺CD39⁺ population. (**A,B**) Representative dot plots showing CD71 positivity in (**A**) Ctrl NK cells, (**B**) effcNK and eML NK cells. (**C**) Pooled data indicating percentage positivity for CD71 in Ctrl, effcNK, and eML NK cells. Data are (**A,B**) representative or (**C**) mean $\pm$ SEM of N=3 donors. Statistical analysis was performed using a paired t-test. (* $p < 0.05$).

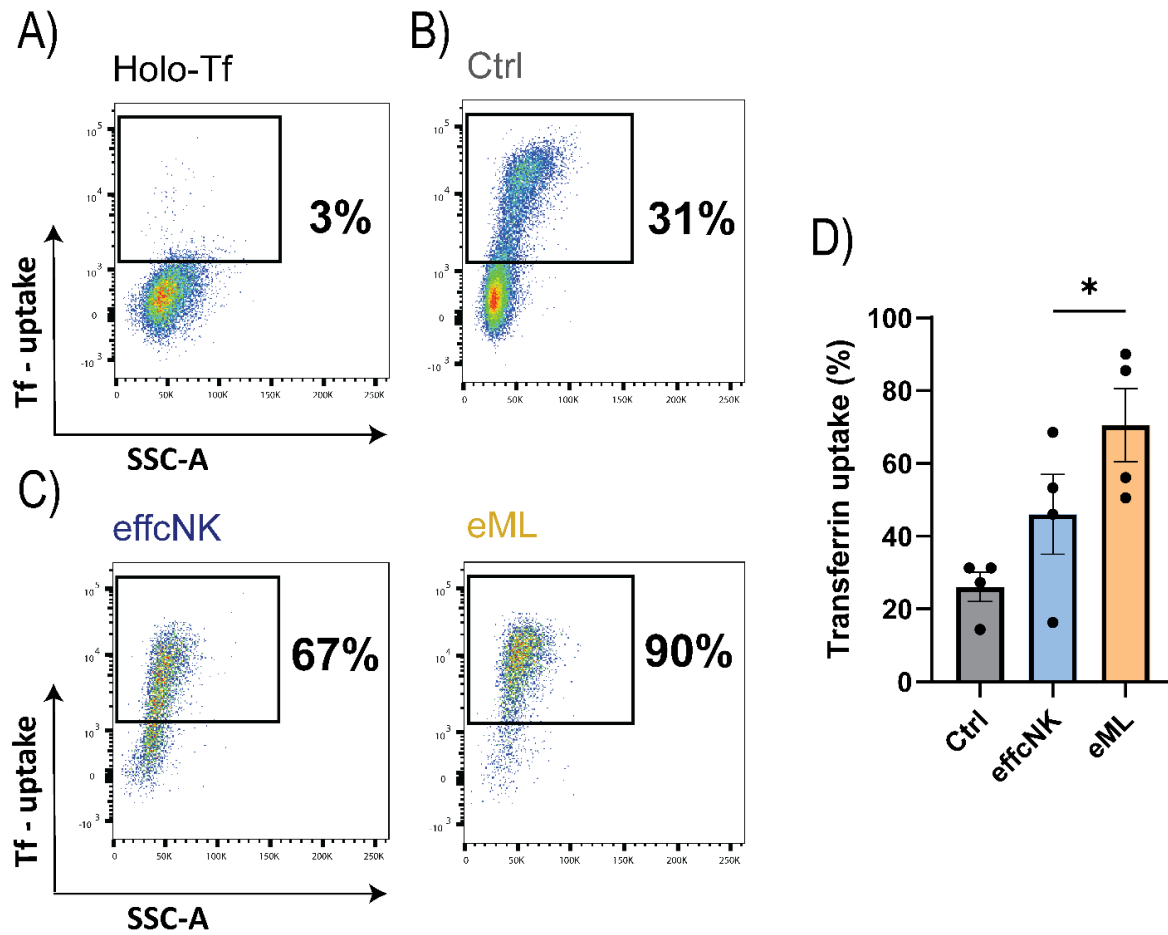


Figure 5.21. eMLs have increased transferrin uptake.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Transferrin (Tf) uptake was determined by administering transferrin conjugate (AF657) to cells at a final concentration of 5 μ g/ml. Tf uptake was also performed on cells treated with Holo-transferrin (Holo-Tf) at a final concentration of 500 μ g/ml as a competition control. **(A-D)** Flow cytometry analysis of Tf uptake measured by percentage positivity in competition, Ctrl, effcNK cells, and eML NK cells. Primed “effcNK” cells identified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells identified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. **(A-C)** Representative dot plots showing Tf positivity in **(A)** Holo-Tf treated bulk CIML cells, **(B)** Ctrl NK cells, **(C)** effcNK and eML NK cells. **(D)** Pooled data indicating percentage positivity for Tf in Ctrl, effcNK, and eML NK cells. Data are **(A-C)** representative or **(D)** mean $\pm$ SEM of N=4 donors. Statistical analysis was performed using a paired t-test. (* p <0.05).

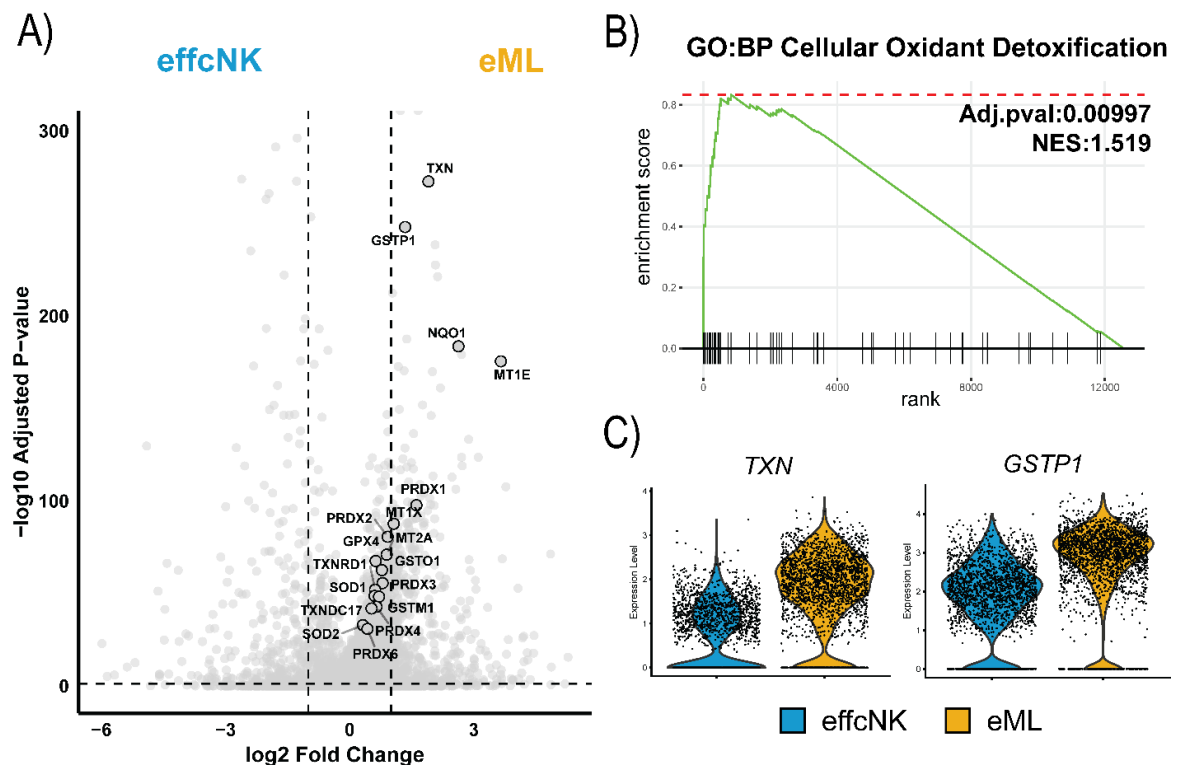


Figure 5.22. eMLs have increased antioxidant-associated gene expression.

