Investigating the impact of Natural Killer (NK) cell metabolism on NK cell effector function

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

School of Biochemistry and Immunology

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Declaration

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Vanessa Zaiatz Bittencourt
For my Mom and Dad
Publications

Metabolic Reprogramming Supports IFN-γ Production by CD56<sup>bright</sup> NK Cells.

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

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Publications

Canonical TGF-β Signaling Pathway Represses Human NK Cell Metabolism.

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Abstract

It is now known that metabolism, in addition to providing energy and biochemical building blocks, also regulates immune cell function. Over the last few years, we have increased our understanding of how NK cell metabolism impacts on murine NK cell function; however, nothing was known about human NK cells. In this study, we defined the metabolic changes that occur in human peripheral blood NK cells in response to overnight stimulation with either innate cytokines, IL-12 and IL-15, or IL-2, a cytokine generally associated with adaptive T cell responses. We also investigated NK cell metabolic changes after a more prolonged exposure (5 day) to IL-2. Analysis showed that NK cells upregulated glycolysis and oxidative phosphorylation (OxPHOS) in response cytokine. Many of the metabolic changes observed, particularly in the 5 day cultures were dependent on mTORC1. We also defined metabolic differences in important NK cell subsets. CD56\textsuperscript{bright} cells were more metabolically active compared to CD56\textsuperscript{dim} cells as they preferentially upregulated nutrient receptors and differed substantially in terms of their glucose metabolism. Our data showed that elevated levels of OxPHOS were required to support both cytotoxicity and IFN\textgamma production in NK cells and while elevated glycolysis was not required directly for NK cell degranulation, limiting the rate of glycolysis significantly impaired IFN\textgamma production by the CD56\textsuperscript{bright} subset of cells.

We also investigated how NK cell metabolism might be down-regulated after activation in chronic inflammatory conditions. We demonstrated that transforming growth factor beta (TGF-\textbeta), known to inhibit IFN\textgamma production by NK cells, inhibited cytokine induced (18h) increases in OxPHOS, maximum respiration and glycolytic capacity of human NK cells. TGF-\textbeta-mediated suppression of NK cell metabolism or function did not involve the inhibition of mTORC1 in these experiments, and inhibition of the canonical TGF-\textbeta signaling pathway was able to restore almost all metabolic and functional responses. Disruption of cytokine induced changes in NK cell metabolism by prolonged exposure to either TGF-\textbeta or rapamycin resulted in dysfunctional NK cells suggesting that impaired metabolic changes could contribute to NK cell dysfunction during chronic inflammation. Interestingly, although the
canonical signalling pathway was still the most active, TGF-β could suppress mTORC1 activity in these longer cultures.

Our data support that human NK cells undergo metabolic changes in response to cytokine and that mTORC1 is important for sustained reprogramming of NK cell metabolism. Metabolism is important for human NK cell functions and may be dysregulated in pathological situations. These findings will help in the development of new ways to manipulate NK cell function during inflammation, virus infection or cancer.
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Abbreviations

2DG  2 deoxy-D-glucose
ADCC  Antibody dependent cell cytotoxicity
APC  Antigen presenting cell
BM  Bone marrow
BSA  Bovine serum albumin
CTLs  Cytotoxic T lymphocyte, CD8+ T cell
CMV  Cytomegalovirus
DCs  Dendritic Cells
ECAR  Extracellular acidification rate
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
GATA3  GATA-binding protein 3
G6P  Glucose-6-phosphate
HLA  Human leukocyte antigen
HSC  Hematopoietic stem cells
iDCs  Immature dendritic cells
IFN-γ  Interferon-γ
Il2  Innate helper 2 cells
IL-2Rβ  Interleukin-2 receptor-β
ILCs  Innate lymphoid cells
ION  Ionomycin
ITAM/ITIM  Immunoreceptor tyrosine-based activation (or inhibitory) motifs
IFN  Interferon
KIR  Killer cell immunoglobulin like receptor
LTi  Lymphoid tissue inducer
MHC  Major histocompatibility complex
mTORC  Mammalian target of rapamycin complex
NADPH  Nicotinamide adenine dinucleotide phosphate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-NBDG</td>
<td>2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2</td>
</tr>
<tr>
<td>N-CAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural Killer cells</td>
</tr>
<tr>
<td>NKR-P1</td>
<td>NK cell receptor protein 1</td>
</tr>
<tr>
<td>OCAR</td>
<td>Oxygen consumption rate</td>
</tr>
<tr>
<td>OxPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pS6</td>
<td>S6 ribosomal protein</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose phosphate pathway</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombinant-activating gene</td>
</tr>
<tr>
<td>RORα</td>
<td>Retinoic acid receptor-related orphan receptor-α</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
</tbody>
</table>
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Chapter 1
Introduction
1.1 NK cells

Our first line of defense against foreign organisms are barrier tissues such as skin, which stops the entry of organisms in our bodies. If, however, these barriers are damaged, the body contains circulating cells that respond quickly to the presence of the invader by recognizing them with their different kinds of receptors that specifically recognize a pathogen. These cells include dendritic cells (DC), macrophages, neutrophils and Natural Killer (NK) cells, which have different functions. These cells can engulf the foreign organisms or activate inflammatory processes to kill the invading pathogens. This is the innate immune system which is continually ready to respond to an invasion but has a limited repertoire of germline-encoded receptors and do not express variable receptors generated by the recombination-activating gene (RAG)\(^1\).

The other division of our immune system is called adaptive immune system. It can eliminate pathogens and is composed of highly specialized lymphocytes, T cells and B cells. These adaptive properties are possible because of genetic recombination of antigen receptor gene segments and somatic hypermutation\(^2\).

Innate lymphoid cells (ILCs) are considered to be important for the regulation of the innate immunity and to have a fundamental role in tissue remodeling. These cells lack rearranged antigen receptors when compared to lymphoid cells. The ILC family has been divided into three groups by Vivier et al. in 2013. The Group 1 includes cells that produce interferon-γ (IFNγ) and include NK cells; group 2 consist of cells that produce type 2 cytokines (for example, IL-5 and IL-13) and are dependent on retinoic acid receptor-related orphan receptor-α (RORα) and GATA-binding protein 3 (GATA3) for their development and function. Example of group 2 ILCs are the natural helper cells, monocytes and Innate helper 2 (In2) cells. Group 3 comprises cells that are dependent on the transcription factor RORγt for their development and function and have the ability to produce IL-17 and/or IL-22. Example of group 3 cells are Lymphoid tissue inducer (LTi) cells (Table 1)\(^3\).
Table 1.1 The ILC family modified from Cupedo et al (2013).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Function</th>
<th>Signature cytokine produced by cells</th>
<th>Major stimulating cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK cells (cytotoxic ILCs)</td>
<td>Innate immunity against viral infections, tumor immunosurveillance</td>
<td>Interferon-γ</td>
<td>IL-18, IL-12, and IL-15</td>
</tr>
<tr>
<td>Type 2 ILCs (ILC2)</td>
<td>Innate immunity against extracellular parasites</td>
<td>IL-5 and IL-13</td>
<td>IL-25 and IL-33</td>
</tr>
<tr>
<td>RORγt+ ILCs</td>
<td>Lymphoid tissue formation and repair, innate immunity against bacteria</td>
<td>IL-17 and IL-22</td>
<td>IL-1β and IL-23</td>
</tr>
</tbody>
</table>

NK cells were considered “non-specific” lymphocytes since their discovery in 1975, being classified as innate immune cells as they do not express variable receptors generated by genes. These cells are called natural killer cells because of this ability of directly killing a tumor cell or a virus-infected cell without previous specific activation, also known as natural cytotoxicity of NK cells. NK cells can eliminate infected or transformed cells based on the alteration or absence of MHC class I molecules (“missing self”)\. Depending on the stimulation, NK cells can kill the target cell and produce many cytokines and chemokines. On their surface, they express a wide array of variable stimulatory and inhibitory NK receptors. IL-15 is critical for development of mature NK cells and maintenance of NK cells in the periphery. This cytokine is also necessary for expansion and proliferation of NK cells in vitro.

NK cells are capable of interacting with many other immune cells. They can increase T cell and macrophage responses by releasing IFNγ; can kill immature DCs (iDCs) through engagement of NKp30 natural cytotoxicity receptor; promote DCs
maturation by releasing IFNγ and TNFα and reduce immune cells activity by releasing IL-10.

NK cells are important immune cells and understanding how NK cells are equipped for their specialized functions is important not only in terms of understanding the basic biology of these cells, but it will also contribute to the development of new treatments against cancer cells and virally infected cells.

1.1.1 Development and Distribution of NK cells

In the bone marrow (BM), CD34+ hematopoietic stem cells (HSC) will give rise to NK cells. Full maturation of NK cells can occur in the BM because it offers a mix of cytokines needed for NK cell development. Firstly, NK cells acquire the expression of interleukin-2 receptor-β (IL-2Rβ) (indicated as commitment stage in Figure 1.1). Later on, during the maturation stage they acquire the expression of NK cell receptor protein 1 (NKR-P1) molecules (which in murine is called NK1.1 and in humans is called CD161). Non-lytic NK cells are recognized by the expression of CD2 on their surface. Ability of cytolysis by NK cells is only attained when CD56 (in humans) and DX5 (in mice) is expressed. CD94-NKG2 complex is expressed on NK cells surface and to complete the maturation process, cells express MHC-specific receptors. In humans, these receptors are called killer-cell immunoglobulin-like receptors (KIRs) and in mice, they are known as Ly49. After maturation, cells can be found in the blood, all lymphoid organs (which may also be significant sites of NK cell development) and some parenchymal tissues (lungs and liver), the mechanism behind NK cells trafficking to these distinct locations has not been completely characterized. In human peripheral blood, NK cells comprise 10% – 15% of total circulating lymphocytes and may constitute up to 5% of the total lymphocyte population in the red pulp of the spleen\cite{8,9,10}.
Figure 1.1 Stages of NK cell maturation.

NK cells during the commitment stage acquire the expression of IL-2Rβ. Later on, during the maturation stage they acquire the expression of NKR-P1 molecules. Adapted from Colucci et al (2003)°.
1.1.2 Phenotype of NK Cells

Human NK cells express CD56 in their surface. It is a 140-kD polypeptide that has similar structure to neural cell adhesion molecule (N-CAM). What differentiates them from T cells is that they do not express CD3 antigen (T cell co-receptor). Human NK cells are also negative for CD4 (glycoprotein present in T helper cells, monocytes, macrophages, dendritic cells), CD14 (co-receptor that recognizes pathogen-associated molecular patterns such bacterial lipopolysaccharide), CD19 and CD20 (expressed on B-cells). Expression of CD25 (alpha chain of the IL-2 receptor), CD69 (also present in T cells and induced upon activation) and Human leukocyte antigen (HLA) class II antigens are strongly upregulated on activated NK cells.

During infections or tumor growth, macrophages and DCs can activate NK cells through the production of many cytokines like IFN-α, IFN-β, IL-12, IL-18 and IL-15. In either human or murine NK cells, these cytokines can stimulate different receptors however, it induces similar biological effects like cytotoxicity and cytokine production. Better understanding of signaling pathways in NK cells would help us to manipulate NK cells for treatment of disease. Murine and human NK cells have differences in receptor expression and recognition of proteins. For instance, murine NK cells lack the KIR family receptors while express the MHC-recognizing C-type lectin Ly49, which is not present on human NK cells. Murine and human NK cells express the NKG2D receptor complex however, murine NKG2D recognizes retinoid acid early transcript 1 (Rae-1) and the histocompatibility antigen H-60 while human NKG2D can recognize MICA, MICB, ULBP1, ULBP2, ULBP3 proteins. In humans, there are a diverse number of distinct NK cells populations with different features in many compartments of the human body. Freshly isolated human NK cells can be classified into distinct subsets based on the expression of CD56 antigen on their surface with no equivalents among the murine cells.

1.1.2.1 NK CD56dim cells and CD56bright cells subset

In peripheral blood, human NK cells can be divided into two subsets based on the level of surface expression of CD56 as measured by flow cytometry. These subsets
are called CD56\textsuperscript{dim} and CD56\textsuperscript{bright} cells. CD56\textsuperscript{dim} cells are more cytolytic, express high levels of FcyRIII (CD16) that allow them to target antibody-opsonized cells. Nearly 90\% of the total human NK cells account for CD56\textsuperscript{dim} cells. While expressing weakly or not expressing FcyRIII, CD56\textsuperscript{bright} cells account for 10\% of human NK cell in peripheral blood and are primarily found in secondary lymphoid tissues. When activated, CD56\textsuperscript{bright} cells are capable of producing cytokines, chemokines and IFN\gamma (Figure 1.2\textsuperscript{14, 15}).

The lineage link between CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells is still not clear. Recently it has become more accepted that CD56\textsuperscript{bright} are an immature form of CD56\textsuperscript{dim} cells. However, there are studies that believe that the subsets come from different origins, explaining their differences in function\textsuperscript{16}.

CD56\textsuperscript{bright} cells express constitutively high affinity IL2-R\alpha\beta\gamma (similar to antigen activated T cells); have high expression of C-type lectin CD94/NKG2 receptor family and low expression of KIR. These cells express high levels of L-lectin and C-C chemokine receptor type 7 (CCR7) and CD62L which has been shown to control the migration of immune cells to secondary lymphoid organs (e.g. lymph nodes).\textsuperscript{15, 17}

CD56\textsuperscript{bright} cells are present in human lymph nodes and Fehniger et al (2003) showed that these cells can produce IFN\gamma by interacting with T-cell-derived IL-2 which will bind specifically to the high affinity IL-2R\alpha\beta\gamma receptor, indicating that the adaptive immunity can also influence the innate immunity\textsuperscript{15}.

Maria et al (2011) stimulated NK cells subsets for 16 hours and analyzed the production of IFN\gamma. It was notable that CD56\textsuperscript{dim} cells produce IFN\gamma earlier than CD56\textsuperscript{bright} cells; however, this production was quick and not detectable after the 16 hours stimulation. This data suggests that CD56\textsuperscript{dim} cells are capable of responding quickly to stimuli, leading the innate responses after cell activation whereas CD56\textsuperscript{bright} cells are responsible for regulating the immune response in a later period pointing out an important difference between these two subsets\textsuperscript{18}. 

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Figure 1.2 CD56\textsuperscript{dim} cells vs CD56\textsuperscript{bright} NK cell subset adapted from Cooper et al.
1.1.3 NK Cell Regulation

1.1.3.1 NK cell receptors

NK cells are regulated by the combination of several signals. The cytokine microenvironment in combination with interactions with other immune cells, such as macrophages, DCs and T cells allows NK cells to be activated and release cytotoxic compounds and start a proinflammatory response\(^{19}\). NK cells surface express a wide array of variable germline-encoded stimulatory and inhibitory receptors that control activation status of the cell against target cells. Human NK cell markers and receptors are shown in Table 1.2\(^{20}\).