(A-C) Foltz et al. isolated single cells from CIML NK cells by flow cytometry based on expression of CD117, CD57, NKG2A, and CD39 markers. Primed “effcNK” cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. NK cells were then barcoded and sequenced by 10X Genomics scRNA-seq. **(A-C)** Expression of genes involved in redox management in effcNK cells and eML NK cells. **(A)** Volcano plot of differentially expressed genes in effcNK cells and eML NK cells, the plot represents the $-\log_{10}$ of the adjusted p-value versus $\log_2$ of the fold change of gene expression. Differentially expressed genes have a $\log_2$ fold change >1 and a $-\log_{10}(\text{adj.p-value}) > 1.35$. Genes of interest are highlighted with a black border. **(B)** GSEA was performed on the entire gene expression datasets, independent of differential expression thresholds. Normalised enrichment scores (NES) are shown, with positive values indicating enrichment of the GO:BP Cellular oxidant detoxification (GO:0098869) pathway. **(C)** Violin plots visualising expression of highly differentially expressed antioxidant genes *TXN* and *GSTP1* in effcNK and eML NK cells. N=1 donor. Raw data available at [DOI: 10.1126/sciimmunol.adk4893](https://doi.org/10.1126/sciimmunol.adk4893).

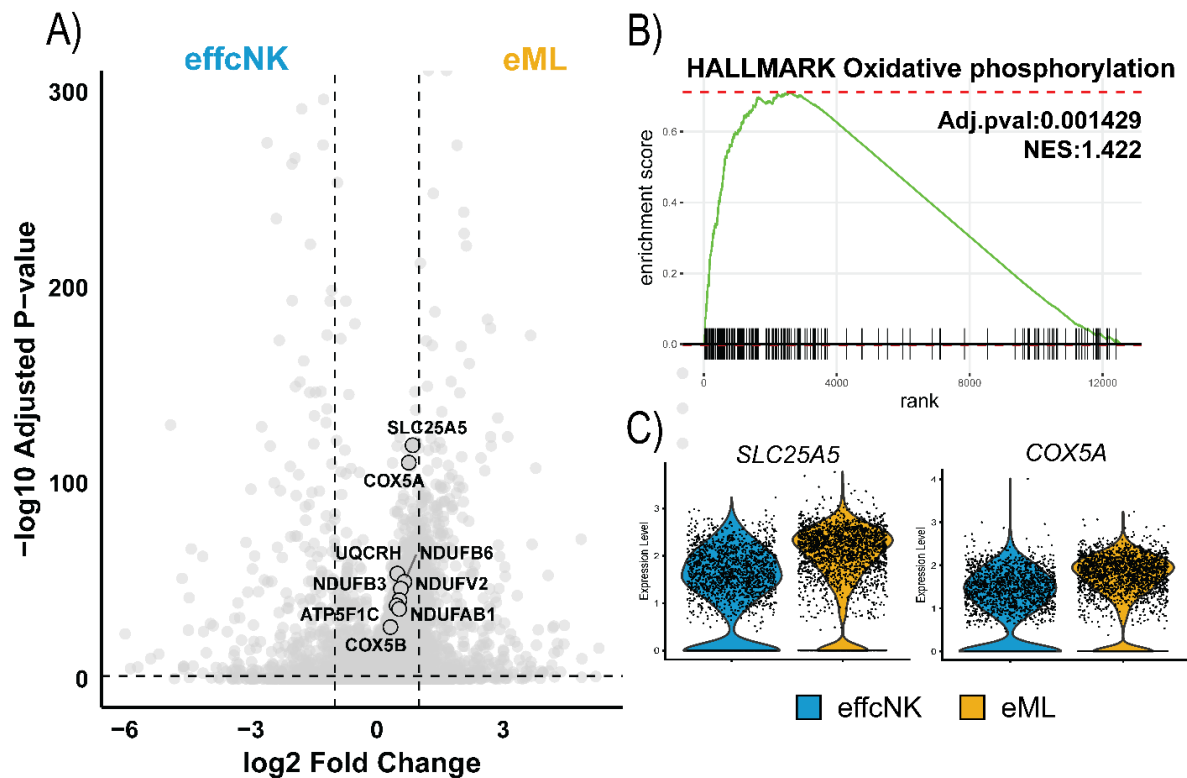


Figure 5.23. eMLs have increased OXPHOS-associated gene expression.

(A-C) Foltz et al. isolated single cells from CIML NK cells by flow cytometry based on expression of CD117, CD57, NKG2A, and CD39 markers. Primed “effcNK” cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells purified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. NK cells were then barcoded and sequenced by 10X Genomics scRNA-seq. (A-C) Expression of genes involved in OXPHOS in effcNK cells and eML NK cells. (A) Volcano plot of differentially expressed genes in effcNK cells and eML NK cells, the plot represents the -log₁₀ of the adjusted p-value versus log₂ of the fold change of gene expression. Differentially expressed genes have a log₂ fold change >1<-1 and a -log₁₀(adj.p-value) > 1.35. Genes of interest are highlighted with a black border. (B) GSEA was performed on the entire gene expression datasets, independent of differential expression thresholds. Normalised enrichment scores (NES) are shown, with positive values indicating enrichment of the HALLMARK Oxidative phosphorylation (M5936) pathway. (C) Violin plots visualising expression of OXPHOS genes *SLC25A5* and *COX5A* in effcNK and eML NK cells. N=1 donor. Raw data available at [DOI: 10.1126/sciimmunol.adk4893](https://doi.org/10.1126/sciimmunol.adk4893).

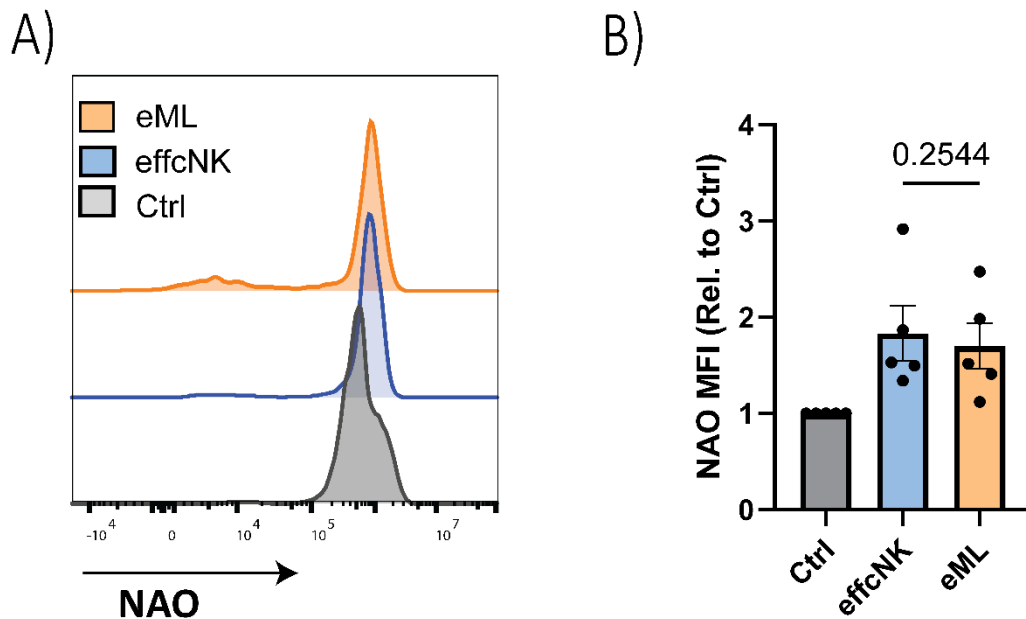


Figure 5.24. effcNK and eML NK cells have similar mitochondrial mass.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Mitochondrial mass was determined by staining with the nonyl acridine orange (NAO) probe. Cells were incubated with 50 nM NAO for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A-D)** Flow cytometry analysis of NAO staining on Ctrl, effcNK cells, and eML NK cells. Primed “effcNK” cells identified as the CD117⁻CD57⁻NKG2A⁻CD39⁻ population and trained “eML” NK cells identified as the CD117⁻CD57⁻NKG2A⁺CD39⁺ population. **(A,B)** Flow cytometry analysis of NAO staining on Ctrl, effcNK, and eML NK cells. **(A)** Representative histogram showing NAO staining signal and **(B)** pooled MFI of NAO signal in effcNK and eML NK cells relative to Ctrl. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=5 donors. Statistical analysis was performed using a paired t-test.