Detection of insufficient amount of major histocompatibility complex (MHC) 1 can be sensed by NK cells (also known as “the missing self”)\(^{21}\), that can occur in virally infected or transformed cells (Figure 1.3) The “missing-self” adapted from Lanier et al (2005).\(^{22}\) NK cell receptors can be encoded by genes clustered in the NK gene complex and includes, for example: NKG2D, NKG2A/C/E, CD94, Ly49 receptors and leukocyte receptor complex (which includes, for example KIRs). Subsets of NK cells express different receptors and they may be expressed on other cell types, particularly T cells. Various NK cell receptors specifically recognize HLA molecules like the members of the lectin family (NKG2A/C/E in association with CD94) and the Ig superfamily (toll-like receptors and KIR family of receptors)\(^{22,23}\).

The inhibitory receptors fine-tune NK-cell reactiveness based on the expression level and detection of aberrations in MHC I class molecules and is responsible for controlling self-tolerance. When a cell goes through a cellular stress, these cells downregulate the expression of MHC I molecules, which leads to NK cell recognition by the the NK cells receptors called NK cell stimulatory (or activating) receptors. The stimulatory receptors can also recognize viral protein ligands\(^{23}\).

Same mechanisms involved in the recognition of target cells by NK cells was possible with the discovery of the wide range of NK cell activating receptors\(^{24}\). NK cell activating receptors induce diverse signaling cascades in contrast to inhibitory receptors, which use the common immunoreceptor tyrosine-based inhibitory motif (ITIM)-dependent mechanism.
CD16 is an important activating receptor. It is coupled with CD3ζ and FcRγ signal transduction polypeptides and has intracytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs). Other strong NK cell receptors that are also connected to ITAM-bearing CD3ζ, FcRγ, or DAP12 activating receptors include NKp46, NKp44 and NKp30 receptors.

When the Fc portion of an antibody engages with CD16, it activates NK cells and allows NK cells to perform antibody dependent cell cytotoxicity (ADCC). Increased cytotoxicity in resting NK cells can occur with the crosslinking of CD16 receptor but not crosslinking NKp46, NKG2D, 2B4, DNAM-1, or CD2.

Table 1.2 Human NK cell markers and receptors, adapted from Montaldo et al (2013).

<table>
<thead>
<tr>
<th>NAME</th>
<th>CLUSTER OF DIFFERENTIATION</th>
<th>LIGANDS</th>
<th>EXPRESSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA-1</td>
<td>CD11a/CD18</td>
<td>ICAM-1, ICAM-2, ICAM-3</td>
<td>CD56dim NK cells</td>
</tr>
<tr>
<td>LFA-2</td>
<td>CD2</td>
<td>CD58, CD48</td>
<td>From the pNK stage on</td>
</tr>
<tr>
<td>LFA-3</td>
<td>CD58</td>
<td>CD2</td>
<td></td>
</tr>
<tr>
<td>αMβ2</td>
<td>CD11b/CD18</td>
<td>C3bi, fibrinogen, x factor, ICAM-4</td>
<td>80% of circulating NK, up-regulated upon activation (fundamental in synapse formation)</td>
</tr>
<tr>
<td>αXβ2</td>
<td>CD11c/CD18</td>
<td>C3bi, fibrinogen, ICAM-1</td>
<td>Heterogeneous expression</td>
</tr>
<tr>
<td>N-CAM</td>
<td>CD56</td>
<td>FGRF1 (stimulating maturation)</td>
<td>From the iNK stage on</td>
</tr>
<tr>
<td>Human NK-1 (HNK-1)</td>
<td>CD57</td>
<td>GlyCAM-1 MadCAM-1</td>
<td>CD56dim− NK subset</td>
</tr>
<tr>
<td>L-selectin</td>
<td>CD62L</td>
<td>CD56bright and subsets of CD56dim NK</td>
<td></td>
</tr>
</tbody>
</table>

| LIR/ILT2              | CD85j                      | HLA-I, UL-18 (CMV)            | Subsets of CD56dim NK          |
| KIR2DL1               | CD158a                     | HLA-C2                        | subsets of CD56dim NK          |
| KIR2DL2/3             | CD158b                     | HLA-C1                        | subsets of CD56dim NK          |
| KIR2DL4*              | CD158d*                    | HLA-G                         | All mature CD56dim NK?         |
| KIR3DL1               | CD158e1                    | HLA-Bw4                       | Subsets of CD56dim NK          |
| KIR2DL5               | CD158f                     | ???                           | Subsets of CD56dim NK          |
| KIR3DL2               | CD158k                     | HLA-A*03 and *11, CpG-ODN    | Subsets of CD56dim NK          |
| NKG2A/KLRD-1          | CD159a/CD94                | HLA-E                         | Heterodimer present from the iNK stage on |
| IRp60                 | CD300a                     |                               | Virtually all mature NK cells  |
| p75/AIRM1 (Siglec7)   | CD328                      | a2,8-linked disialic acid    | Virtually all mature NK cells  |
### Activating Receptors

<table>
<thead>
<tr>
<th>Name</th>
<th>Cluster of Differentiation</th>
<th>Ligands</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2Rα (high affinity receptor)</td>
<td>CD25</td>
<td>IL-2</td>
<td>CD56bright NK cells</td>
</tr>
<tr>
<td>IL-2Rββ/IL-2Rγ (intermediate affinity receptor)</td>
<td>CD122/CD132</td>
<td>IL-2 and IL-15</td>
<td>CD56bright NK cells</td>
</tr>
<tr>
<td>c-Kit</td>
<td>CD117</td>
<td>SCF (KL)</td>
<td>CD56bright NK cells</td>
</tr>
<tr>
<td>IL-7Rz</td>
<td>CD127</td>
<td>IL-7</td>
<td>CD56bright and CD56dim NK cells</td>
</tr>
<tr>
<td>CXCR1</td>
<td>CD128</td>
<td>IL8</td>
<td>CD56bright and CD56dim NK cells</td>
</tr>
<tr>
<td>CXCR3</td>
<td>CD183</td>
<td>CXCL9, CXCL10, CXCL11</td>
<td>CD56bright and CD56dim NK cells</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CD184</td>
<td>CXCL2</td>
<td>CD56bright and CD56dim NK cells</td>
</tr>
<tr>
<td>CCR4</td>
<td>CD194</td>
<td>CCL2, MIP-1β (CCL4), RANTES, CCL17, CCL22</td>
<td>CD56bright NK cells</td>
</tr>
<tr>
<td>CCR7</td>
<td>CD197</td>
<td>CCL19, CCL21</td>
<td>CD56bright NK cells</td>
</tr>
<tr>
<td>IL-18R</td>
<td>CD218a</td>
<td>IL18</td>
<td>CD56dim NK cells</td>
</tr>
<tr>
<td>ChemR23</td>
<td>CD29</td>
<td>Chemerin</td>
<td>CD56dim NK cells</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>CD333</td>
<td>Fractalkine</td>
<td>CD56dim NK cells</td>
</tr>
</tbody>
</table>

### Cytokines and Chemokine Receptors

<table>
<thead>
<tr>
<th>Name</th>
<th>Cluster of Differentiation</th>
<th>Ligands</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fas or Apo1</td>
<td>CD95</td>
<td>CD95L</td>
<td>Activated NK (causes self apoptosis)</td>
</tr>
<tr>
<td>Fas ligand</td>
<td>CD95L</td>
<td>CD95</td>
<td>Activated NK (induces target apoptosis)</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD154</td>
<td>CD40</td>
<td>Activated NK (induces target apoptosis)</td>
</tr>
<tr>
<td>TRAIL (TNF-related apoptosis-inducing ligand)</td>
<td>CD253</td>
<td>DR4 (TRAIL-R1), DR5 (TRAIL-R2)</td>
<td>Activated NK (induces target apoptosis)</td>
</tr>
</tbody>
</table>

### Other Antigens

<table>
<thead>
<tr>
<th>Name</th>
<th>Cluster of Differentiation</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAMP-1</td>
<td>CD107a</td>
<td>Marker of degranulation, expressed on the inner surface of the cytotoxic granule membrane</td>
</tr>
<tr>
<td>TNFRSF7</td>
<td>CD27</td>
<td>CD56bright cells</td>
</tr>
</tbody>
</table>
Figure 1.3 The “missing-self” adapted from Lanier et al (2005).
1.1.3.2 NK Cells and Cytokines

The homeostasis and function of NK cells is affected by a number of cytokines, including type I interferon (IFNs), IL-2, IL-12, IL-15 and IL-18. Identified initially as NK cell stimulatory factor, IL-12 induce cytotoxicity and IFNγ production by NK cells\(^{29}\). Macrophage-derived IL-12 is responsible for NK cell activation and increased production of IFNγ\(^{30}\). IL-12 in combination with IL-2 can synergize increasing the production of IFNγ. It is thought that IL-12 is capable of enhancing the high affinity IL-2 receptor subunit CD25 allowing robust IL-2 signaling in mice\(^{31}\). In combination with IL-12, IL-15 synergistically facilitates NK cell activation and achievement of effector functions\(^{32,33}\).

IL-18 has potent effect on NK cell effector functions. IL-18 is responsible for stabilizing IFNγ mRNA transcripts through the MAPK p38 pathway in NK cells\(^{34}\). It can also increase the expression of CD25\(^{35}\) and facilitate the clearance of tumor in mouse model treated with pre-activated NK cells with IL-12/15/18\(^{36}\).

Tolerant NK cells that reside in the liver and regulatory NK cells that reside in the uterus are regulated mainly by IL-10 and by the transforming growth factor beta (TGF-β) immunoregulatory cytokine\(^{37,38}\). The cytotoxicity receptors NKp30 and NKG2D have reduced expression on NK cell surface in presence of TGF-β, which leads to a reduction on NK cell function\(^{39}\). Conversely, NK cells cytotoxicity is enhanced if anti-TGF-β antibodies are used\(^{40}\). Further details regarding TGF-β are explained at section 1.4.

1.1.4 Anti-viral NK cell response

The interaction of many cell types of the innate and adaptive immune systems are required for the immune response to virus. NK cells are quickly recruited to sites of intracellular infection where they execute cytotoxic activities or cytokine secretion. NK cells not only recognize and kill virally infected cells, but they prevent viral associated inflammatory disorders through the removal of activated immune cells. NK cells recognize virally infected cells through the engagement of activating receptors with viral associated antigens. NK cells have been shown to be critical for control of cytomegalovirus, influenza virus, HIV-1 and hepatitis C virus in humans.
Limited NK cell function has been correlated to increased susceptibility to viral infections in humans. For instance, patients with immunodeficiency syndromes with impaired NK cell functions are more susceptible to infections by herpes simples virus, varicella zoster virus, cytomegalovirus (CMV) and human papilloma virus. This topic has been extensively reviewed elsewhere.

1.1.5 NK cells immunosurveillance

NK cells play important roles in cancer and in the immune response to infection, in particular viral infection as previously described (section 1.1.4). While NK cells are generally considered part of the innate immune system, evidence is emerging that they regulate and respond to the adaptive immune response where they continue to function as effector cells. NK cells can control the fate of various cells of the innate and adaptive immune system through the release of immune-modulating cytokines like IFNγ (pro-inflammatory cytokine) and IL-10 (anti-inflammatory cytokine).

NK cells recognize transformed cells via the interaction of specific ligands on the tumor cells with the NK cells receptors (section 1.1.3) in order to promote their elimination. This initial recognition is extremely important to avoid uncontrolled growth of tumor cells in the body. NK cell tumor immunosurveillance is gaining more attention as they are essential in the elimination of these transformed cells in the body.

NK cells in the tumor environment are less understood giving us a lack of knowledge of the basic biology of these cells. Understanding how NK cells in the peripheral blood distinguish from NK cells in the tumor environment is vital to comprehend NK activity and further develop new treatments. Manipulating functions of NK cells is an attractive mechanism to provide therapy for a range of disease states. Harnessing the function of NK cells has great potential as an anti-cancer therapy and modulating NK function could be beneficial for a range of autoimmune diseases and could enhance the efficacy of vaccination protocols.
1.1.5.1 NK cells and cancer

NK cells are considered the major effector cell of the innate immunity responsible for immunosurveillance with removal of spontaneously arising transformed cells in vivo. NK Studies have shown that due to the downregulation of MHC class-I and upregulation of stress antigens on the surface of the tumor cells, NK cells are able to kill these tumor target cells and that the depletion of NK cells leads to accelerated tumor growth. Cancer immunotherapy is a therapy that exploits the immune system to target tumour cells by inducing an antitumor immune response. Examples of cancer immunotherapies include administration of cytokines, monoclonal antibodies, vaccines, adoptive cell transfers (T, NK and NKT) and Toll-like receptor (TLR) agonists.\(^{45}\)

While, NK cell therapies have had some success for the treatment of hematological malignancies there has been less success in the treatment of solid tumours.\(^{46, 47}\) Indeed, NK cells found infiltrating solid tumours show decreased effector functions including decreased cytotoxicity.\(^{48, 49}\) There are many possible reasons for the observed decrease in NK function in the tumour microenvironment including the release of suppressive factors from tumour cells (e.g TGF-β)\(^{50}\) and tumour hypoxia (which can regulate NK function).\(^{51}\) However, the tumour microenvironment also becomes depleted of nutrients (glucose, amino acids) due to the rapid uptake of nutrients into cancer cells and the release enzymes that metabolize available amino acids.\(^{52, 53}\) There is good evidence that disrupting cellular metabolism can effect the functions of other immune cells such as T cells and little is known about NK cell metabolism in cancer microenvironment.\(^{54}\)

Use of NK cells for cancer immunotherapy is still an attractive model of treatment as there is evidence that high concentrations of NK cells inside tumors correlates with improved prognosis in different cancers. However little improvement has been made in the past few years. This is likely due in part to the tumor microenvironment, which has many mechanisms of resistance and immunosuppression.\(^{55}\) Further, NK cell access to solid tumors may be difficult. Tumor infiltrating NK cells exhibit higher expression of the molecule CXCR3 on their surface compared to cells of the peripheral blood.\(^{56}\) Tumor infiltrating NK cells respond robustly to malignant cells and are able to recruit more NK cells to the tumor area. However, tumor infiltrating
NK cell still have reduced activity compared to peripheral NK cells from patients\textsuperscript{55}. Down regulation of effector responses of tumor infiltrating NK cells is not completely understood. The general idea is that activator receptors are blocked by ligands released from the tumor cells (for example TGF-β, PGE2, macrophage migration inhibitory factor) which reduces the cytokine production and cytotoxic ability of NK cells. Other possibilities include that NK cells receptors are down-regulated after being chronically stimulated. Some cancers express different surface molecules that can directly block NK cells activity, for example, the glycoprotein MUC16 from ovarian cancer cells. Enzymes in the tumor microenvironment, like the indoleamine 2, 3-dioxygenase (IDO) released by different cancers like melanoma, helps the tumor cells to be protected from NK cell activity\textsuperscript{55}. Downregulation of the NKG2D receptor by cancerous cells expressing MICA has been linked to impair NK cell killing\textsuperscript{56}. Unveiling mechanisms involved in tumor downregulation of NK cells effector functions is an attractive approach to develop new cancer immunotherapy.

\textbf{1.1.5.2 NK cell immunotherapy}

For cancer patients, immunotherapy is becoming one of the major therapeutic options. Immunotherapy consist of many categories: use of cytokines, use of immune checkpoint inhibitors, vaccines, antibodies and also the use of \textit{in vitro} expanded and trained cells\textsuperscript{57, 58}.

The knowledge that T cells could be used to treat murine tumors started in the 1960s. Since then, T cells have been largely investigated to be used as treatment against cancer. Usually, tumor-infiltrating lymphocytes from the patient are expanded \textit{in vitro} in the presence of IL-2. When pure cultures are obtained they can be tested for reactivity against the original tumor and infused back into the patient. Tumor-infiltrating lymphocytes from melanoma have given best results against the original tumor and more work is required for other solid cancers\textsuperscript{59}.

In the effort to improve the T cell responses against cancer and expand its capacity of recognizing different malignant cells, the use of gene modifications started to develop. T cells can be manipulated \textit{in vitro} and genes encoding naturally occurring T cell receptors or chimeric antigen receptor (CAR) against a specific antigen can be
introduced in cell DNA. Challenges include selecting the best genes to be introduced and the best T cell subpopulation in which to perform these genetic modifications. Another concern is to develop cells with reduced or no targeting of normal cells and that show low toxicity to the patient.\textsuperscript{59}

NK cells have a variety of properties that allow them to become great tools for the development of new treatments against cancer.\textsuperscript{58} NK cells eliminate tumor cells without the need to recognize specific antigens as is the case with T cells, which require presentation, an advantage over these cells. Research on NK cells activation, inhibition and how these cells respond to cytokines opens doors to further improvements on adoptive cell therapy or allogeneic stem cell transplantation.\textsuperscript{58} Expansion of specific NK cells, for example, expressing a single KIR receptor on the cell surface to be used in cancer therapy has shown advantageous results. Methods on how to obtain the purest NK cell population \textit{in vitro} is being refined.\textsuperscript{58, 60} Use of drugs or antibodies to reduce expression of inhibitory receptors on NK cell surface, antibodies that increase NK cell effector functions and drugs that modify tumor cell receptors responsible for down-regulation of inhibitory receptors or up-regulation of death receptors on NK cells show positive outcomes. To further increase NK cell performance during treatment, scientists have suggested that use of adoptive cell transfer combined with drugs or antibodies would benefit patient outcome.\textsuperscript{61}

Many years of NK cell research and their transplant into patients indicated that NK cells experience weak responses and normally do not last long in the blood stream. In an attempt to increase NK cell function in order to enhance the patient recovery, scientists started culturing murine splenocytes or PBMC with the cytokine IL-2.\textsuperscript{62} Cells that quickly eliminated tumor cells were developed and shown positive effects in murine models, however, when this technology was transferred to humans the efficacy was limited and many side effects were observed.\textsuperscript{62, 63} In addition to side effects, IL-2 used in high doses induces Treg cells expansion which can limit the IL-2 availability and release of TGF-β, a well-known NK cell inhibitor.\textsuperscript{62, 64, 65} In addition to IL-2, the cytokine IL-15 became important in the development of the adoptive transfer technology. IL-15 enhances NK cell survival \textit{in vivo} and together with IL-2 may induce NK cell expansion and survival.\textsuperscript{62}
In order to improve NK cell responses, a combination of different cytokines to pre-stimulate NK cells started to be tested. The discovery that NK cells show “memory” upon stimulation with cytokines led researchers to develop new cell culture systems and in vivo experiments to test NK cells improvements

Jing et al demonstrated that in murines when NK cells were pre-treated for 16 hours with IL12 (10ng/mL)+IL-15 (20ng/mL)+IL-18 (100ng/mL) they had better IFNγ production, better killing of cancer cells prior and after transferring into mice. These pre-activated cells also showing high levels of demethylation on CNS1 in the Ifnγ locus after transfer (a crucial Ifnγ gene transcription enhancer in Th1 cells). IL-15 (50 ng/mL) pretreated NK cells presented weaker responses, reduced IFNγ production and no demethylation on CNS1. Interestingly, the authors identified CD4+T cells and IL-2 to be important in the enhancement of IFNγ production by the cytokine pre-treated NK cells, interestingly this enhancement was not linked to demethylation of CNS1. The stimulation with IL12/15/18 also increases expression of CD25 and STAT5 which also binds to CNS1 facilitating IFNγ production. Taken this data together, stimulation with IL12/15/18 activates STAT4 (which have been shown to assist DNA demethylation by limiting the recruitment of DNA methyltransferase) and STAT5 that induces Ifnγ transcription and also epigenetically modulate the Ifnγ locus.

Besides using cytokines to increase NK cells activation, studies on how to reverse the negative effect of inhibitory cytokines on NK cell functions have been done as well. Recent studies on adoptive cell treatment are focus on inhibiting TGF-β activity in culture to increase NK cell activity prior to return these cells to the patient. Recently it was published that expanding human NK cells with EW-7197, a TGF-β ALK5 receptor inhibitor, in vitro for 21 days increased NK cell cytotoxic and reduced tumor size in vivo when transferred to mice with liver metastasis. A similar approach was done to treat neuroblastoma. Adoptive cells were treated with galunisertib that inhibits TGF-β receptor 1. Expanded NK cells treated with galunisertib were transferred with anti-disialoganglioside (anti-GD2) mAB dinutuximab (GD2 is highly expressed on neuroblastoma cells) to a mouse model of neuroblastoma. These expanded cells showed a significant recovery of the mice.
These studies are really promising and hopefully in the future they will be assessed in humans to evaluate safety and efficacy.

Recently, Rouce et al identified TGF-β1 as an important suppressor of NK cell activities in children with B-cell acute lymphoblastic leukemia. In these patients, NK cells expressed more inhibitory receptors than activating receptors which facilitated blasts to escape NK cell immunosurveillance. The group co-cultured NK cells from healthy donors with leukemia blasts. Healthy cells stopped producing IFNγ and had high SMAD2/3 activity. To confirm cell impairment was due to TGF-β released by the blasts, NK cells from healthy donors were co-cultured with a TGF-β blocking antibody and function was restored and by ELISA it was confirmed that these cells produce large quantity of TGF-β1. The researchers also evaluated SMAD2/3 activity in patients and it was significantly higher compared to healthy controls. Patients at remission had NK cells partially recovered their phenotype and function associated with normalization of SMAD2/3.

The discovery that cellular metabolism can impact on NK cell effector functions means that it is a new unexplored avenue for improving NK cell based immunotherapies.

1.2 Immunometabolism

Immunometabolism is the new emerging scientific field combining immunology and metabolism. Crucial metabolic changes in pro-inflammatory immune cells are required for the normal effector functions and differentiation of cells. During increase of metabolism, specific metabolites can be made fulfilling the requirements of the activated immune cell. Cells require the energy-carrying molecule adenosine triphosphate (ATP) for cellular processes. Generation of ATP comes from glycolysis and OxPHOS pathways. During glycolysis, glucose is metabolized to pyruvate in the cytosol and generates two molecules of ATP. Pyruvate can be further metabolized in the mitochondria to CO₂, NADH and FADH₂ by the TCA cycle. OxPHOS and ATP synthase activity are induced generating up to 34 ATP per molecule of glucose. Intermediates generated during glycolysis are used in different pathways for synthesis of biomolecules that support biosynthetic processes. For example,
glucose-6-phosphate (G6P) generated in the beginning of glycolysis can enter the pentose phosphate pathway (PPP) pathway that will further synthesize nucleotides. G6P also generates NADPH, a cofactor essential for lipid synthesis, nucleic acids and other biosynthetic processes\textsuperscript{71}. Glucose that is converted into cytoplasmic acetyl-CoA via citrate in the TCA cycle generates cholesterol and fatty acids. Large quantities of glucose-derived carbon are produced and secreted from the cell in the form of lactate. Lactate is important for increased rates of glycolytic flux and regeneration of NAD\textsuperscript{+}, which is essential for the sixth step of glycolysis\textsuperscript{71}.

1.2.1 Different metabolism profiles of immune cells

Pro-inflammatory immune cells (M1 macrophages, CD8\textsuperscript{+} cytotoxic T cells, Th1/Th17 CD4 T cells, activated dendritic cells) increase glucose uptake and the levels of glycolysis and the production/secretion of lactate – a metabolic signature that is termed aerobic glycolysis (glucose metabolised to lactate despite the presence of abundant oxygen)\textsuperscript{72}. In contrast, regulatory and memory immune cells (Tregs, M2 macrophages, CD8\textsuperscript{+} memory T cells) do not utilize elevated levels of glycolysis but instead rely upon OxPHOS metabolism\textsuperscript{72}. A summary of the metabolic signatures of different lymphocyte subsets was recently published by our laboratory with different signaling molecules that promote or inhibit metabolism in these cells (Figure 1.4)\textsuperscript{73}.

Pro-inflammatory immune cells engage in aerobic glycolysis as the elevated glucose uptake and glycolytic flux generates abundant glycolytic intermediates that can be shuttled into various biosynthetic pathways (e.g. PPP) to generate amino acids, nucleic acids, fatty acids, i.e. the building blocks for macromolecular synthesis\textsuperscript{73}. Thus, a glycolytic metabolism allows pro-inflammatory immune cells to meet the energy and biosynthetic demands placed upon them by large scale cytokine production, chemokine production, proliferation, migration and the production of various other effector molecules (e.g. perforin and granzymes)\textsuperscript{73}. Progress has also been made in revealing the molecular mechanisms underpinning the regulation of immune cell metabolism. For example, in CD8\textsuperscript{+} cytotoxic T cells (CTL), the mammalian Target of Rapamycin (mTORC1)/Hypoxia-inducible Factor 1\textalpha\ (HIF1\textalpha) signaling axis is critical in maintaining CTL metabolism and function. In the absence
of mTORC1 signaling or in the absence of HIF1α expression, CTL cannot sustain elevated glucose uptake and glycolysis and so do not maintain the tools required to migrate and kill target cells (virally infected cells or tumour cells). Therefore, it is becoming clear that cellular metabolism is closely linked to the effector functions of immune cells.

Advances in the field of metabolism have shown the importance of metabolic pathways for immune cell function. These metabolic changes, cell fate and function can be potentially manipulated and used, for example to improve anti-cancer immunotherapy.
Figure 1.4 Different metabolic changes occur in lymphocytes based on cell fate.

Pro-inflammatory immune cells like Th1, Th17, Th2, CTL and activated NK cells depend on aerobic glycolysis for the effector functions. Conversely, Treg, memory T cell rely on lipid oxidation to maintain their normal functions. Signaling molecules that promote metabolism are in green and the molecules that inhibit metabolism are in red. Figure adapted from Donnelly et al\textsuperscript{73}. 

\textsuperscript{73} Donnelly et al.
1.2.2 NK cell metabolism

Until relatively recently, little was known about metabolism within NK cells. NK cells share many attributes of CTL. NK cells and CTL are both responsive to similar cytokines that control their function (IL-2, IL-12, IL-15, IL-18), produce the effector cytokines Interferon γ (IFNγ) and Tumour necrosis factor α (TNFα) and engage in the direct killing of virally infected cells and tumour cells. Our group hypothesizes that NK cell metabolism, like that of CTL, might be dynamically regulated and be closely linked to NK cell effector functions.

Our lab has demonstrated that glycolysis and OxPHOS is required for murine NK cell effector functions. One key molecule identified for metabolic control of NK cells is the mammalian target of rapamycin complex 1 (mTORC1) and this will be further discussed in detail in this thesis. Most studies to date have been performed in murine NK cells and this work was, to our knowledge, the first detailed study on human NK cell metabolism.

1.3 Mammalian target of rapamycin (mTOR)

In order to preserve cellular homeostasis and metabolism, cells have mechanisms to control anabolic and catabolic pathways depending on the availability of nutrients and energy. The mammalian target of rapamycin (mTOR), is an evolutionarily conserved serine/threonine protein kinase that acts as a molecular sensor of the cellular microenvironment. mTOR sense environmental signals and translate them into appropriate cellular responses. Discovery of mTOR signaling advanced after discovery of rapamycin. Rapamycin is an anti-fungal macrolide with properties of blocking cell growth and proliferation extracted from the bacterial species Streptomyces hygroscopicus found in the soil of Easter Islands in the 1970s. Inhibition of mTORC signaling and activity takes place when rapamycin binds to the FKBP12 endogenous cellular protein, this complex binds from the N-terminal to the C-terminal of mTOR kinase domain.75, 76
1.3.1 mTOR complexes and signaling pathway

mTOR interacts with many proteins forming mainly two distinct signaling complexes which are defined as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is a nutrient-sensitive pathway. It promotes anabolic cellular metabolism (lipid and nucleotide synthesis), suppresses catabolic processes, suppresses autophagy, is sensitive to rapamycin in acute treatments and is associated with at least five major signals (growth factors, genotoxic stress, energy status, oxygen and amino acids) (Figure 1.5). Rapamycin is widely used as a specific mTORC1 inhibitor in different studies. Regulation of energy metabolism and many immune responses are linked to mTORC1 activity. Adverse conditions e.g. lack of nutrients or growth factors or cellular stress (like hypoxia, low energy, ER stress, and reactive oxygen species – ROS) leads to the reduction of mTORC1 activity. Some of the known mTORC1 substrates are S6K, 4EBP, IRS-1, ULK1, Lipin1, TFEB and Grb10. What is well established is that, when activated, mTORC1 phosphorylates the translation-initiation factor eIF4E-binding protein 4EBP1 and the S6 ribosomal kinase (S6K) for cell proliferation, protein synthesis and cell growth. Studies shown that with the treatment of rapamycin, mTORC1 is unable to phosphorylate S6K1 (p70 ribosomal protein S6 kinase 1) and able to only partially phosphorylate 4EBP1 (eukaryotic initiation factor 4E binding protein 1). This shows that mTORC1 possibly has a different substrate access to the kinase active site.

Gene expression may be controlled by mTORC1. It has been shown that mTORC1 is involved in the control of transcription factors important for the glycolysis like HIF-1α and c-Myc. Transcription factors important for regulation of the Sterol regulatory element binding protein (Srebp) like SREBP1 and PPARγ (which are responsible for driving the expression of enzymes within the cholesterol and fatty acid synthesis pathways) have also been shown to be regulated by mTORC1.

mTORC2 is controlled by growth factors and upstream signaling is relatively undiscovered (Figure 1.5). It is capable of regulating metabolism, cell survival and organization of the actin cytoskeleton by phosphorylating Akt, SGK1, and PKCα. If mTORC2 is inactivated, mitochondrial metabolism, cell survivor and mitochondrial-associated endoplasmic reticulum membrane integrity are reduced. mTORC2 is not sensitive to rapamycin when treated for short time with
this inhibitor; however, mTORC2 can be suppressed and its function compromised when treated for a long time because of the disruption of the complex integrity. 