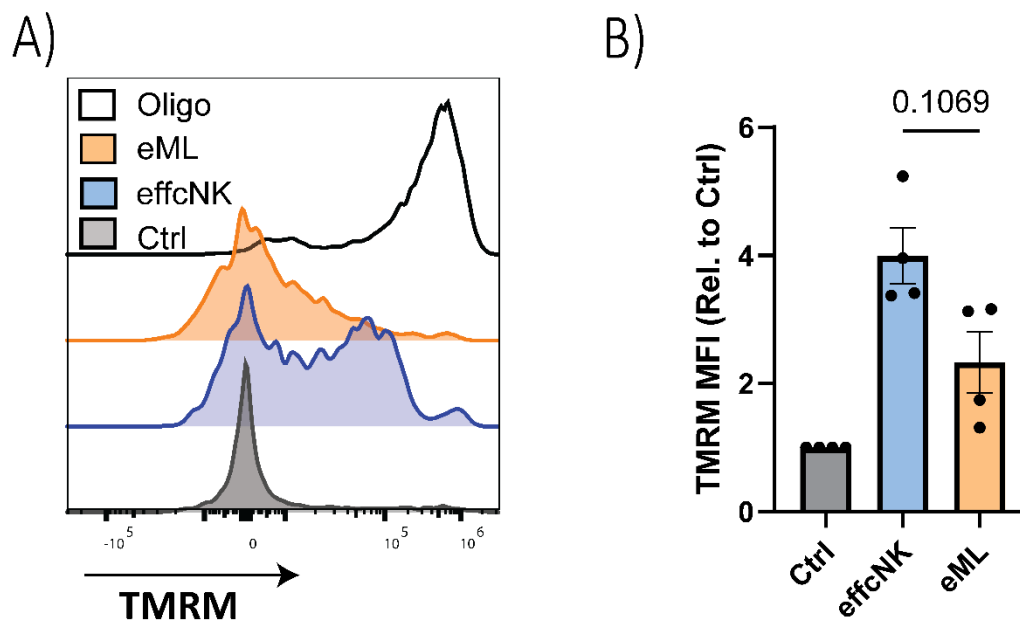


Figure 5.25. effcNKs have increased mitochondrial membrane potential.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Mitochondrial membrane potential was determined by staining with tetramethylrhodamine methyl ester (TMRM) dye. Cells were incubated with 3 nM TMRM for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A-D)** Flow cytometry analysis of TMRM staining on Ctrl, effcNK cells, and eML NK cells. Primed “effcNK” cells identified as the CD117⁻CD57⁻NKG2A⁻CD39⁻ population and trained “eML” NK cells identified as the CD117⁻CD57⁻NKG2A⁺CD39⁺ population. **(A,B)** Flow cytometry analysis of TMRM staining on Ctrl, effcNK, and eML NK cells. **(A)** Representative histogram showing TMRM staining signal and **(B)** pooled MFI of TMRM signal in effcNK and eML NK cells relative to Ctrl. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=4 donors. Statistical analysis was performed using a paired t-test.

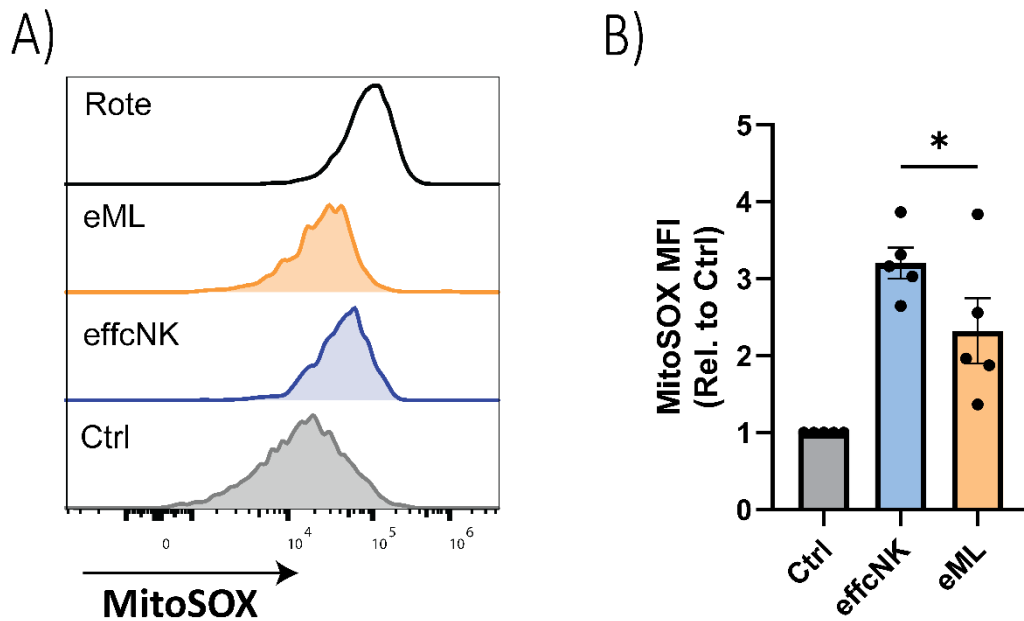


Figure 5.26. eMLs have significantly less mitochondrial ROS compared to effcNKs.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were analysed. Mitochondrial ROS was determined by staining with mitoSOX dye. Cells were incubated with 2 μ M mitoSOX red dye for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A-D)** Flow cytometry analysis of mitoSOX staining on Ctrl, effcNK cells, and eML NK cells. Primed “effcNK” cells identified as the CD117⁻CD57⁻NKG2A⁻CD39⁻ population and trained “eML” NK cells identified as the CD117⁻CD57⁻NKG2A⁺CD39⁺ population. **(A,B)** Flow cytometry analysis of mitoSOX staining on Ctrl, effcNK, and eML NK cells. **(A)** Representative histogram showing mitoSOX staining signal and **(B)** pooled MFI of mitoSOX signal in effcNK and eML NK cells relative to Ctrl. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=5 donors. Statistical analysis was performed using a paired t-test.

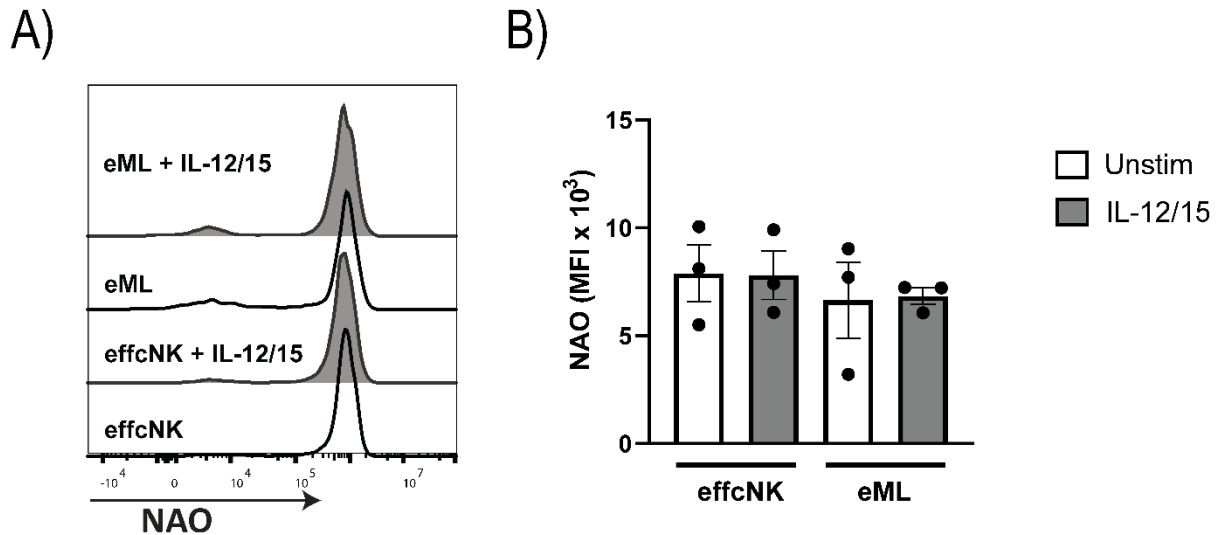


Figure 5.27. Mitochondrial mass is unchanged in eMLs and effcNKs cells post restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. Mitochondrial mass was determined by staining with the nonyl acridine orange (NAO) probe. Cells were incubated with 50 nM NAO for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A-D)** Flow cytometry analysis of NAO staining on Ctrl, effcNK cells, and eML NK cells. Primed “effcNK” cells identified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells identified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. **(A,B)** Flow cytometry analysis of NAO staining on Ctrl, effcNK, and eML NK cells. **(A)** Representative histogram showing NAO staining signal and **(B)** pooled MFI of NAO signal in effcNK and eML NK cells relative to Ctrl. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=3 donors.