81.
Figure 1.5 mTORC1 and mTORC2 signaling pathways. Adapted from Saxton et al.82.
1.3.2 mTORC1 regulation of immune cell metabolism

mTORC activity has been of interest for more than 2 decades since its discovery. Shown to be a key regulator of cell growth and proliferation, only recently the role for mTORC in cell metabolism has started to be investigated. Complex metabolic changes to provide optimal effector functions of immune cells are dependent of mTORC1. mTORC1 promotes lipid synthesis by regulation of transcription factor sterol regulatory element binding protein (SREBP) required to control the glycolytic enzyme glucose-6-phosphate dehydrogenase (G6PDH) necessary for generation of RNA and DNA synthesis, along with generation of NADPH required for lipid synthesis and growth. mTORC1 regulates cell metabolism shift from OxPHOS to glycolysis necessary to generate biomolecules for cell growth and effector function as discussed previously in this thesis. mTORC1 regulates HIF1-α and cMyc which are important regulatory transcription factors of glucose metabolism. mTORC1 senses endoplasmic reticulum stress which activates transcription factors that controls metabolic homeostasis. mTORC1 also senses energy stress and controls mitochondrial oxidative function through PGC1-α transcription factor responsible for regulation of mitochondria biogenesis.

1.3.2.1 mTORC1 activity and NK cells

mTORC1 activation through IL-15 cytokine was shown to be important for murine NK cell development in the bone marrow and function in the periphery. NK cells lacking mTORC1 had smaller size, reduced glucose uptake and loss of the receptors CD71 and CD98 compared to the normal cells. These changes were regulated transcriptionally and were related to impaired early NK cell development.

Our laboratory has demonstrated that mTORC1 in murine NK cells is a key regulator of glucose metabolism. NK cells stimulated with cytokines increased mTORC1 activity and the production of effector molecules like IFN-γ and granzyme B were dependent on mTORC1. mTORC1 activity was also necessary to maintain an elevated glycolytic state. Other publications confirmed this finding, showing that murine NK cells exposed to high dose of IL15 have higher expression of mTOR and increased metabolism, and without mTORC1 activity cells, were unable to sustain effector functions.
Most of this studies were performed on murine NK cells and mTORC1 activity in human NK cells has not been well characterized.

**1.4 The immunoregulatory protein TGF-β**

The immune system has the ability to recognize and distinguish our body's own tissues from pathogens and maintain the body healthy. There are diverse mechanisms that control the equilibrium in the human body\(^8^6\). Negative regulation has a central role and is needed to be able to shut down immune responses so as not to cause pathology or harm. Different molecules have roles in downregulating cell function such as interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and programmed cell death 1 (PD1). Not only T\(_{\text{reg}}\) cells have the ability to produce suppressive molecules, other cells like regulatory B cells, myeloid-derived suppressor cells also have regulatory functions\(^8^6\).

The TGF-β superfamily is evolutionarily conserved and there are more than 33 distinct proteins that play important roles in cell growth and differentiation. TGF-β superfamily ligands include TGF-βs, activins, inhibins, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), anti-mullerian hormone (AMH), and nodal growth differentiation factor (NODAL). Seven type I (i.e., ACVRL1 (ALK1), ACVR1 (ALK2), BMPR1A (ALK3), ACVR1B (ALK4), TGFBR1 (ALK5), BMPR1B (ALK6), and ACVR1C (ALK7)) and five type II receptors (i.e., TGFBR2 (TGFβRII), ACVR2 (ACTRII), ACVR2B (ACTRIIB), BMPR2 (BMPRII), and AMHR2 (MISRII)) have been identified\(^8^7\).

TGF-β is a potent immunoregulatory protein and it is involved in many cellular processes like controlling differentiation, proliferation, apoptosis, recognition, migration, wound healing and activation of immune cells. It is produced by leukocytes like lymphocytes, macrophages, dendritic cells and NK cells. In mammals, TGF-β family is comprised of three members (TGF-β1, TGF-β2, TGF-β3) and TGF-β1 is mostly expressed by hematopoietic cells. TGF-β2 and TGF-β3 are considered to be involved in development and hormonal signals and not strongly important in immune responses although recent report have shown that TGF-β3 can regulate B cells\(^8^8, 8^9, 9^0\).
When synthesized, TGF-β is inactive and is only able to bind to receptors after going through acidification and changes on the latency-associated protein (LAP) structure. TGF-β signaling pathway is highly conserved between species which covers the phosphorylation of TGF-β type II receptor (TβRII) and consequently phosphorylation of the cytoplasmic domain of the TGF-β type I receptor (TβRI). Further, Smad2 and Smad3 proteins are phosphorylated and become a complex that binds to the Smad4 and translocate into the nucleus where can bind to DNA acting as transcription factors activating or inactivating particular genes. This pathway is known as the canonical signaling pathway (Figure 1.6)\(^{88}\).

There are also other TGF-β pathways that do not use Smads signaling molecules, which might induce responses unrelated to transcription. Examples of non-smad pathway are: TAK1 (TGF-β associated kinase 1), Erk (extracellular signal-regulated kinase), p38 MAPK (mitogen-activated protein kinase), and Akt which further signal through the mTOR pathway\(^91\). However, it has been shown that activation of Erk and JNK pathways through TGF-β can phosphorylate and regulate Smad. Also, activation of Ras/Erk MAPK signaling can increase the expression of TGF-β1, thus increasing the TGF-β response. The opposite is also possible, Smads can interfere with this signaling. For example, Smad6 can downregulate TAK1 activity and Smad7 can induce and sustain JNK activation. These differences in pathways indicate that some cellular responses to TGF-β implicates an equilibrium between activation of Smads and MAPK pathways (Figure 1.7)\(^92\).
The latent TGF-β complex circulates in the plasma, upon activation, it specifically binds to type II (TβRII) and type I (TβRI) receptors. Type II receptors then transphosphorylate the type I receptors, which propagate the signal into the cell by phosphorylating TGF-β receptor-specific SMADs (R-SMADs: SMAD2 and SMAD3). They form heteromeric complexes with the common mediator SMAD (co-SMAD: SMAD4) and translocate to the nucleus. Once in the nucleus, the R-SMAD–co-SMAD complex preferentially associate with the genomic SMAD-binding element (SBE) in a sequence-specific manner. The nuclear proteins SKI and SNO (also known as SKIL) antagonize the transcriptional regulation by SMADs. An inhibitory SMAD (I-SMAD), SMAD7, inhibits the TGF-β pathway through multiple mechanisms, including by mediating the degradation of the type I receptor, inhibiting phosphorylation of R-SMADs by the type I receptor kinase or inhibiting the formation of the R-SMAD–co-SMAD complex. In addition to regulating transcription, R-SMADs can modulate microRNA (miRNA) biogenesis by facilitating the processing of primary miRNA into precursor miRNA in the nucleus. The co-SMAD is not required for the regulation of miRNA biosynthesis by R-SMADs. 'mG' and 'AAAAA' represent 5′ capping and 3′ polyadenylation of mRNAs, respectively. Adapted from Akhurst et al.\textsuperscript{93}.
Figure 1.7 The non-canonical pathway of TGF-β signalling.

In the non-canonical pathway, the activated TGF-β receptor complex transmits a signal through other factors, such as TNF receptor associated factor 4 (TRAF4) or TRAF6, TGFβ-activated kinase 1 (TAK1), p38 mitogen-activated protein kinase (p38 MAPK), RHO, phosphoinositide 3-kinase (PI3K)–AKT, extracellular signal-regulated kinase (ERK), JUN N-terminal kinase (JNK) or nuclear factor-κB (NF-κB). TGF-β signalling can be influenced by pathways other than the canonical and non-canonical TGF-β signalling pathways, such as the WNT, Hedgehog, Notch, interferon (IFN), tumour necrosis factor (TNF) and RAS pathways. The crosstalk between TGF-β and other pathways defines the activities of TGF-β to propagate spatial- and temporal-specific signals. miRNA, microRNA; ROCK, RHO-associated protein kinase; R-SMAD, receptor-specific SMAD; TβR, TGFβ receptor. ‘mG’ and ‘AAAAA’ represent 5′ capping and 3′ polyadenylation of mRNAs, respectively. Adapted from Akhurst et al.93.
1.4.1 TGF-β and the immune system

TGF-β is involved in many cellular processes and most immune cell types can be regulated by TGF-β (Figure 1.8). It is not difficult to predict that whenever TGF-β is over expressed or its activity is impaired this can lead to complications in the body. Indeed, TGF-β is frequently found overexpressed in countless diseases e.g. cancer, fibrosis and inflammation. TGF-β promotes changes in cell phenotype, cell growth and migration promoting disease progression. TGF-β is a complex cytokine which can behave differently in distinct cell types and different cell culture conditions can affect TGF-β activity. TGF-β expression differs among diseases and disease stages and also the genetic divergence between individuals play an important role when considering studying this molecule. Many drugs with the properties of inhibiting TGF-β signaling directly, or monoclonal antibodies and inhibitors of gene expression are being developed and even reaching Phase III clinical trials for treatment of fibrosis and cancer\(^93\).

Recent work in the immunology field shows immune cells depend on metabolism not only for survival but for their function therefore, investigation of the effects of TGF-β on cell metabolism is really promising not only for a better understanding on how TGF-β alter cells function but also possible identification of new drug targets\(^94\).

Recent studies indicate that TGF-β regulates mitochondrial bioenergetics and oxidative stress responses\(^95\). TGF-β is found in high levels in adipose tissue during morbid obesity, cardio vascular disease, hypertension, stroke, cancer, diabetes which are all conditions where mitochondria metabolism is impaired. Yadav et al shown that TGF-β inhibits PGC-1α which is a master regulator of mitochondria biogenesis\(^96\). TGF-β and SMAD3 are linked to reduce glucose and insulin tolerance in obesity and type 2 diabetes. TGF-β can also reduce anti-oxidant enzymes (e.g. superoxide dismutase-2) and reduce cytochrome C activity indicating that TGF-β is linked to suppression of mitochondria function and biogenesis\(^95\). TGF-β induces mitochondria fragmentation but the molecular mechanism is unknown\(^95\).
Figure 1.8 TGF-β and its effects on different immune cell types. (adapted from Akhurst et al)⁹³.
1.4.2 TGF-β and NK cells

It is long known that TGF-β impairs NK cell effector functions in human and in mice. TGF-β can be detected in high concentrations in some cancers and chronic diseases, which may explain the reduced activity of NK cells in these conditions\textsuperscript{37, 93, 97}. Inhibiting TGF-β signaling and reducing its immunosuppressive effects on NK cells is an important niche of study which is constantly being investigated. Thirty years ago exogenous TGF-β has been shown to directly inhibit NK cell cytotoxicity and reduce IFNγ production\textsuperscript{37}. NK cells can produce TGF-β and the inhibition of the action of endogenous TGF-β resulted in increased IFN-γ production\textsuperscript{98}. TGF-β also inhibits NK cell perforin polarization to the immune synapse and NK cell proliferation, development and differentiation\textsuperscript{64}. Furthermore, many NK cell receptors are downregulated by TGF-β e.g. NKG2D, NKG2C, NKp30 and NKp44 while PD-1 (inhibitory receptor) is upregulated\textsuperscript{70, 99, 100}. The canonical TGF-β pathway has been shown to be important on downregulation of NK cell effector functions. For instance, inhibition of both ADCC and CD16 mediated IFNγ production were dependent of SMAD3 signaling\textsuperscript{101}. Furthermore, IFNγ downregulation by TGF-β is restored by inhibiting the canonical pathway in different \textit{in vitro} models\textsuperscript{68, 69}.

TGF-β affects human NK cell differentiation by inhibiting hematopoietic progenitors of NK cells. Interestingly, TGF-β converted CD56\textsuperscript{bright}CD16\textsuperscript{+} NK cells into CD56\textsuperscript{bright}CD16\textsuperscript{−} cells in peripheral blood, and the use of an anti-TGF-β antibody, allowed cells to preserve CD16 expression. CD56\textsuperscript{dim} cells did not had their CD16 expression changed, indicating that TGF-β affects more CD56\textsuperscript{bright} CD16 expression, however both subgroups had reduced proliferation\textsuperscript{99}.

TGF-β has been shown to downregulate NK cells in many diseases. For instance, HIV infected patients have higher TGF-β in their blood stream. In these patients, TGF-β promotes NK cell apoptosis by downregulation of the protein Bcl-2, which is an antiapoptotic protein\textsuperscript{102}. Lee et al shown that TGF-β1 was present in high concentrations in serum taken from cancer patients (lung cancer and colorectal cancer). The presence of TGF-β1 strongly inhibited NKG2D expression\textsuperscript{103}. Shaim et al also shown that the contact of glioblastoma cells with NK cells resulted in release
of TGF-β by the glioma cells which reduced NK cell activity through the SMAD pathway\textsuperscript{104}.

Moreover, it has been suggested that TGF-β inhibits murine NK cell metabolism through the mTORC pathway. NK cell impairment promoted by rapamycin and TGF-β presented similar outcomes, reduced IFNγ production and decreased NK cell killing\textsuperscript{105}.

Much research has been done on NK cells and TGF-β, however nothing was known about TGF-β effects on metabolism of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells.
Aims

The overall goal of this work was to understand metabolic changes that human NK cells undergo in response to cytokine exposure. Our hypothesis was that upon activation, human NK cells undergo metabolic reprogramming which is required for optimal NK cell effector functions.

Specific aims by chapter:

**Chapter 3 Metabolic changes in cytokine stimulated NK cells**

1. Investigate changes in NK cell metabolism after cytokine stimulation
2. Identify metabolic differences between CD56\textsuperscript{bright} NK cells and CD56\textsuperscript{dim} NK cells
3. Define the importance of mTORC1 activity in human NK cells
4. Investigate the importance of metabolism for optimal NK cell effector function

**Chapter 4 Investigation of the effects of TGF-β on human NK cell metabolism and function**

1. Assess the impact of TGF-β on NK cell metabolism
2. Determine if TGF-β inhibits mTORC1 activity
3. Investigate the importance of TGF-β canonical pathway in human NK cells

**Chapter 5 Investigating the impact of prolonged metabolic disruption on IL-2 induced metabolism and function in human NK cells**

1. Investigate the impact of longer term (5 day) stimulation with IL-2 on NK cell metabolism
2. Determine the requirement of mTORC1 activity for sustained metabolic and functional activation of human NK cells
3. Explore the effects of TGF-β on metabolism in long term stimulated NK cell
4. Investigate if TGF-β inhibits NK cell function and metabolism through the canonical pathway
Chapter 2

Materials and Methods
2.1 Materials

2.1.1 Chemicals
Dulbecco’s phosphate buffered saline (PBS), 2-Deoxy-D-glucose (2-DG), glucose, galactose, oligomycin, rotenone and antimycin A were purchased from Sigma, Ireland. Trypan Blue solution and rapamycin from Fischer Scientific. Glucose-free medium, RPMI medium 1640 GlutaMax®, Penicillin-Streptomycin solution, Gibco® Minimum Essential Medium (Mem) vitamin solution (100X), Insulin-Transferrin-Selenium-G Supplement (100X) were purchased from Biosciences. IL-15 and IL2 were purchased from National Institutes of Health (NIH, USA). IL-12 and human NK cell purification kit were purchased from Miltenyi Biotech, UK. Foetal Bovine Serum (FCS) was purchased from Labtech International. Golgi plug, Cytofix/Cytoperm reagent and Celltak® were purchased from BD Pharmingen, Ireland. Methanol and Absolute Alcohol were purchased from the school stores. Seahorse Media was purchased from Agilent Technologies, USA. ATP assay determination kit was purchased from Perkin Elmer, Ireland.