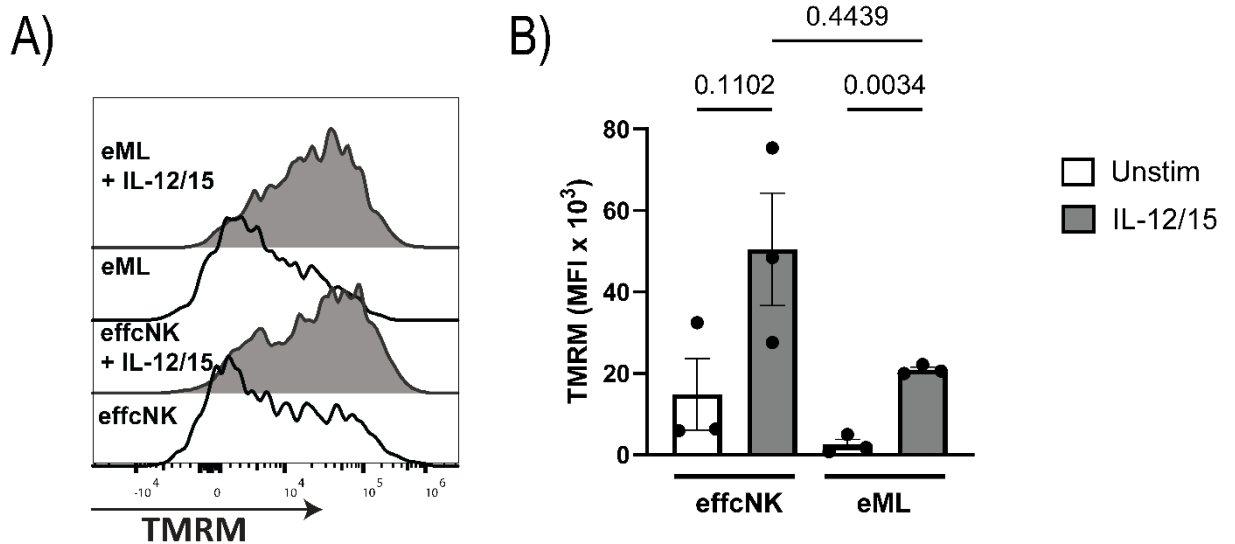


Figure 5.28. Changes in mitochondrial membrane potential in eMLs and effcNKs post-restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. Mitochondrial membrane potential was determined by staining with tetramethylrhodamine methyl ester (TMRM) dye. Cells were incubated with 3 nM TMRM for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A-D)** Flow cytometry analysis of TMRM staining on Ctrl, effcNK cells, and eML NK cells. Primed “effcNK” cells identified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population and trained “eML” NK cells identified as the CD117⁺CD57⁺NKG2A⁺CD39⁺ population. **(A,B)** Flow cytometry analysis of TMRM staining on Ctrl, effcNK, and eML NK cells. **(A)** Representative histogram showing TMRM staining signal and **(B)** pooled MFI of TMRM signal in effcNK and eML NK cells. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=3 donors. Statistical analysis was performed using a one-way ANOVA with a Sidak’s multiple comparisons post hoc test.

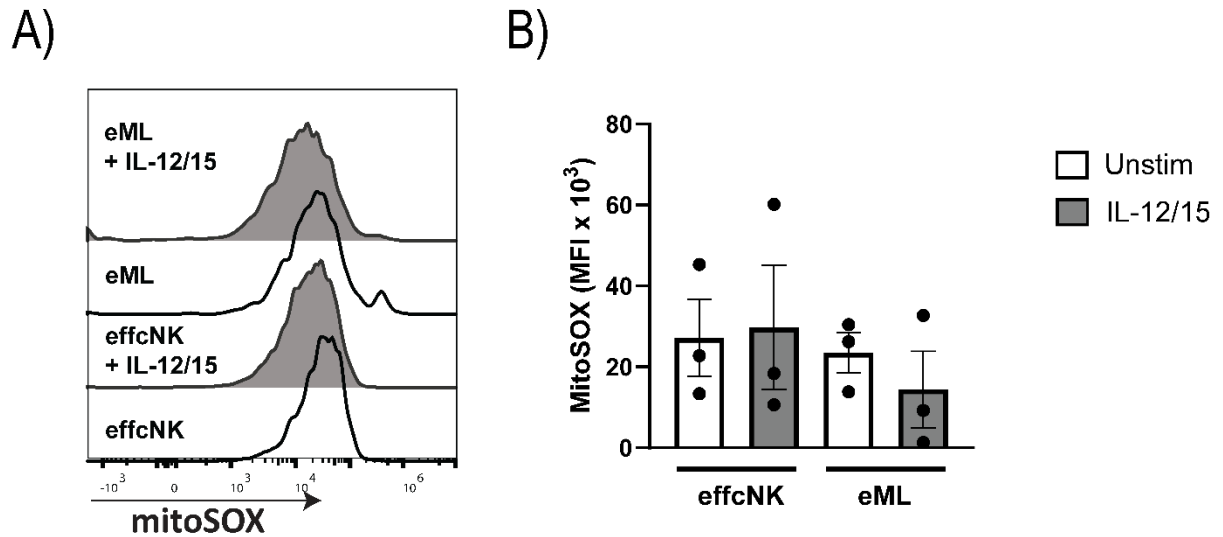


Figure 5.29. Mitochondrial ROS in eMLs and effcNKs cells post restimulation.

PBMC were isolated from fresh healthy human blood, and NK cells were isolated by FACS sorting. Control (Ctrl) NK cells were treated with a low concentration of IL-15 (1 ng/ml), and CIML NK cells were generated by preactivation with IL-12 (30 ng/ml), IL-15 (100 ng/ml), and IL-18 (50 ng/ml) for 18 hours. After 7 days, the Ctrl and CIML NK cells were left unstimulated or restimulated with IL-12 (30 ng/ml) and IL-15 (100 ng/ml) for 6 hours and then analysed. After 7 days, the Ctrl and CIML NK cells were analysed. Mitochondrial ROS was determined by staining with mitoSOX dye. Cells were incubated with 2 μ M mitoSOX red dye for 30 minutes at 37°C and subsequently stained for surface markers and acquired live on Cytex Aurora. **(A-D)** Flow cytometry analysis of NAO staining on Ctrl, effcNK cells, and eML NK cells. Primed “effcNK” cells identified as the CD117⁻CD57⁻NKG2A⁻CD39⁻ population and trained “eML” NK cells identified as the CD117⁻CD57⁻NKG2A⁺CD39⁺ population. **(A,B)** Flow cytometry analysis of mitoSOX staining on Ctrl, effcNK, and eML NK cells. **(A)** Representative histogram showing mitoSOX staining signal and **(B)** pooled MFI of mitoSOX signal in effcNK and eML NK cells. Data are **(A)** representative or **(B)** mean $\pm$ SEM of N=3 donors.

5.7 Discussion.

This chapter aimed to investigate the functional and metabolic differences between the recently identified effcNK and eML NK cell subsets within the bulk CIML NK cell population. Foltz et al. describe eMLs as transcriptionally and epigenetically distinct from effcNKs and conventional NK cells with enhanced production of IFN γ upon restimulation [219]. However, the Foltz et al. study did not investigate the metabolic differences between these subsets. As eMLs meet the criteria for trained immunity, it was hypothesised that they may display a unique metabolic profile compared to that of effcNK cells. To investigate this, single-cell RNA sequencing data from isolated effcNK and eMLs presented in the Foltz et al. paper were interrogated, and supporting experiments were performed using flow cytometry.