2.1.2 Equipment
Pipettes, pipette tips, filtered tips, tissue culture dishes (6, 12, 24 and 48 well), cell scraper and tissue culture flasks were obtained from Fisher. FlowJo software was obtained from TreeStar. XF 24-well microplate was from Agilent Technologies. LS Macs purification columns and quadroMacs cell separator were from Miltenyi Biotec. Grant JB Series Water Bath was obtained from Amlab Services. Steri-Cycle CO2 Incubator from Thermo Forma. Heraeus Fresco 21 and Heraeus Pico 17 Centrifuges from Thermo Scientific. Legend RT Centrifuge from Sorvall. 1500 Standard Fumehood from Phoenix Controls Corporation. Bioair Topsafe Fumehood from Crowthorne HiTec Services. Bright-Line Hemacytometer from Hausser Scientific.
2.1.3 Antibodies

Antibodies used for flow cytometry: CD56(HCD56/NCAM16.2) CD3(SK7/UCHT1) Granzyme B(GB11); IFNγ(B27); CD71(M-A172); CD69(L78); CD98(UM7F8); NKp44(p44-8.1); TRAIL(RiK-2); CD107a(H4A3) (eBioscience and BD Pharmingen). Glut1 RBD ligand (Metafora Biosystems), pS6 ribosomal protein phosphorylated on serine 235/6 and 4EBP1 ribosomal protein phosphorylated on threonine 37/46 (Cell Signaling Technologies), ATP5B (35D) (Abcam).

2.2 Methods

2.2.1 Cell culture

Blood samples were obtained from normal healthy donors from whom written consent had been obtained. Ethics for this study was provided by the Research Ethics Committee of School of Biochemistry and Immunology in Trinity College Dublin and by the committee of St. James Hospital, Dublin, Ireland. Peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep (Axis-Shield) gradient. 15 mL of Lymphoprep was added into a 50 mL Falcon tube and 35 mL of diluted blood (1:2) in PBS was added to the tube and centrifuged for 30 minutes at 1200 RPM. After the centrifugation, the buffy coat was removed using a Pasteur pipette and transferred to new tubes. The collected cells were subjected to red blood cell lysis then washed with PBS (centrifuged for 10 minutes at 300g) and resuspended in media for counting. Cell viability was assessed by trypan blue exclusion.

Cell culture for chapter 3: Unless stated otherwise, 5x10^6 cells/ml PBMC were incubated at 37°C for 18 hours in RPMI 1640 Glutamax medium (Gibco, Invitrogen) supplemented with 10% FCS, 1% Penicillin/Streptomycin (Invitrogen) and with IL2 (500U/ml) or IL12 (30ng/ml) and IL15 (100ng/ml). Where indicated cells were cultured +/- the inhibitors rapamycin (20nM) or oligomycin (40nM) or galactose (10 mM) was substituted for glucose (10 mM).

Cell culture for chapter 4: Unless stated otherwise, 5x10^6 cells/ml PBMC were incubated at 37°C for 18 h in RPMI 1640 GlutaMAX medium (Life Technologies, Invitrogen) supplemented with 10% FCS, 1% penicillin/streptomycin (Invitrogen),
and with IL-2 (500 U/ml, NCI or IL12 (30 ng/ml; Miltenyi) and IL15 (100 ng/ml; NCI). In some experiments, lower concentrations of IL2, IL12 and IL15 were used, as indicated in the relevant figures. Also, cells were cultured with or without TGF-β (10ng/mL; R&D Systems), SB431542 (5µM; Sigma), Rapamycin (20nM; Fisher) and AZD8055 (1 µM; Source Biosciences, UK).

Cell culture for chapter 5: PBMCs were incubated at 37°C for 5 days in RPMI 1640 Glutamax medium (Gibco, Invitrogen) supplemented with 10% FCS, 1% Penicillin/Streptomycin (Invitrogen) and with IL2 (100U/ml), IL15 (1ng/ml) as survival factor, rapamycin (20nM), TGF-β (10ng/mL), SB431542 (5µM). After 5 days, cells were counted with trypan blue, resuspended at 5x10^6 cells/ml and incubated at 37°C for 18 hours with or without IL12 (30ng/ml) and IL15 (100ng/ml). Where indicated cells were cultured +/- the inhibitors rapamycin (20nM), TGF-β (10ng/mL), SB431542 (5µM). Where indicated, after the 5 day cultures, cells were stimulated for 4 hours with PMA (50ng/mL, Sigma) and Ionomycin (Ion) (1 µg/mL, Sigma). For metabolism experiments, NK cells were purified using an NK isolation Kit II (Miltenyi Biotec) as per manufacturer’s instructions; purity was routinely >95% CD56+CD3- NK cells.

2.2.2 Flow Cytometry analysis

2.2.2.1 Surface and intracellular staining
Cells were stained for 30 minutes at 4°C with saturating concentrations of titrated antibodies CD56(HCD56/NCAM16.2) CD3(SK7/UCHT1) Granzyme B(GB11); IFNγ(B27), CD71(M-A172); CD69(L78); CD98(UM7F8); NKp44(p44-8.1); TRAIL(RiK-2); CD107a(H4A3) (eBioscience or BD Pharmingen). Analysis was performed using the gating strategy for live cells according to their forward scatter and side scatter (Figure 2.1), acquired by FACS Canto or Fortessa (Becton Dickinson) and FlowJo software (TreeStar).
2.2.2.2 Phospho-S6 and 4EBP1 ribosomal protein staining
Following stimulation cells were washed in FACS buffer and stained for 20 minutes for surface staining (CD56 and CD3). After being washed again, cells where fixed and permeabilized with the anti-phospho-S6 ribosomal protein Ser 235/236 (Cell Signalling Technologies) for 30 min at RT. Cells were washed and data acquired on a FACSCanto flow cytometer. As a negative control, rapamycin (20nM, 20 min, and 37°C) was used to inhibit S6 phosphorylation.

2.2.2.3 Glut1 receptor staining
Cells were transferred to FACS tube with cap, centrifuged at 300g for 3 minutes, resuspended at 10% of human AB serum block buffer was added for 10 minutes at 4°C (100 µL). Centrifuge cells at 300g for 3 minutes and resuspend in 200 µL Peptide Master Mix (Glut1 RBD ligand - Metafora Biosystems) which was used 4 µL peptide in 200 µL of Facs Buffer. Cells were incubated at 37°C for 30 minutes (shake the tubes at 15 minutes). Cells were washed twice with washing buffer (1x PBS + 2% FCS). Secondary antibody AF488 was added in a 1/1000 dilution in 200 µL with the surface staining and further incubate for 20 minutes at 4°C. Cells were fixed.

2.2.2.4 Nutrient uptake assay with 2-NBDG
1x10⁶ isolated cells were washed with glucose free media and re-suspended in pre-warmed glucose-free media: RPMI media supplemented with 10% dialyzed FCS
(Fisher), 2mM glutamine (Invitrogen/Biosciences), 1mM sodium pyruvate (Gibco®), 1x concentration of MEM Vitamin Cocktail (Invitrogen/Biosciences), 1x concentration of selenium/insulin/transferrin cocktail (Invitrogen/Biosciences) and 1% Penicillin/Streptomycin (Invitrogen/Biosciences), at a concentration of 5x10^6 cells/ml. After stimulation overnight cells were centrifuged and re-suspended in 1 ml of supplemented Glucose-free media with 50 µM of the fluorescently labelled glucose analogue 2-NBDG (Life technologies), or in glucose-free media alone as a control. Cells were then incubated for 1 hour at 37°C after which they were washed twice in supplemented glucose-free media and analysed by flow cytometry.

2.2.2.5 Mitochondria staining

Mitochondria potential analysis was performed by staining cells with TMRM (tetramethylrhodamine, methyl ester - TermoFisher) and detecting the fluorescence through flow cytometry. TMRM is a cell-permeant dye that accumulates in active mitochondria with intact membrane potentials. If the cells are healthy and have functioning mitochondria, the signal will be bright. Upon loss of the mitochondrial membrane potential, TMRM accumulation will cease and the signal will dim or disappear. Cells were stained for 30 minutes with TMRM (0.1µM). After washing, surface markers and live dead antibody was used to eliminate dead cells. Membrane potential was also analyzed with JC-1 dye which accumulates in mitochondria. The dye is a monomer at low concentrations and yields green fluorescence, at higher concentrations the dye exhibits a broad excitation spectrum (become more red/orange color with emission maximum around 590nm). Cells were incubated with JC-1 (1 µM) for 30 minutes at 37 °C incubator. Cells were washed with FACS buffer and surface staining of the cells was completed prior acquisition.

ATP synthase analysis was performed by detecting the expression of a subunit of the enzyme called ATP5B (Abcam). ATP5B antibody is stained together with other intracellular markers after cell fixation.

Mitochondria superoxide levels were detected by MitoSOX (ThermoFisher). Rotanone (20µM), was used as positive control (increase ROS) and Mitotempo (2.5
µM) added to negative control (decrease ROS). Controls were incubated at 37 °C incubator with rotenone or mitotempo for 30 minutes. MitoSox (1.5 µM) was added to samples and control and incubated for 15 minutes at 37 °C incubator. Cells were washed with FACS buffer and surface staining and fixation of the cells was completed prior acquisition.

2.2.3 ATP assay

For ATP analysis, 200uL of purified NK cells were stimulated in a 96 well plate at 5x10^6 cells/mL for 18 hours in triplicate. Cells were lysed and relative ATP levels in mM per 10^6 cells were measured using an ATP assay determination kit (Perkmin Elmer) as per manufacturer's instructions on a FLUROstar OPTIMA luminescence reader. ATP is a marker for cell viability because it is present in all metabolically active cells and the concentration declines very rapidly when the cells undergo necrosis or apoptosis. The ATPlite assay system is based on the production of light caused by the reaction of ATP with added luciferase and d-luciferin. The emitted light is proportional to the ATP concentration within certain limits. Data is not shown in this thesis but experiment was performed to confirm that cells in oligomycin were not in energy crisis.

2.2.4 Metabolic analysis

A XF-24 Extracellular Flux Analyzer (Seahorse Bioscience) simultaneously interrogates the two major energy producing pathways of the cell – mitochondrial respiration and glycolysis in a microplate, in real-time. It determines the real-time in vitro oxygen consumption rate (OCR), and proton production rate (PPR), in order to assess cellular functions such as oxidative phosphorylation and glycolysis of NK cells cultured under various conditions. MACS purified NK cells were stimulated in different stimulations for 18 hours and for 5 or 6 days. To get the seahorse plate ready for the addition of cells 200µl Celltak® (BD Pharmingen) was added and incubated at room temperature for 20 minutes and washed off with double distilled water. Following the indicated stimulation time NK cells were centrifuged at 300g, the supernatant poured off and the cells were washed in 2-3
mL of pre-warmed seahorse media (containing 10mM Glucose). The cells were centrifuged down again and resuspended at a concentration of 7.5x10^6 cells/ml in seahorse media supplemented with relevant treatments. 100 µl (0.75x10^6 cells) media was added to each well and centrifuged with the break off at 300g for 3 minutes. Plates were incubated in a non-CO₂ incubator for 20 minutes. Following incubation wells were topped up with 500 µl seahorse media containing relevant treatments. Plates were incubated for a further 40 minutes, while incubating inhibitors were made up and loaded into the relevant injection ports. Sequential measurements of OCR following the addition of the inhibitors oligomycin (2µM), fluoro-carbonyl cyanide phenylhydrozone (FCCP 0.5µM), rotenone (100nM) plus antimycin A (4µM) and 2DG (30mM) allows for the accurate calculation of the oxygen consumption due to OxPHOS and the proton production due to glycolysis.

For the determination of glycolytic rates (proton excretion related to glycolysis, also called ECAR – extracellular acidification rate), glucose is used as the glycolysis substrate, oligomycin as the ATP synthase inhibitor and 2DG as the glycolysis inhibitor. Basal rates of glycolysis can be determine by the change in acidification in the media over time by release of protons during glycolysis. Oligomycin inhibits ATP synthase thus blocking OxPHOS and increasing Glycolysis to maintain ATP production, this allows us to determine the maximum amount of glycolysis any given cell can undergo i.e. its glycolytic capacity. Lastly, 2DG, a direct glycolytic inhibitor blocks all glycolysis and thus any change in acidification of the media can be determined to be from a process other than glycolysis. This allows us to calculate the actual rates of glycolysis.

For the determination of oxidative phosphorylation rates (that is cell respiration, also known as OCR – oxygen consumption rate), we use oligomycin as the ATP synthase inhibitor, FCCP as the electron transport chain accelerator, antimycin A as the electron transport chain complex III inhibitor and rotenone as the electron transport chain complex I inhibitor. Basal rates of OxPHOS can be determine by the amount of oxygen being consumed over time. While Oligomycin inhibits ATP synthase, FCCP uncouples it which will dissipate the gradient of protons across the inner mitochondrial membrane and thus maximising OxPHOS. Antimycin A and
rotenone inhibit the Electron transport chain thus any residual oxygen consumption can be discarded. This allows us to calculate the actual rates of OxPHOS.
Figure 2.2 Determining Glycolytic rates using the Seahorse metabolic flux analyser.

Basal rates of glycolysis can be determined by the change in acidification in the media over time by release of protons during glycolysis. Oligomycin inhibits ATP synthase thus blocking OxPhos and increasing Glycolysis to maintain ATP production, this allows us to determine the maximum amount of glycolysis any given cell can undergo i.e. its glycolytic capacity. Finally 2DG, a direct glycolytic inhibitor blocks all glycolysis and thus any change in acidification of the media can be determined to be from a process other than glycolysis. This allows us to calculate the actual rates of glycolysis. Figure adapted from Seahorse Bioscience.
Figure 2.3 Determining OxPhos rates using the Seahorse metabolic flux analyser.