As discussed, eMLs and effcNKs have no surface expression of CD57, which is used as a marker for long-lived CD56^{dim} NK cells. CD57 has been identified as a marker of terminal NK cell differentiation and is associated with lower proliferative capacity and senescence in T cells [339-341]. CD57⁺ NK cells have also been shown to be increased in PBMC and bone marrow in Leukemia patients and are associated with resistance to antibody therapy in HER2⁺ breast cancer [342, 343]. Therefore, it may be clinically relevant to remove CD57⁺ NK cells from the CIML NK cell population. Interestingly, eMLs do have significantly increased expression of NKG2A and CD39, markers associated with immunosuppression in NK cells. NKG2A positivity in NK cells has been associated with impaired function and exhaustion in numerous diseases, such as Graves' disease and liver cancer and its genetic deletion was shown to improve anti-tumour responses [344-347]. However, a recent study reported that NKG2A maintains the ability of human NK cells to proliferate *in vitro* and acts as a checkpoint molecule for NK cell proliferation over several weeks [348]. This suggests that although NKG2A positivity is generally associated as a marker of impaired function *in vivo*, its expression in *in vitro* settings may confer proliferative competency. NK cells expressing CD39 have also been associated with worse outcomes in patients with human immunodeficiency virus (HIV), where CD39-positive NK cells were associated with disease progression, acting as negative regulators of T cell proliferation [349]. Furthermore, blockade of CD39 has been shown to result in

improved NK cell cytotoxicity against gastric cancer *in vitro* and in murine models, as well as NK-92 (NK cell line) cytotoxicity against AML [350, 351]. CD39 expression on tumour-infiltrating NK cells is also a predictor of poor prognosis in individuals with esophageal cancer [352]. Thus, although eML NK cells show enhanced functionality *in vitro*, upon transfusion into patients, these receptors may have a detrimental effect on eML cellular activities. CD39 is an ectonucleotidase that can act in synergy with CD73 to cleave ATP into adenosine, which is a potent immunosuppressant [353, 354]. As CIML NK cells are yet to show efficacy against solid tumours, and the TME is associated with high levels of ATP, blockade of CD39 may be a useful strategy to improve anti-tumour function in the clinic [355].

Differential expression analysis, GSEA, and pathway enrichment analyses revealed that eML NK cells have a highly proliferative gene expression signature, with enrichment of E2F target genes and cell cycle-associated pathways as well as increased expression of Ki67. This supports data outlined in Chapter 3 that identified enrichment of E2F target proteins in bulk CIML NK cells compared to control NK cells, which may, in fact, be a result of the expansion of eMLs within the CIML NK cell population. As discussed in Chapter 3 regarding immunotherapy efficacy, markers identified in this chapter, such as *MCM5* and *MYBL2*, could also be used for eML immunotherapy tracking through peripheral blood sampling.

Foltz et al. reported that effcNKs and eMLs have similar cytotoxicity against K562s and that eMLs exhibit higher IFN γ production upon restimulation. Data generated in this chapter demonstrate that although effcNKs have increased RNA expression of activating receptors such as CD16 compared to eMLs, they also have significantly increased inhibitory receptors, including TIGIT and TGFBR2. TIGIT has long been associated with NK cell exhaustion and impaired function, and its blockade can rescue these abrogated effector functions [356-359]. Similar characteristics have been defined for TGFBR2, which serves as a receptor for TGF β , and its deletion in mice results in retention of NK cell functional activity in the presence of TGF β [360]. This milieu of inhibitory receptors may impact effcNK efficacy in the clinic, thus combination therapy with antibody blockade may improve cytotoxicity. Similarly, although eMLs have decreased

expression of CD16 and other activating receptors, this could be circumvented by cell engineering methods. In mice, NKp46 overexpression leads to increased viral clearance and tumour metastasis control [361]. Human iPSC-derived NK cells can be transduced to express the high-affinity CD16a protein, which shows superior ADCC against multiple tumours [362]. Ultimately, effcNKs and eMLs can be specifically tailored using various combination therapies or through cell engineering to enhance their capabilities in the clinic.

As well as showing differences in proliferative and functional capacity, results outlined in this chapter also demonstrate that there are metabolic differences between effcNKs and eMLs. Increased glucose, PPP, and purine metabolism were observed and are in line with observations from Chapter 3. However, it was also noted that eMLs have significantly increased iron metabolism compared to effcNKs based on their gene expression signature, and by CD71 expression and transferrin uptake assays. Iron overload has been shown to be detrimental to NK cell function and is associated with iron-induced NK cell ferroptosis in cancer. Therefore, eMLs may be better equipped for handling iron-rich environments in such disease states, owing to their constitutively high expression of iron-sequestering genes [193]. Conversely, iron deficiency anaemia is very common in many cancers due to either the cancer itself or treatments against the cancer [363-365]. Iron is known to be important for NK cells for functional responses, such as those against viral infections, and is widely accepted as a necessary element for cellular mitochondrial biogenesis and fitness, as well as replication [191, 366, 367]. Thus, in patients with excessive or deficient iron levels due to conditions such as hemochromatosis or anaemia, respectively, CIML NK cell therapy may be hampered, resulting in impaired eML proliferation and function. This observed increase in iron uptake may also be driving the increased expression of antioxidant genes in eMLs, such as *HMOX1*, *MT1E*, and *NQO1*, the induction of which can be triggered by heavy metal-induced oxidative stress [368, 369].

Finally, this chapter investigated mitochondrial differences between effcNKs and eMLs. Interestingly, eMLs had a strong OXPHOS gene expression signal when compared to effcNKs, but upon analysis by flow cytometry, effcNKs had increased mitochondrial membrane potential. This discrepancy between RNA

levels and other readouts, such as flow cytometry analyses, can be a common phenomenon that could be explained by numerous factors, such as post-transcriptional regulation, translation efficiency, and the analysis technique itself [370, 371]. Nevertheless, effcNKs had a trending increase in membrane potential compared to eMLs at rest, whereas eMLs had lower mitochondrial ROS production. The decreased mitochondrial ROS production could be owed to the overall reduced mitochondrial membrane potential, or to the enhanced antioxidant gene expression in eMLs [123]. Both effcNK and eML NK cells had comparable mitochondrial mass, and both had a trend towards increased mitochondrial membrane potential generation upon restimulation, indicating the mitochondrial features of accumulation and rapid OXPHOS induction outlined in Chapters 3 and 4 may be common between both primed and trained CIML NK cell subsets.

Overall, integrated scRNA-seq analysis and flow cytometry identified further functional and metabolic differences between effcNKs and eMLs. This was characterised by increased cytotoxic potential, glycolysis, PPP, purine metabolism, iron metabolism, and redox management in eMLs. Comparatively, effcNKs show enhanced gene expression of activating and inhibitory receptors, as well as potentially enhanced mitochondrial activity at rest and upon restimulation. Observations made in Chapters 3 and 4 may be owed to the differences between effcNKs and eMLs. Together, these data demonstrate that metabolic heterogeneity exists within CIML NK cell subsets.

Chapter 6. General discussion.

The data presented in this thesis investigated the metabolism of CIML NK cells, combining proteomic, metabolomic, publicly available scRNA-seq data, and functional analyses. To date, most of the metabolic analyses that have been performed on CIML NK cells have employed techniques with lower resolution, scope, and complexity, such as flow cytometry and Seahorse-based analysis. Proteomic analysis allows for broad untargeted surveying of samples for global patterns, while targeted metabolomics allows for molecular tracking of known nutrients of interest, such as glucose. This offers system-wide, unbiased profiling of molecular function, which gives a more comprehensive and high-throughput snapshot of CIML NK cell metabolism [372-374]. In this thesis, flow cytometry was used as a targeted approach to validating predefined observations in high-resolution proteomic, metabolomic, and scRNA-seq analyses, rather than an exploratory technique. Quantitative proteomic and metabolomic analyses of CIML NK cells revealed a metabolic reprogramming that favours a proliferative phenotype, characterised by enhanced glycolysis, PPP, purine metabolism, and accumulation of mitochondrial mass. CIML NK cells mobilise this preestablished mitochondrial mass for rapid OXPHOS-associated protein synthesis and generation of mitochondrial membrane potential post-stimulation. The data presented herein suggests that CIML function at rest and post stimulation is likely to be managed by PPP metabolism, placing the PPP as a central regulator of general CIML NK cell metabolic reprogramming. This thesis also considers metabolic and functional heterogeneity between recently identified CIML NK cell subsets, including primed and bona fide-trained CIML NK cell phenotypes, that may allow for selective expansion of subsets with enhanced immunotherapeutic potential.