Basal rates of OxPhos can be determine by the amount of oxygen being consumed over time. While Oligomycin inhibits ATP synthase, FCCP uncouples it which will dissipate the gradient of protons across the inner mitochondrial membrane and thus maximising OxPhos. Antimycin A and Rotenone inhibit the Electron transport chain thus any residual oxygen consumption can be discarded. This allows us to calculate the actual rates of OxPhos. Figure adapted from Seahorse Bioscience.
2.2.5 Statistical analysis

GraphPad Prism 6.00 (GraphPad Software) was used for statistical analysis. Data was tested and if a non-normal distribution was found, a non-parametric test was used. If there were insufficient numbers to test for normality, a non-parametric test was also used. In general, a non-parametric one-way ANOVA test was used with the Kruskal-Wallis post-hoc test. Paired student’s t-test with the Wilcoxon matched-pairs signed rank test was used as appropriate when there were only 2 data sets for comparison. Fold changes in MFI were compared with a one-sample t-test against a theoretical mean set to 1.00.
Chapter 3

Metabolic changes in cytokine stimulated NK cells
3.1 Introduction

NK cells are lymphocytes with important roles in cancer and in the immune response to infection. Although NK cells are generally considered part of the classical innate immune system, evidence is emerging that they regulate and respond to the adaptive immune response and continue to function as effector cells. As mentioned in the introduction, human NK cells in the peripheral blood can be divided into at least two functional and phenotypically different NK cells subsets based on expression levels of CD56 on their surface. These cells retain distinct phenotypic and functional differences suggesting that both are likely to play important roles during the NK cell immune response\textsuperscript{106}. In brief, CD56\textsuperscript{dim} cells are predominant in peripheral blood and are strongly cytotoxic. In contrast, the CD56\textsuperscript{bright} cells are predominantly found in secondary lymphoid organs and are not strongly cytotoxic but are capable to produce significantly more IFN\textgreek{g} per cell then CD56\textsuperscript{dim} cells. Understanding how NK cells are equipped for their specialized functions is important not only in terms of understanding the basic biology of these cells and their ontogeny, but can also inform optimal conditions for \textit{ex vivo} culture of immunocompetent NK cells for immunotherapy.

Dynamically regulated cellular metabolism is now recognized as an important factor that contributes to a successful immune response\textsuperscript{107}. Metabolism is important to maintain energy homeostasis and to supply cells with the building blocks for macromolecular synthesis, but cellular metabolism can also directly influence immune cell function and differentiation. Different immune cell subsets have very different metabolic demands that are accommodated by different types of glucose metabolism\textsuperscript{108}. Some lymphocytes predominantly use mitochondrial OxPHOS to efficiently generate ATP, a process that requires oxygen. In contrast, effector lymphocyte subsets metabolize large amounts of glucose by aerobic glycolysis, a process in which glucose is metabolized to lactate in the presence of oxygen. This is a metabolic signature that is common to highly proliferative cells because it provides biosynthetic precursors for the synthesis of nucleotides, amino acids, and lipids. Aerobic glycolysis can also directly impact upon the functions of effector lymphocytes\textsuperscript{108}. 
Our laboratory has reported that cytokines upregulate glucose metabolism in expanded murine NK cells and that rates of both glycolysis and OxPHOS are increased in these activated cells\textsuperscript{31}. mTORC1 was important for both metabolic and functional changes in these activated NK cells, and elevated glycolysis directly affected multiple key NK cell effector functions. Because there are important differences between murine and human NK cells including potential for immunotherapy, divergent receptor systems, and intrinsic genetic and phenotypic variability\textsuperscript{109, 110}, we undertook experiments to investigate whether 18 hour cytokine stimulation induced changes in metabolism in human NK cells and whether these affected immune functions. We also investigated the importance of mTORC1 activity and metabolic requirements for optimal NK cell effector functions.

*All experiments from chapter 3 were carried out in collaboration with Dr. Sinead Keating.*
3.2 Results

3.2.1 CD56\textsuperscript{bright} NK cells preferentially upregulate metabolism in response to cytokine

Our lab has previously reported that murine NK cells upregulate metabolism in response to cytokine stimulation and that glycolysis impacts on IFN\(\gamma\) production by these cells\textsuperscript{31}. In order to investigate if human NK cells behave in similar fashion, we explored the cellular metabolism of human primary NK cells in response to either IL-2 or IL-12/15 combination. In peripheral blood, human NK cells can be divided into two subsets based on the surface expression of CD56. CD56\textsuperscript{dim} cells are generally considered more cytotoxic while the CD56\textsuperscript{bright} cells are potent producers of IFN\(\gamma\). After 18 hours stimulation, we can still confidently assign NK cells freshly isolated from blood to CD56\textsuperscript{dim} or CD56\textsuperscript{bright} subpopulations. To support this, the frequency of CD56\textsuperscript{dim} and CD56\textsuperscript{bright} cells pre- and post-cytokine activation data is demonstrated in Table 3.1.

To investigate changes in human NK cell metabolism we initially focus on the transferrin receptor, CD71. CD71 was absent or expressed at very low levels on resting NK cells and increased in expression in response to cytokine stimulation (Figure 3.1 A-B). Interestingly, CD71 was preferentially upregulated on the CD56\textsuperscript{bright} subset of NK cells but only on a subset of CD56\textsuperscript{dim} NK cells (Figure 3.1 A-B). CD98, a component of the L-amino acid transporter, was expressed on all NK cells and expression levels increased in response to cytokine (Figure 3.1 C-D). Was notable that CD56\textsuperscript{bright} cells had less CD98 expression on their surface however, after cytokine stimulation, CD56\textsuperscript{bright} cells expressed more CD98 than CD56\textsuperscript{dim} cells (Figure 3.1 C-D).
In terms of glucose metabolism, we measured expression of Glut1, the glucose transporter, thought to be primarily involved in supporting aerobic glycolysis in lymphocytes. Without cytokine, CD56^dim^ NK cells had relatively low levels of Glut1 but strikingly CD56^bright^ cells expressed high levels of this glucose transporter (Figure 3.2 A-B). As levels of Glut1 were high in the CD56^bright^ cells, this did not change substantially after cytokine stimulation. However, both IL-2 and IL-12/15 cytokine combinations significantly upregulated Glut1 on CD56^dim^ NK cells (Figure 3.2 D).

Because of the increase of Glut1 receptor after cytokine stimulation, we hypothesized that these cells would consequently increase their nutrient uptake. To confirm this, nutrient uptake was measured using the fluorescent glucose analogue 2-(N-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl) Amino)-2-Deoxyglucose (2-NBDG) after stimulation with cytokines. Cytokine stimulated CD56^bright^ NK cells, but not CD56^dim^ NK cells, significantly increased the rate of nutrient uptake (Figure 3.3 A-B). These data suggest that CD56^bright^ cells have the machinery in place to facilitate a more rapid nutrient uptake response after cytokine stimulation than their CD56^dim^ counterparts.

Furthermore, we performed the 2-NBDG uptake experiment with NK cells that were sorted into CD56^dim^ and CD56^bright^ cells subsets from PBMC. Sorted subsets were stimulated overnight with cytokine and 2-NBDG, as a direct readout associated with increased glycolysis, was measured. While there were relatively modest increases in CD56^dim^ cells, CD56^bright^ cells increased nutrient uptake dramatically, in particular in response to IL-12/IL15 cytokine combination (Figure 3.3 C-D). These data directly demonstrate preferential nutrient uptake in CD56^bright^ cells compared to CD56^dim^ NK cells. These observations suggests that CD56^bright^ NK cells are more metabolically active then CD56^dim^ cells in response to cytokine.
Table 3.1 Frequency of CD56\textsuperscript{dim} and CD56\textsuperscript{bright} cells pre- and post-cytokine activation from 10 random experiments.

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<td><strong>7.8±4.0</strong></td>
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Figure 3.1 CD56\textsuperscript{bright} NK cells show higher expression of metabolic markers than CD56\textsuperscript{dim} cells in response to cytokine.

PBMC were stimulated for 18 hours with either IL-2 (500 U/mL), IL-12 (30 ng/ml)+IL-15 (100 ng/mL), or left unstimulated. Cells were analyzed for CD71 and CD98 expression by flow cytometry. (A, B) Representative dot plots (A) and pooled data (B) (stratified into CD56\textsuperscript{bright} and CD56\textsuperscript{dim} subsets) of NK cells expressing CD71, the transferrin receptor are shown. Bars show percentage of NK cells (CD56\textsuperscript{+}CD3\textsuperscript{-}) that are positive for CD71 expression and error bars shown from 6 repeats. (C, D) Representative dot plots (C) and pooled data (D) (stratified into CD56\textsuperscript{bright} and CD56\textsuperscript{dim} subsets) of NK cells expressing CD98,
a component of the L-amino acid transporter are shown. Bars show mean fluorescence intensity (MFI) of NK cells (CD56+CD3-) expressing CD98 and SEM error bars from separated experiments shown from 6 repeats. Avarage data is shown as mean±SEM from separated experiments. Data were compared using a non-parametric one way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$.

Figure 3.2 CD56$^{\text{bright}}$ cells express higher levels of Glut1 receptor then CD56$^{\text{dim}}$ cells.

PBMC stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. (A) Representative dot plot of Glut1 expression on unstimulated NK cells is shown. (B,C) Representative histogram (B) and pooled data (C) of
Glut1 expression on unstimulated CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cell subsets. (D) Glut1 expression on cytokine stimulated CD56\textsuperscript{bright} (left) and CD56\textsuperscript{dim} (right) NK subsets. (n=5-8 donors). Average data is shown as mean±SEM from separated experiments. Samples were compared using a non-parametric one way ANOVA or a Student's t-test when appropriated. * $P \leq 0.05$, ** $P \leq 0.01$.

Figure 3.3 CD56\textsuperscript{bright} NK cells are more metabolically active than CD56\textsuperscript{dim} cells in response to cytokine.

PBMC stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated in the presence of 2-NBDG. (A,B) Representative histograms (A) and pooled data (B) of nutrient uptake by CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK subsets analysed using the fluorescent glucose analogue, 2-NBDG by flow cytometry (n=6 donors). (C,D)
CD56\textsuperscript{dim} and CD56\textsuperscript{bright} cells were sorted from freshly isolated PBMC and stimulated overnight with IL-2 or IL-12/15 in the presence of 2-NBDG. After 18 hours, nutrient uptake was measured by flow cytometry (n=3). Average data is shown as mean±SEM from separated experiments. Samples were compared using a non-parametric one way ANOVA. * \( P \leq 0.05 \).

### 3.2.2 mTORC1 activity is induced in cytokine activated NK cells

Our lab has reported that mTORC1 was activated in murine NK cells after stimulation with cytokine\textsuperscript{31}. Given that mTORC1 is a key metabolic regulator, we investigated if mTORC1 was activated in human NK cells in response to these different cytokine combinations. mTORC1 activity in NK cells subsets was determined by measuring phosphorylation of the S6 ribosomal protein (pS6), a downstream target of mTORC1 signalling. pS6 levels were significantly elevated in both CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells in response to either IL-12/15 or IL-2 cytokine stimulations (\textbf{Figure 3.4} A-D). These pS6 levels were substantially inhibited by the mTORC1 inhibitor, rapamycin, demonstrating that mTORC1 is active in these cells (\textbf{Figure 3.4} A,C).

Our lab has reported that in murine NK cells CD71 and CD98 are mTORC1 dependent therefore, we investigated if this is also true in human NK cells. Indeed, rapamycin treatment decreased the expression of CD71 in CD56\textsuperscript{bright} cells, the subset that predominantly expresses this surface receptor (\textbf{Figure 3.5} A-B). Additionally, rapamycin significantly abolished the IL-2 (\textbf{Figure 3.5} C-D) and IL-12/15-induced (\textbf{Figure 3.5} E-F) increases in CD98 expression in CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells. These data demonstrate that mTORC1 can regulate nutrient receptor expression on human NK cell, as previously seen in murine NK cells\textsuperscript{31,80}.
Figure 3.4. mTORC1 activity increase after cytokine stimulation in human NK cells.

PBMC were stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml) + IL-15 (100 ng/ml), or left unstimulated. Rapamycin (20 nM) was added as indicated. Cells were then analyzed for pS6 levels by intracellular flow cytometry staining. (A-D) Representative histograms (A and C) and pooled (B and D) data are shown for IL-2 or IL-12/15 stimulated NK cells stratified into CD56\textsuperscript{bright} (left panel) and CD56\textsuperscript{dim} (right panel) subsets. (A and C) NK cells were treated with rapamycin for the final 20 minutes of stimulation at 37°C to provide
a negative control of pS6 levels in the absence of mTORC1 activity. (n=6 donors). Data were compared using a Student's t-test. *P ≤ 0.05.

Figure 3.5 mTORC1 regulates expression of NK cell metabolism markers.

PBMC were stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml) +IL-15 (100 ng/ml), or left unstimulated. Rapamycin (20 nM) was added as indicated. (A-B) Representative histograms of IL-2 or IL-12/15 induced expression of CD71 expression on
CD56\textsuperscript{bright} cells (A) and paired responses +/- rapamycin (B) are shown (n=10 for IL-2 and n=6 for IL-12/15). (C-D) Representative histograms of IL-2 induced expression of CD98 expression on CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells (C) and paired responses +/- rapamycin (D) are shown. (E-F) Representative histograms of IL-12/15 induced expression of CD98 expression on CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells (E) and paired responses +/- rapamycin (F) are shown. Samples were compared using a paired Student’s t-test analysis. *P \leq 0.05.

### 3.2.3 Cytokines upregulate glycolysis and oxidative phosphorylation in human NK cells

Given the increased expression of Glut1 and nutrient uptake in CD56\textsuperscript{bright} and a subset of CD56\textsuperscript{dim} NK cells, we undertook a detailed analysis of glucose metabolism. As mTORC1 is well characterized in murine cells to be a key regulator of glucose metabolism\textsuperscript{31, 80}, we also investigated its importance in the metabolic changes observed. Purified NK cells were stimulated with cytokines overnight in the presence or absence of rapamycin as treatment, before metabolic analysis. One caveat of these analyses is that the high number of cells required precluded independent analysis of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} subsets and results reflect changes in the overall NK cell population. IL-2 and IL-12/15 stimulations both increased the rate of glycolysis in NK cells to similar levels (Figure 3.6 A-B and Figure 3.7 A). While the observed ECAR values are quite low compared to those published for other activated lymphocytes\textsuperscript{31, 80, 112}, this likely reflects the heterogeneous nature of NK cells as discussed above. Interestingly, rapamycin inhibited increases in glycolysis induced by IL-2 but not by IL-12/15 stimulation (Figure 3.6 A-B and Figure 3.7 A).

The maximum glycolytic rates for cytokine stimulated NK cells were also determined. Cytokines increased the glycolytic capacity up to 5 fold indicating increased expression of glycolytic machinery, a process termed glycolytic capacity (Figure 3.7 B). While both IL-2 and IL-12/15 increased the glycolytic capacity equivalently, IL-2 but not IL-12/15 stimulated glycolytic capacity was significantly decreased by rapamycin treatment (Figure 3.7 B).

The rate of mitochondrial oxygen consumption (OCR), which represents OxPHOS levels, was also measured. Both cytokine stimulations increased the rate of oxygen
consumption (Figure 3.7 C). However, changes in oxidative phosphorylation were insensitive to rapamycin.