Through a combination of proteomic, metabolomic, and flow cytometric analysis, Chapter 3 reveals that CIML NK cells have increased protein content of enzymes involved in glycolysis, the PPP, and purine biosynthesis. This study is the first to perform ¹³C glucose tracing on CIML NK cells, which indicated enhanced glycolysis by significantly increased labelled lactate secretion into supernatants. The enhanced glucose uptake committed more glucose-derived carbons to

purine biosynthesis compared to control NK cells, and as the PPP is the primary source of R5P in cells, purine biosynthesis and glycolysis are likely to be linked by the PPP. As discussed in Chapter 3, CIML NK cells undergo a proteomic shift toward a proliferative phenotype, and anabolic processes like glycolysis and purine biosynthesis would be essential for providing coenzymes, biosynthetic intermediates, nucleotides, and the energy needed for the formation of new cells. It was also discovered here that CIML NK cells have significantly more dysfunctional mitochondria compared to control NK cells, producing significantly more ROS and generating low amounts of mitochondrial membrane potential relative to mass. This dysfunctional mitochondrial accumulation may be a major source of oxidative stress in cells, and CIML NK cells displayed significantly increased expression of proteins involved in redox management. As the PPP provides a substantial amount of NADPH for reducing reactions, this thesis demonstrates that the PPP may not only act as a mediator of purine synthesis but also as a mechanism for ROS management in CIML NK cells and their mitochondria.

This is one of the first studies to show a dual mechanistic role for the PPP in NK cells and presents the PPP as a central node for CIML NK cell metabolism through maintenance of increased ROS-producing mitochondrial mass and purine biosynthesis. It would be of interest to investigate if the increase in redox potential is triggered by mitochondrial ROS production during the training stimulus or if early initiation of PPP metabolism increases antioxidant capacity, facilitating the accumulation of dysfunctional mitochondria over time. This could have strong implications for cancer immunotherapy, given that the metabolic changes that CIML NK cells undergo may result in detrimental outcomes upon adoptive transfer into patients. For example, it is demonstrated that the *in vitro* culture of CIML NK cells results in the accumulation of dysfunctional mitochondria producing increased ROS, which is likely to be mitigated by the PPP. However, adoptive transfer of these cells into a nutrient-restricted environment, such as the blood of cancer patients or the TME, may induce numerous forms of cellular oxidative stress [375-377]. Glucose restriction and subsequent decreased PPP flux may cause a collapse in NADPH pools *in vivo*, and the resulting unmanaged ROS generation from accumulated mitochondria could drive stress responses,

arrest proliferation, or even result in cell death [378]. However, with cell engineering becoming more commonplace in cellular therapies, these issues could be circumvented by genetic manipulations. Overexpression of nutrient receptors like GLUT1 would improve the ability of CIML NK cells to compete with other cells for glucose in the TME and has been demonstrated to enhance metabolism, redox potential, proliferation, and antitumour potency in murine and human CAR T cells [379, 380]. Other studies have demonstrated that overexpression of the antioxidant enzyme PRDX1 in CAR NK cells had a protective effect and improved both antitumour immunity and resistance to oxidative stress in murine models [305]. Similarly, overexpression of catalase (CAT) in CAR NK cells resulted in enhanced resistance to hydrogen peroxide and hypoxic conditions *in vitro* [381]. Genetic manipulation of pathways identified in this thesis could theoretically improve CIML NK cell viability and function in environments with exogenous sources of oxidative stress and nutrient competition, such as the bloodstream or the TME. Overall, the data presented in Chapter 3 reveal that CIML NK cells undergo metabolic reprogramming that favours a proliferative profile supported by glycolysis-fuelled purine biosynthesis and PPP/redox metabolism, offering biomarkers and targetable mechanisms for improved CIML NK cell therapy (Figure 6.1).

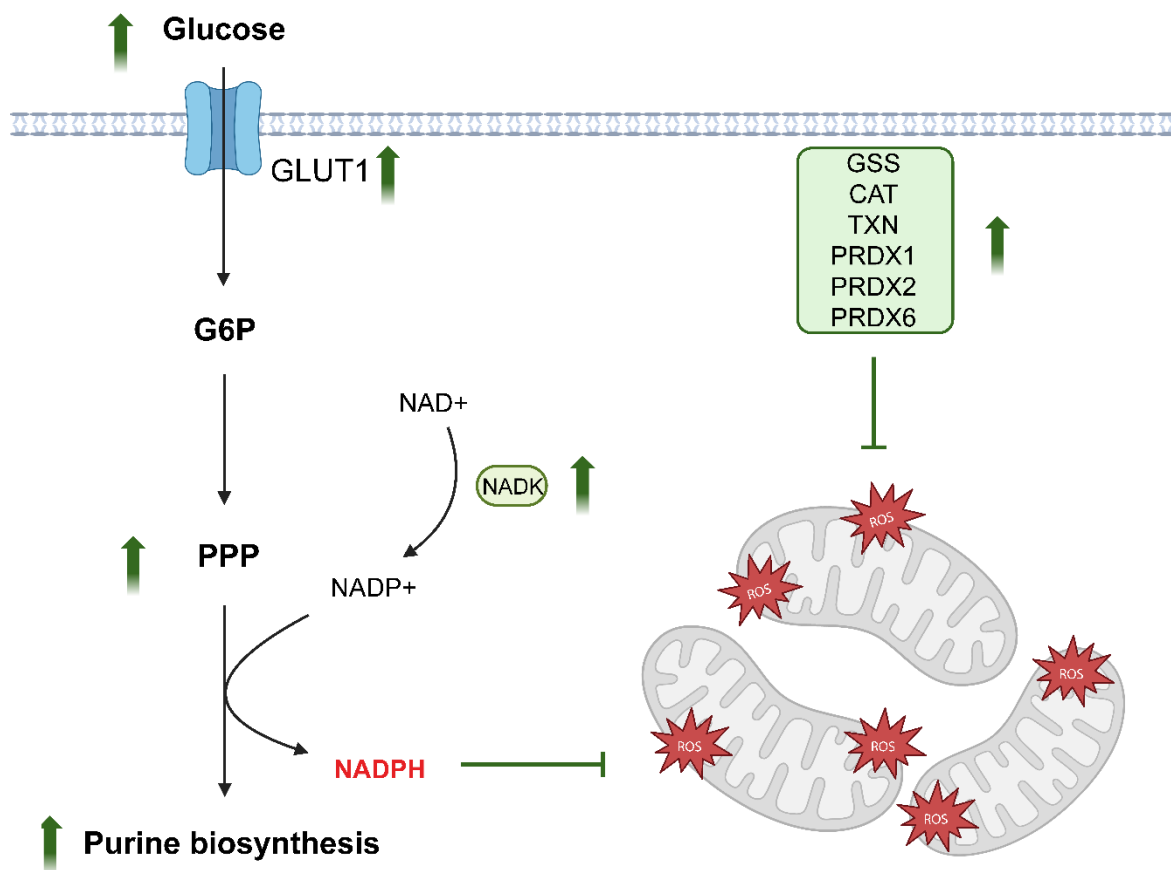


Figure 6.1. Illustration of proposed metabolic reprogramming in resting CIML NK cells.

CIML NK cells demonstrate increased glycolysis and PPP metabolism, resulting in purine biosynthesis and NADPH production. This is supported by cytosolic NADK that converts NAD⁺ to NADP⁺, which is then reduced in the PPP to NADPH. Increased NADPH pools and antioxidant protein expression converge to manage ROS production from accumulated dysfunctional mitochondria. Illustration made using Biorender.