We used 2-NBDG uptake to confirm, on a single cell basis, the requirement for mTORC1 signaling for uptake of nutrients necessary to upregulate glucose metabolism in NK cell subsets. Rapamycin treatment abolished the elevated levels of nutrient uptake observed in IL-2 stimulated CD56\(^{\text{bright}}\) NK cells (Figure 3.8 A-B). While the increase in nutrient uptake in IL2-stimulated CD56\(^{\text{dim}}\) NK cell was minimal, rapamycin nonetheless significantly decreased these rates (Figure 3.8 A-B). However, there were no changes in IL-12/15 induced glucose uptake in either CD56\(^{\text{dim}}\) or CD56\(^{\text{bright}}\) subset (Figure 3.8 C-D). This data argues for an important role for mTORC1 signaling in promoting elevated levels of nutrient uptake and glycolysis in the CD56\(^{\text{bright}}\) subset of NK cells following IL-2, but not IL-12/15, cytokine stimulation.

Taken together, this data shows that cytokine activated NK cells undergo metabolic changes to increase both rates of glycolysis and OxPHOS. However, it appears that IL-2 induced glycolysis is regulated in part by mTORC1 while IL-12/15 induced glycolysis is independent of mTORC1.
Figure 3.6 Human NK cells upregulate glycolysis in response to cytokine.
NK cells were purified and stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml) +IL-15 (100 ng/ml), or left unstimulated. Rapamycin (20 nM) was added as indicated. Detailed metabolic analysis was performed using the Seahorse extracellular flux analyzer. (A-B) Representative traces for the extracellular acidification rate (ECAR) in response to IL2 (A) or IL12/15 (B) +/- rapamycin are shown.

Figure 3.7 Human NK cells upregulate glycolysis and OxPhos in response to cytokine.

NK cells were purified and stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Rapamycin (20 nM) was added as indicated. Detailed metabolic analysis was performed using the Seahorse extracellular flux analyser. (A-B) Pooled data for the glycolysis (A) and glycolytic capacity (B) in response to IL-2 or IL-12/15 +/- rapamycin are shown. (A, n=5 donors; B, n=3 donors) (C) Pooled data for oxygen consumption rate (OCR) are shown for IL-2 (n=4 donors) and IL-12/15 (n=3 donors). Data is mean±SEM from separated experiments. Samples were compared using
either a one way ANOVA followed by a Kruskall Wallis post-hoc test or a non-parametric paired Student’s t-test analysis as appropriate. ns = not significant. *P ≤ 0.05.

Figure 3.8 Uptake of the nutrient analogue 2-NBDG in IL-2 stimulated NK cells is impaired by rapamycin treatment.

PBMC stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Nutrient uptake was analyzed using the fluorescent glucose analogue, 2-NBDG by flow cytometry. (A-B) Representative histograms (A) and individual paired responses (B) of IL-2 induced 2-NBDG uptake by CD56bright (left panel) and CD56dim
(right panel) NK cells +/- rapamycin (n=6 donors). (C-D) Representative histograms (C) and individual paired responses (D) of IL-2 induced 2-NBDG uptake by CD56bright (left panel) and CD56dim (right panel) NK cells +/- rapamycin (n=6 donors). Samples were compared using a non-parametric paired Student’s t-test analysis. ns = not significant. *P ≤ 0.05

3.2.4 Cytokine induced effector functions of human NK cells at 18 hours are independent of mTORC1

Having observed differences in the requirements for mTORC1 for short-term metabolic reprogramming in IL-2 and IL-12/15 stimulated NK cells we investigated whether mTORC1 is required for NK cell effector functions induced by these cytokines in this timeframe. Both IL-2 and IL-12/15 induced robust expression of the activation antigen CD69 on NK cells (Figure 3.9 A). The activating natural cytotoxicity receptor NKp44 and death receptor, TRAIL, were preferentially expressed on CD56bright NK cells in response to cytokine as expected113, 114. None of these responses were inhibited by rapamycin (Figure 3.9 A-C). In terms of effector functions, we measured granzyme B and CD107a degranulation as markers associated with NK cell cytotoxicity. Although cytokines potently caused NK cells to degranulate in presence of target cells as increase of CD107a expression was observed, this was independent of mTORC1 (Figure 3.9 D). Granzyme B is expressed in all NK cells; the CD56dim subset constitutively contained more granzyme B, but upon cytokine activation, both IL-2 and IL-12/15 induced a strong upregulation of granzyme B in all CD56bright NK cells to levels comparable with CD56dim. Inclusion of rapamycin did not affect granzyme B upregulation in CD56bright cells (Figure 3.10 A-B).

Additionally, we investigated whether mTORC1 signalling is required for NK cell production of IFNγ in response to cytokine. Rapamycin treatment did not affect the frequency of either CD56dim or CD56bright NK cells producing IFNγ in response to IL-12/15 stimulation (Figure 3.11 and Figure 3.12 A,C). While there was a trend towards decreased IFNγ production per CD56bright NK cell, as determined by the mean fluorescence intensity (MFI) of IFNγ+ NK cells, with rapamycin treatment, this effect was not statistically significant (Figure 3.12 B,D). While IL-2 is not a potent
stimulus for the production of IFN\(\gamma\) in CD56\(^{\text{dim}}\) or CD56\(^{\text{bright}}\) NK cells, rapamycin inhibited IL-2-induced IFN\(\gamma\) production in both NK cell subsets, in terms of the frequency of NK cells producing IFN\(\gamma\) (Figure 3.11 and Figure 3.12 A,C). As seen with IL-12/15 stimulations, there was a trend towards decreased IFN\(\gamma\) per CD56\(^{\text{bright}}\) NK cells, though this was not statistically significant (Figure 3.11 and Figure 3.12 B,D). Thus, this data identifies a discrete role for mTORC1 signalling in IFN\(\gamma\) production in response to IL-2 but not IL-12/15 cytokine stimulation.

Finally, based on the observation that CD56\(^{\text{bright}}\) NK cells upregulated general metabolic markers and produced more IFN\(\gamma\), we hypothesised that the NK cells that produce IFN\(\gamma\) are those that have induced a metabolic response. Indeed, when IL-12/15 stimulated CD56\(^{\text{bright}}\) and CD56\(^{\text{dim}}\) NK cells were stratified based on IFN\(\gamma\) production, it was clear that NK cells that were producing IFN\(\gamma\) were more metabolically active and had significantly more CD71 expression (Figure 3.13 A-B) than those that were not producing any cytokine.
Figure 3.9 Cytokine induced increases in expression of CD69, NKp44, TRAIL and CD107a of human NK cells are independent of mTORC1.

PBMC were stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml) + IL-15 (100 ng/ml), or left unstimulated. Rapamycin (20 nM) was added where indicated. (A) Expression of the CD69 surface antigen on NK cells is shown (n=4 donors). (B, C) Frequency of expression of NKp44 (B) or TRAIL (C) on CD56bright NK cells (n=4 donors). (D) NK cell degranulation as determined by CD107a positivity following incubation with target cells is shown (n=4-7 donors). Data is mean±SEM from separated experiments. Samples were compared by a one way ANOVA followed by a Kruskall Wallis post-hoc test. ns = not significant.
Figure 3.10 Cytokine induced upregulation of granzyme B in human NK cells is independent of mTORC1.

PBMC were stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Rapamycin (20 nM) was added where indicated. (A-B) Representative dot plots (A) and pooled data (B) of Granzyme B expression (MFI) on CD56^{bright} NK cells (n=6 donors). Data is mean±SEM from separated experiments. Samples were compared by a one way ANOVA followed by a Kruskall Wallis post-hoc test. ns = not significant.
Figure 3.11 NK cell subsets stimulated with IL-2 depend on mTORC1 activity for optimal IFNγ production.

PBMC were stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Rapamycin (20 nM) was added where indicated. Representative dot plots of IFNγ production by NK is shown.
Figure 3.12 IL-2 stimulated effector functions of human NK cells at 18h are dependent of mTORC1.

PBMC were stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Rapamycin (20 nM) was added where indicated. (A) Frequency of IFNγ producing CD56bright NK cells with individual paired responses for IL-2 (right panel) and IL-12/15 (left panel) +/- rapamycin is shown. (B) Pooled data for expression (MFI) of IFNγ producing CD56bright NK cells stimulated with IL-2 or IL-12/15 +/- rapamycin. (C) Frequency of IFNγ producing CD56dim NK cells with individual paired responses for IL-2 (right panel) and IL-12/15 (left panel) +/- rapamycin is shown. (D) Pooled data for expression (MFI) of IFNγ producing CD56dim NK cells stimulated with IL-2 or IL-12/15 +/- rapamycin. (n = 9 donors). Data is mean±SEM from separated experiments. Samples were compared either with a one way ANOVA followed by a Kruskall Wallis post-hoc test or paired Student’s t-test as appropriate. ns = not significant. *P ≤ 0.05.
Figure 3.13 NK cell subsets that produce cytokines are metabolically active.

PBMC were stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml)+IL-15 (100 ng/ml). CD56bright cells (from data in Figure 3.12) were stratified based on IFNγ + staining. Expression of CD71 on these different subsets was then analysed. (A) Representative histogram of CD71 expression by IFNγ positive or IFNγ negative cells. (B) CD56dim NK cells were analysed as in (A) and pooled data for NK cells stimulated with IL-2 (left panel) and IL-12/15 (right panel) is shown. Data is mean±SEM from separated experiments. Paired Student's t-test was used to compare the data. **P ≤ 0.01.
3.2.5 Elevated OxPHOS is required for cytokine-induced NK cell function

We next investigated whether the increases in NK cell metabolism associated with cytokine stimulation were important for NK cell effector functions. Cytokine stimulation induced a pronounced increase in cellular OxPhos, suggesting that increased ATP synthesis is important in these activated cells (Figure 3.7 C). To determine the importance of OxPhos, we included the ATP synthase inhibitor, oligomycin, in our experiments. We used a relatively low dose of oligomycin that limits the rate of mitochondrial ATP synthesis and OxPhos without causing an energy crisis in the cells, an approach previously described by others54.

Oligomycin treatment did not inhibit cytokine induced granzyme B expression in NK cell subsets (Figure 3.14 A-B). However, oligomycin inhibited degranulation induced by IL-2 in both CD56dim and CD56bright subsets but inhibition of degranulation induced by IL-12/15 cytokines was only observed in the CD56dim subset (Figure 3.14 C-D).

Elevated OxPhos was also required for the IFNγ response in IL-12/15 stimulated NK cells. Oligomycin treatment resulted in a reduced frequency of CD56dim and CD56bright NK cells producing IFNγ in response to IL-12/15 stimulation (Figure 3.15 A). Additionally, the level of IFNγ produced per cell, by CD56bright subset (the main subset that produces IFNγ) was also reduced by oligomycin treatment (Figure 3.15 B). The relative small IL2-induced IFNγ response was not affected by oligomycin treatment (data not shown). Overall, oligomycin inhibited most NK cell functions supporting the tenet that increased ATP production due to mitochondrial OxPhos is important for fueling NK cell functions.
Figure 3.14 OxPhos is required for NK cell effector functions.

PBMC were stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Oligomycin (40nM) was added for the duration of cultures where indicated. (A, B) Pooled data for granzyme B expression (MFI) in CD56\textsuperscript{bright} (A) and CD56\textsuperscript{dim} NK cells (B) is shown with individual paired responses (right panel) for IL-2 and IL-12/15 stimulated cells +/- oligomycin (right panel). (C, D) NK cell degranulation as determined by CD107a expression following incubation with target cells is shown (left panel) for CD56\textsuperscript{bright} (C) and CD56\textsuperscript{dim} cells (D), with individual paired responses for IL-2, IL-12/15 stimulated cells +/- oligomycin (right panel). Data is mean±SEM from separated experiments (n=6 donors). Paired Student’s t-test was used to compare the data. ns = not significant. *P ≤ 0.05.
Figure 3.15 Human NK cells require OxPhos for optimal IFNγ production and expression.

PBMC were stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Oligomycin (40nM) was added for the duration of cultures where indicated. (A-B) Pooled data for frequency of IFNγ producing NK cells (A) and MFI (B) of IFNγ+ NK cells is shown for CD56\textsuperscript{bright} and CD56\textsuperscript{dim} cells (left panels), with individual paired responses for IL-2 and IL-12/15 +/- oligomycin (right panels) is shown. Data is mean±SEM from separated experiments (n=6 donors). Paired Student’s t-test was used to compare the data. ns = not significant. *P ≤ 0.05.
3.2.6 Elevated rates of glycolysis are required for maximal INFγ production

Our lab has previously reported that glycolysis is important for IFNγ production in murine NK cells\textsuperscript{31}. In order to investigate if glycolysis is important for human NK cell effector functions, we carried out experiments in the presence of galactose as an alternate carbon fuel source to glucose that cannot support elevated glycolysis\textsuperscript{31,54}. Inhibiting glycolysis in this way had only modest inhibitory effects on NK cell degranulation (CD107a) or granzyme B induction in CD56\textsuperscript{bright}, but not CD56\textsuperscript{dim} cells (Figure 3.16 A-D). In terms of INFγ production, limiting the rate of glycolysis had no effect on IL-2-simulated INFγ production (Figure 3.17). However, when the rate of glycolysis was limited in the IL-12/15-stimulated NK cells, there was a trend towards a decreased frequency of CD56\textsuperscript{bright} NK cells making cytokine with a significant reduction in the amount of INFγ being produced per cell (Figure 3.18 A-C). While the INFγ response is variable in donor PBMC, the relative amounts of INFγ in galactose cultured cells were significantly decreased compared to those cultured in glucose. Thus, this data argues that elevated glycolysis in human NK cells is required for maximal INFγ responses.
Figure 3.16 Glycolysis is not required for Granzyme B production and CD107a expression in NK cells.

PBMC were stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Cultures were carried out in glucose replete medium or medium in which galactose (10 mM) replaced glucose. (A, B) Granzyme B expression (MFI) in CD56<sup>bright</sup> (A) and CD56<sup>dim</sup> cells (B) is shown. (C, D) NK cell degranulation as determined by CD107a positivity following incubation with target cells is shown for CD56<sup>bright</sup> (C) and CD56<sup>dim</sup> cells (D). Data is mean±SEM from separated experiments of 6 donors. Samples were compared using a non-parametric one way ANOVA. ns = not significant.
Figure 3.17 IFNγ production by CD56bright NK cells does not require high rates of glycolysis.

PBMC were stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Cultures were carried out in glucose replete medium or medium in which galactose (10 mM) replaced glucose. Pooled data of IFNγ production in IFNγ+ CD56bright cells with the change, glucose versus galactose. Data is mean±SEM from separated experiments (n=6 donors). Samples were compared using one-way ANOVA followed by a Kruskal-Wallis post hoc test. ns = not significant.
Figure 3.18 Glycolysis is required for maximal IFNγ expression by CD56\textsuperscript{bright} NK cells.