Chapter 4 investigated the differences in control and CIML NK cells post stimulation, revealing that CIML NK cells rapidly mobilise their preestablished mitochondrial mass for rapid OXPHOS. This was accompanied by effective mitochondrial ROS management in CIML NK cells post-stimulation, which was not observed in CIML NK cells at rest. This indicates that CIML NK cells are better equipped for mitochondrial energy production upon restimulation compared to control NK cells. This may be a result of epigenetic imprinting on OXPHOS-associated genes that occurs during the training stimulus, or throughout the 7-day quiescence period, which would account for the rapid induction of OXPHOS proteins observed unique to CIML NK cells. The ability of stimulated CIML NK cells to manage mitochondrial ROS effectively was not observed in other studies that have demonstrated significant increases in NK cell mitochondrial ROS after stimulation with IL-12, IL-15, and IL-18 [223]. This may also be due to epigenetic imprinting on genes such as the mitochondrial glutathione importer, *SLC29A40*, which was observed to be rapidly induced at the protein level in CIML NK cells compared to control NK cells. The data presented in chapter 4 also suggests that the inhibition of machinery important for mitochondrial energy production, such as ATP synthase and PDH, may result in decreased CIML NK cell enhanced secondary effector functions such as granzyme B and IFN γ production. Nutrients such as pyruvate derived from glucose and fatty acids may drive the TCA in CIML NK cells upon restimulation, facilitating the observed increase in OXPHOS, which in turn supports the synthesis of effector molecules. Again, this demonstrates an essential role for nutrients such as glucose and fatty acids for competent effector functions. Although these pathways are necessary during stimulation of CIML NK cells, overexpression or agonism of the genes involved prior to infusion into cancer patients could perturb CIML NK cell metabolism in an unwanted manner. To address this, new technologies such as focused ultrasound (FUS) have been shown to selectively activate immune cells through localised heating. CIML NK cells could be engineered with heat-shock-protein (Hsp) promoters that would drive expression of genes such as GLUT1, PDH, or FAO-associated enzymes. This would allow for selective activation of CIML NK cells in localised areas such as the TME using FUS-mediated activation of Hsp-driven gene expression. This approach could prevent unwanted metabolic reprogramming of CIML NK cells due to constitutive overexpression or agonism of metabolic genes in resting NK

cells prior to infusion [382-384]. Another strategy to improve CIML NK cell survival *in vivo* could involve enhancing mitochondrial ROS management potential. Overexpression of transporters such as SLC25A40 could enhance glutathione uptake from the cytosol into the mitochondria, and NADK2 or folate metabolism enzymes could be selectively targeted to increase mitochondrial NADPH pools. This could improve the intrinsic ability of mitochondria to detoxify localised ROS production during activation in states of oxidative stress. Overall, this thesis suggests a mechanism by which CIML NK cells rapidly induce mitochondrial translation of OXPHOS-associated proteins and generate increased mitochondrial membrane potential to support the bioenergetic demands of enhanced secondary functions. This increase in OXPHOS may be supported by pyruvate, fatty acid, or amino acid-driven anaplerosis and cytosolic factors such as mitochondrial glutathione uptake and PPP metabolism, again presenting targets for precision CIML NK cell-based immunotherapy (Figure 6.2).

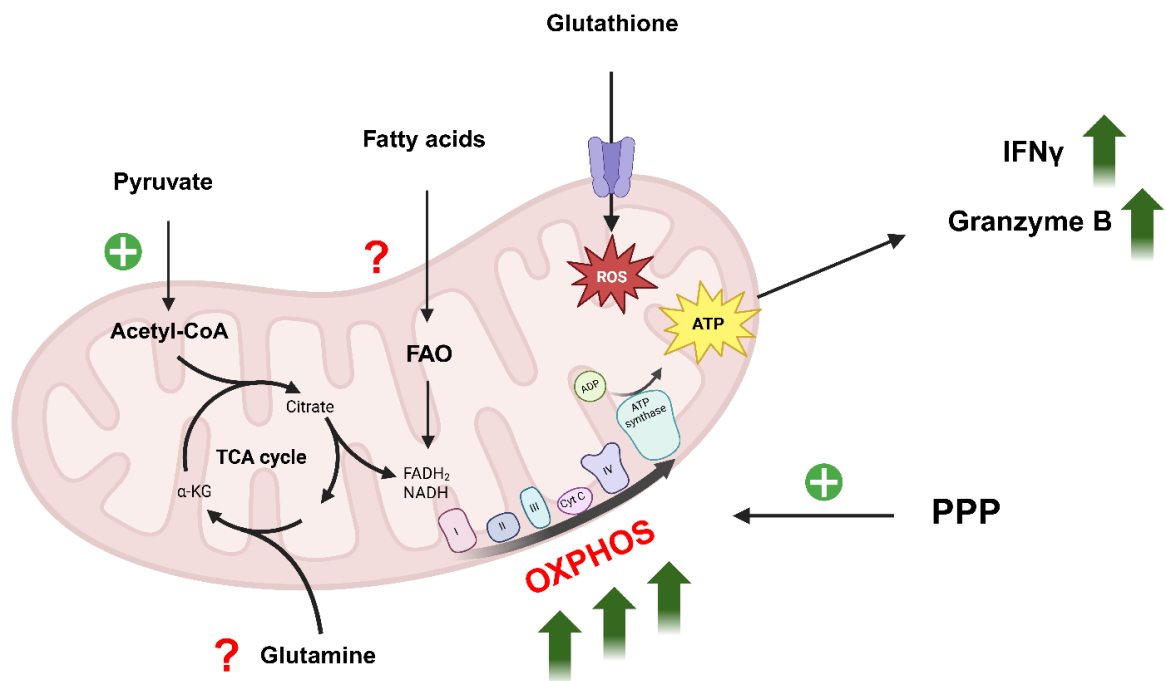


Figure 6.2. Illustration of the proposed bioenergetic mechanism for enhanced secondary functions in CIML NK cells

CIML NK cells exhibit increased IFN γ and granzyme B production upon restimulation, accompanied by enhanced OXPHOS-associated protein expression and mitochondrial membrane potential generation. In this thesis, it was demonstrated that pyruvate-mediated anaplerosis may be important for enhanced secondary responses. Other pathways that drive the TCA cycle and OXPHOS, such as amino acid metabolism and fatty acid oxidation, may also have supporting roles in secondary functions. Furthermore, the PPP may also be important for mitochondrial membrane potential generation and secondary functions, as well as cytosolic-derived reductive factors such as glutathione. This thesis proposes a model whereby enhanced mitochondrial metabolism is supported by alternative pathways that provide the bioenergetic basis necessary for enhanced secondary effector functions. Illustration made using Biorender.

Chapter 5 investigates the functional and metabolic characteristics of recently described CIML NK cells through a publicly available scRNA-seq dataset and supporting flow cytometry. This study revealed functional and metabolic differences between a primed CIML NK cell subset (effcNKs) and a bona fide-trained subset (eMLs). Functional differences included increased granzyme B reservoirs at rest and IFN γ production upon restimulation in eML NK cells, while effcNKs displayed elevated RNA expression of granzyme M and upregulated activating receptors. Differences in metabolism were largely seen in the eML subset as they exhibited increased proliferation, glycolysis, PPP, purine synthesis, iron metabolism and redox-associated pathways. Although eMLs had enhanced OXPHOS at the RNA level, flow cytometric analyses revealed that effcNKs may have more mitochondrial membrane potential generation at rest and post-restimulation compared to eML NK cells. It was also discovered that effcNKs have increased gene expression of inhibitory receptors such as TIGIT and TGFBR2, while eMLs have lower expression of CD16 and are defined by characteristic CD39 and NKG2A expression, markers usually associated with NK cell impairment. This study demonstrates how CIML NK cell subsets, particularly eMLs, could be selected and isolated for expansion. The proliferative and cytotoxic properties of eML NK cells would make them preferential candidates for immunotherapy due to the potential for enhanced survival and functional activity *in vivo*. Although decreased CD16 expression and increased expression of CD39 pose therapeutic issues in terms of eML cytotoxic competency and immune evasion vulnerability, these could be circumvented with gene-targeted overexpression of CD16 for ADCC and anti-CD39 combination therapy [256, 350, 351]. It was also demonstrated here that eMLs have enhanced expression of redox-maintaining genes, which may be advantageous in the TME for oxidative stress resistance. However, expression of these redox genes in eMLs could be driven by their enhanced iron uptake, characterised by increased CD71 expression and transferrin uptake, which may facilitate enhanced function and proliferation. This suggested iron dependency in eMLs raises important considerations for the treatment of solid tumours, especially in iron-deficient TMEs or in patients with low blood iron from iron-deficiency anaemia. Overall, the data presented in Chapter 5 demonstrate functional and metabolic heterogeneity

between CIML NK cell subsets that can be targeted for enhanced bulk CIML NK therapy or selective subset immunotherapy (Figure 6.3).

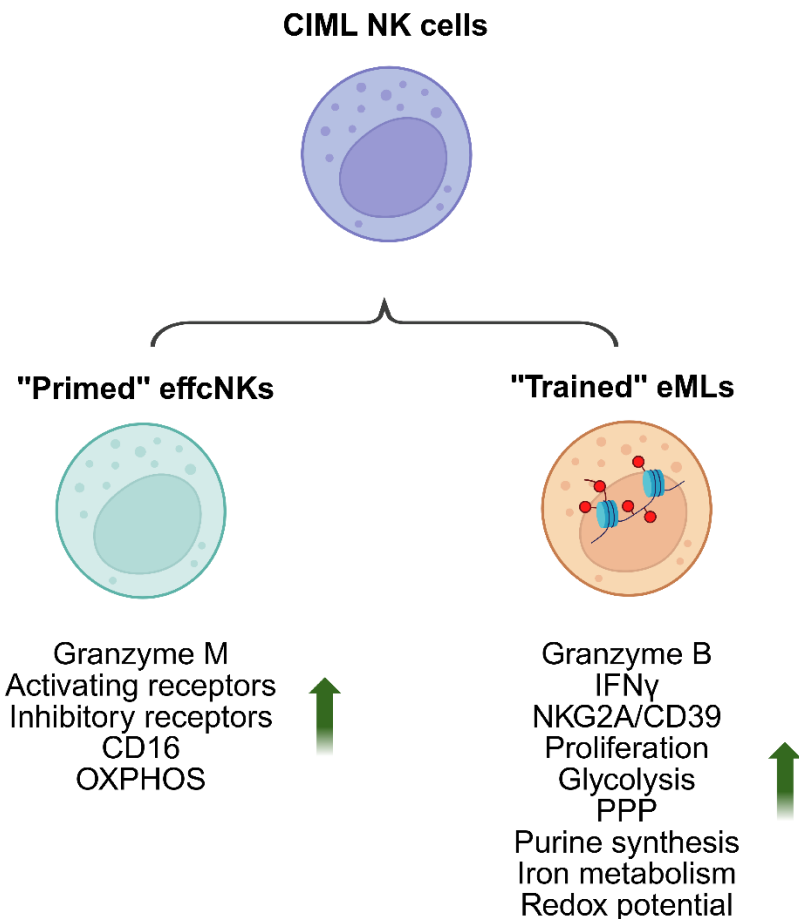


Figure 6.3. Illustration of functional and metabolic heterogeneity within CIML NK cell subsets.

CIML NK cells have been identified to be comprised of primed (effcNK) and trained (eML) NK cell subsets. These subsets have various functional and metabolic differences. Primed, effcNK cells are characterised by increased gene expression of granzyme M, activating receptors, inhibitory receptors, and CD16. Trained, eML, cells have enhanced resting granzyme B stores, enhanced IFN γ production post restimulation, increased NKG2A/CD39 expression and enhanced glycolysis, PPP, purine synthesis, iron metabolism, and redox potential. Illustration made using Biorender.

6.1 Limitations

A key aspect of CIML NK cells is their current use as immunotherapies in the clinic. However, all analyses in this thesis were performed *in vitro*, in optimal and controlled conditions. As discussed, the periphery of humans and TME presents a very different, stressful, and nutrient-deprived environment compared to the cell culture environment. In this study, CIML NK cell secondary functions were only assessed by cytokine restimulation. Assessing CIML NK cell metabolism during activation of cells through contact with target cells like K562s or solid tumour cell lines may provide more clinically relevant insights. Although some trends were observed linking the PPP and mitochondrial metabolism to supporting secondary effector functions, more targeted experiments, such as knockdown of genes in the oxidative/non-oxidative PPP, would be very insightful. Notably, several datasets throughout this thesis demonstrated a trend towards an increase in metabolic pathway utilisation that did not consistently reach statistical significance. This could suggest that the study may be relatively underpowered to detect modest but meaningful metabolic differences, particularly considering the well appreciated donor-to-donor variability in human NK cell biology. Increasing the cohort size could clarify whether these observed trends represent reproducible metabolic shifts. However, the observed variability may also reflect genuine metabolic heterogeneity within the bulk CIML NK cell population. As demonstrated in Chapter 5, distinct CIML NK subsets exhibit phenotypic and functional differences and the ratio of these subsets within the bulk population varies from donor-to-donor. Bulk metabolomic and proteomic analyses may not have the resolution to identify subset specific metabolic signatures, resulting in trends at the population level that do not achieve statistical significance. In this case, high resolution subset orientated metabolic profiling of cells may uncover stronger, mechanistically coherent CIML NK cell pathway dependencies

6.2 Future work

Future work of interest would be to fully understand the role of early metabolic events in shaping CIML NK cell-trained immunity. It would be useful to investigate if early induction of the PPP or mitochondrial accumulation facilitates epigenetic remodeling or the emergence of eML NK cells. These insights may provide

mechanisms for preferentially driving eML differentiation or inducing training in the absence of cytokine stimulation. It would also be useful to explore these findings further *in vivo*. This could be carried out in humanised mouse models or, ideally, in patients receiving CIML NK cells as an immunotherapy. This would be useful for determining if CIML NK cell features, such as proliferation and mitochondrial accumulation, can be sustained *in vivo*. As observed in Chapter 4, CIML NK cells ramp up OXPHOS during enhanced secondary responses, and inhibiting the PPP, mitochondrial ATP synthesis, and pyruvate anaplerosis may impact granzyme B and IFN γ production. Selective enhancement of PDH or fatty acid oxidation through new technologies such as Hsp-induced gene expression would provide precision-based insights in environments like the TME. Targeted modulation of identified metabolic pathways, such as genetic overexpression of antioxidant, PPP or mitochondrial genes for enhanced CIML NK cell function, could be tested in murine cancer models or in TME-replicating cell culture conditions.

Another important consideration for future work is the detailed characterisation of NK cell subpopulations in donors before and after CIML NK cell induction. Factors such as prior HCMV exposure, baseline expression of activation, inhibitory, or exhaustion markers, and the balance of CD56^{bright} and CD56^{dim} NK cells may influence how efficiently CIML NK cells are trained and the extent to which eML populations emerge. CMV-positive donors may have expanded adaptive-like NK cells with unique epigenetic and metabolic profiles, which could either enhance or constrain cytokine-induced training. Likewise, expression of exhaustion or senescence-associated markers such as PD-1, LAG-3, or TIM-3 may impact responsiveness to cytokine stimulation and subsequent effector function. Extensively assessing these markers across donors could help explain variability in CIML NK cell generation and function and may inform strategies to optimise donor selection or pre-conditioning protocols. In the future, this knowledge could also enable sub-cohorting of donors based on training capacity, distinguishing responders from non-responders, and allowing more targeted or personalised approaches to CIML NK cell therapies. Lastly, subset-specific engineering of effcNKs or eMLs to enhance function or combination therapy with

checkpoint inhibitors, such as those against CD39 are experiments with clinical relevance worth pursuing.

6.3 Conclusion

In conclusion, this thesis is one of the first integrated proteomic and metabolomic interrogations of CIML NK cell metabolism. The data presented in this thesis demonstrate that CIML NK cells sustain a proliferative and anabolic phenotype characterised by enhanced glycolysis, PPP, redox management, and accumulate mitochondrial mass poised for rapid mobilisation upon restimulation. This thesis also demonstrates the existence of metabolic heterogeneity in recently identified CIML NK cell subsets. These insights not only add to the general knowledge of NK cell biology but also deepen the understanding of NK cell trained immunity while presenting metabolic targets that can be leveraged for future NK cell immunotherapies.

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