PBMC were stimulated for 18 hours with either IL-2 (500 U/ml), IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Cultures were carried out in glucose replete medium or medium in which galactose (10 mM) replaced glucose. (A) IFNγ expression (MFI) in fold IFNγ+ CD56\textsuperscript{dim} cells is shown (left) with the change, glucose versus galactose (right). (B) IFNγ expression (MFI) in IFNγ+ CD56\textsuperscript{bright} cells is shown (left) with the fold change, glucose versus galactose (right). (C) Representative dot plots of IFNγ production in IL-12/15 stimulated NK cells. Data is mean±SEM from separated experiments of 6 donors. After testing for normal distribution, the fold changes in IFNγ MFI due to galactose were compared with a one-sample \( t \)-test against a theoretical mean set to 1.00. *\( P \leq 0.05 \), ns = not significant.
3.3 Discussion

Metabolic changes on lymphocytes have been shown to be crucial for shaping immune cell response. The mechanism behind these changes linking metabolism and immunity are still being investigated. To our knowledge, this was the first study to define the metabolism of human NK cells and identify that CD56\textsuperscript{bright} cells and CD56\textsuperscript{dim} NK cells subsets in peripheral blood have distinct metabolic phenotypes. We have shown that cytokine stimulation upregulate human NK cell metabolism and that mTORC1, a key metabolic regulator, is highly active in cytokine-stimulated NK cells. We found that mTORC1 activity is important for maintenance of glycolysis and IFN\gamma production by IL-2 stimulated NK cells.

To determine if human NK cells subsets have altered metabolism after stimulation, we analyzed the expression of different surface markers (CD71 and CD98) and uptake of the glucose analogue 2-NBDG which correlates to an elevated metabolic phenotype as shown before in CTL and murine NK cells\textsuperscript{31, 115}. We also investigated specific metabolic changes using the Seahorse extracellular analyzer. Our data show that cytokines can robustly activate NK cell metabolism. Perhaps more important was the observation that CD56\textsuperscript{bright} cells in peripheral blood were different from CD56\textsuperscript{dim} cells in term of their metabolism. CD56\textsuperscript{bright} cells were much more glycolytic and preferentially upregulated metabolism in response to cytokines compared with CD56\textsuperscript{dim} cells. CD56\textsuperscript{bright} cells efficiently upregulated the CD71 and had higher expression of CD98 and higher 2-NBDG uptake. Furthermore, these CD56\textsuperscript{bright} cells are primed to become more metabolically active as they express higher levels of cytokine receptors\textsuperscript{12} and have higher basal expression of Glut1, allowing them to rapidly take up glucose after activation. These changes in metabolism allow CD56\textsuperscript{bright} cells to meet the biosynthetic and energy demands associated with the production of large amounts of IFN\gamma very rapidly upon activation. Indeed, high glycolytic rates have been directly linked to the production of IFN\gamma in murine NK cells\textsuperscript{31}, murine CD4\textsuperscript{+}\textsuperscript{54} and human CTLs\textsuperscript{116}. One mechanism suggested is that glycolytic enzymes can directly modulate IFN\gamma mRNA translation. The glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) catalyzes the first step in the oxidation of triose phosphates. However, GAPDH can also bind to IFN\gamma mRNA reducing IFN\gamma production by the cell. This suggests that in highly glycolytic
cells when GAPDH is being used for the catalysis of the glycolysis, IFN-γ mRNA is free to be translated. This mechanism may also be happening in human NK cells in which high glycolysis is linked to higher IFNγ protein production per cell\textsuperscript{54}. However, there are other glycolytic enzymes that can also bind to mRNA\textsuperscript{117} which could be important for IFNγ production in lymphocytes.

In contrast, CD56\textsuperscript{dim} NK cells are heterogeneous in their metabolic response to cytokine. Only a subset of these cells upregulate CD71, Glut1, and 2-NBDG uptake in response to cytokine. This might be explained by the fact that the principle function for CD56\textsuperscript{dim} NK cells is cytotoxicity, and although cytokine can increase NK cell cytotoxicity, freshly isolated NK cells are able to kill target cells without any additional stimulation. Indeed, they are primed for this function with high constitutive levels of Granzyme B. Therefore, there is not a substantial biosynthetic burden on cytokine-stimulated CD56\textsuperscript{dim} cells. However, it should be noted that a subset of CD56\textsuperscript{dim} NK cells also make substantial amounts of IFNγ, and our data support that these cells also upregulate their metabolism to deal with the associated increased biosynthetic demands as CD56\textsuperscript{dim} NK cells that produced IFNγ had increased surface expression of CD71.

In the present study, we only analyzed the expression of the glucose transporter Glut1 on cell surface of NK cells subsets. Glut1 is known to be highly expressed in glycolytic active cells. However, Glut3 and Glut4 is also able to uptake glucose and are expressed on NK cell surface\textsuperscript{118,119}. Investigation of different glucose receptors and their expression on human NK cells subsets would be valuable to understand their importance in NK cell metabolism.

Quantity of glucose uptake by a cell can be detected by different techniques. In our laboratory we used the glucose analogue 2-NBDG to determine glucose uptake by flow cytometry. Recently, Dr. Doreen Cantrell and her group discovered that 2-NBDG is not uptake by Glut1 (personal communication). Therefore, when our work was published, we used 2-NBDG as indicator of glucose uptake, however now we consider 2-NBDG uptake together with CD71 and CD98 surface markers as a general indicator of increased cell metabolism\textsuperscript{80}.
Taken together the observed metabolic differences between CD56\textsuperscript{bright} cells and CD56\textsuperscript{dim} cells lead to the hypothesis that CD56\textsuperscript{bright} cells upregulate their metabolism at higher levels than their counterparts. Detailed metabolic profile of CD56\textsuperscript{bright} cells and CD56\textsuperscript{dim} cells should be carried out to observe specific metabolic differences between the NK subsets. However, this was not possible due to limitation of cell numbers. Many groups worldwide make use of cells isolated from blood packs which contains high numbers of blood cells however, blood pack regulation for science is different in each country. In Ireland, blood packs are required to be screened for diseases so they are kept in 4°C for usually 2 days prior to delivery. We have investigated using blood packs as a source of NK cells but we observed many differences between freshly isolated NK cells and blood packs. Cells kept in 4°C for 2 days died quickly in culture and had higher variability of IFNγ production (data not shown). These cells might be useful for identifying proteins and specific cellular mechanisms but not for metabolic studies. Therefore, in this work, analysis of metabolic profile of human NK cells after cytokine stimulation was analyzed using bulk population of NK cells together and an overall metabolic change was determined. We observed that cytokine-activated NK cells exhibited increased glycolysis and OxPHOS when compared to resting NK cells and this finding correlates with other lymphocytes that only upregulate metabolism upon stimulation\textsuperscript{73}.

We have shown that elevated levels of OxPHOS are particularly important for the function of human NK cells after short term stimulation with IL-2 or IL-12/15 cytokines. OxPHOS is important to fuel efficient ATP synthesis that is required for activated NK cell function. OxPHOS can also support cellular biosynthesis as it facilitates the conversion of glutamine, and other fuels, into precursors for biosynthetic pathways. In general, activated lymphocytes tend to make more ATP through glycolysis, decreasing their reliance on OxPHOS. In this way, glycolytic lymphocytes can maintain ATP homeostasis when OxPHOS is repressed, as might occur at hypoxic inflammatory sites or within hypoxic tumors. Nonetheless, this does not seem to be the case for 18 hours cytokine-stimulated human NK cells, which need OxPHOS for normal function. However, recent research suggests that the timescale for metabolic reprogramming of murine and human lymphocytes may be different. In human T lymphocytes, glycolytic reprogramming may occur over the
course of 72 hours as opposed to the 24 hours required in murine T cells\textsuperscript{112, 120}. Therefore, cytokine-stimulated human NK cells may further upregulate glycolytic metabolism beyond the 18 hours observed in this study, which might be predicted to decrease their reliance on OxPHOS. Nevertheless, over the time course relevant for early innate immune responses, it is clear that elevated OxPHOS is critical for optimal NK cell responses. It will be of interest to study NK cell metabolism and function in the context of NK cells that function alongside the adaptive immune (see chapter 5). After exploring the importance of OxPHOS we investigated the requirement of glycolysis for NK cells effector functions. We noticed reduced IFN\(\gamma\) production per cell when we provided an alternative carbon source (galactose) and limited the rate of glycolysis in the cell. Cells stimulated with IL-12/15 with reduced glycolysis had decreased frequency of CD56\textsuperscript{bright} cells producing IFN\(\gamma\) and a significant reduction in the amount of IFN\(\gamma\) being produced per cell arguing that elevated glycolysis in human NK cells is required for maximal IFN\(\gamma\) responses.

Our laboratory published data showing that mTORC1, a key metabolic regulator, is increased in cytokine stimulated murine NK cells and its activity is important for their effector functions\textsuperscript{31}. My data indicates that both CD56\textsuperscript{bright} cells and CD56\textsuperscript{dim} cells had elevated levels of mTORC1 activity through measuring the phosphorylation of pS6 after cytokine stimulation. To further confirm the activation and importance of mTORC1 on NK cell metabolism, cells were cultured with cytokines and rapamycin, an mTORC1 inhibitor. In murine NK cells, CD71 and CD98 are dependent of mTORC1 and we observed similar results with human NK cells\textsuperscript{31}. Furthermore, an important finding was that cytokine combinations drive different metabolic mechanisms in human NK cells. Both IL-12/15 and IL-2 upregulated glycolysis and glycolytic capacity to similar levels. However, IL-2 induced increases in glycolysis and glycolytic capacity were mTORC1 dependent, whereas the IL-12/15-induced responses were not. These data argue that IL-2 has the potential to drive mTORC1-dependent changes to glucose metabolism, as we have previously observed with cytokine-expanded murine NK cells and for murine T cells\textsuperscript{31, 83}. However, there are also examples in murine and human lymphocytes where mTORC1-independent mechanism promote increased glycolytic metabolism\textsuperscript{116, 121}. Our study suggests that NK cells can engage both mTORC1-dependent and – independent mechanism in response to distinct cytokine stimulations. Based on our
data, we suggest that there might be temporal changes in NK cell metabolism that are dependent on the nature of cytokines present. Cytokines such as IL-12/15 may upregulate NK cell metabolism and functions for immediate and potent responses, of which IFNγ is particularly important. In contrast, although IL-2 can drive a more modest short-term IFNγ response, it can potentially drive a more sustained NK cell activation that allows NK cells to function in parallel to the adaptive immune response over a longer time period. In this context, controlling NK cell metabolism through mTORC1 potentially provides an additional level of control over NK cell function. This is because mTORC1 activity is acutely sensitive to conditions in the immune microenvironment, most notably the availability of nutrients as discussed in the introduction of this thesis.

In our experiments, to investigate NK cell effector functions we stimulated NK cells with high concentrations of IL-2, IL-12 and IL-15 cytokines. However, these cytokines have been shown to activate NK cell apoptosis pathway, possibly to regulate and limit NK cell functions. In our experiments, cell viability was measured by flow cytometry and no major cell death was observed. However, this does not exclude the possibility that apoptosis pathways are being activated. This may be worth investigating in future experiments.

In summary, this chapter has investigated and described the metabolic difference between NK cells subsets from freshly isolated blood. Our findings indicate that increase in metabolism is required for optimal NK cell effector functions. When OxPHOS was inhibited in cytokine-stimulated NK cells, significant impaired effector functions was observed. Furthermore, optimal rates of glycolysis is important for IFNγ production. Differences were observed between CD56^{bright} cells and CD56^{dim} NK cells, suggesting that CD56^{bright} NK cells are more metabolic active compared to their counterparts. Our findings indicate that NK cells subsets can differ not only in their functions as previously discussed (section 1.1.2) but in their metabolism as well.
Chapter 4
Investigation of the effects of TGF-β on human NK cell metabolism and function
4.1 Introduction

Cellular metabolism impacts significantly on immune cells fate and function\(^{123}\). I have shown in chapter 3 that human NK cells metabolism is upregulated upon cytokine stimulation and this metabolic upregulation is crucial for optimal effector functions. The activity of master regulator of metabolism mTORC1 is increased in cytokine stimulated NK cells and is important for upregulation of glycolysis in IL-2 stimulated NK cells. However, downregulation of metabolic responses is likely to be an important mechanism of immune regulation. Furthermore, we predicted that biological molecules able to regulate NK cell function might also regulate NK cell metabolism.

TGF-β is an important cytokine with pleiotropic effects in the immune system. TGF-β is involved in many cellular processes including cell migration, cell proliferation, extracellular matrix remodeling and immune-suppression. TGF-β signaling pathway can be divided into canonical and non-canonical pathways as explained at section 1.4. Briefly, when TGF-β binds to the TGF-β receptors, phosphorylation of SMAD2 and SMAD3 occur allowing these proteins to form a complex with SMAD4, which translocates and accumulates in the nucleus. This SMAD complex is capable of interacting with various transcription factors leading to activation or repression of genes. In the non-canonical pathway, TGF-β signaling does not require SMADs and activate directly different pathways like PI3K/AKT, MAPK pathways (ERK, JNK, and p38 MAPK), NF-κB and others\(^{92}\).

Different human immune cell types can be regulated by TGF-β (section 1.4.1 and Figure 1.8) and it has long been recognised as a potent inhibitor of NK cell effector functions\(^{64,99}\). Chronic overexpression of TGF-β can be found in cancer, fibrosis and inflammation. High expression of TGF-β in cancer immune environment is related to poor prognosis as TGF-β blocks antitumor functions of CD8+T cells, CD4+T cells, dendritic cells and NK cells. TGF-β inhibits NK cell proliferation, function and the expression of NKp30 and NKG2D receptors, important for killing target cells\(^{70,100,104,124}\). Thus, inhibition of TGF-β is a good target for potentially increasing NK cell immune function. In terms of metabolism, it is reported that TGF-β inhibited IL-15 induced expression of nutrient receptors (CD71 and CD98), and downregulated glycolysis and OxPhos in murine NK cells\(^{105}\). Its key mechanism of action was
proposed to be through inhibition of mTORC1 and not through the canonical TGF-β signalling pathway, as TGF-β directly inhibited mTORC1 signalling induced by IL-15 in murine NK cells\textsuperscript{105}. Given our previous data supporting an important role for mTORC1 in IL-2 induced metabolic reprogramming of human NK cells, we investigated if and how TGF-β regulates metabolic changes in human NK cells.
4.2 Results

4.2.1 TGF-β inhibits NK cell metabolism

It is well known that TGF-β inhibits NK cell functions\textsuperscript{37,64,98}. As we have previously shown that elevated rates of glycolysis and OxPhos are required for cytokine induced NK cell effector function\textsuperscript{31,123}, we undertook a series of experiments to investigate if TGF-β also inhibited NK cell metabolism. Our previous results demonstrated that human NK cells stimulated for 18 hours with IL-2 had increased rates of glycolysis and OxPhos. To investigate the impact of TGF-β on these metabolic pathways, NK cells were purified from PBMC and stimulated with IL-2, in the presence or absence of TGF-β prior to metabolic analysis. TGF-β significantly decreased the rate of IL-2-induced mitochondrial metabolism in human NK cells. It inhibited the rates of OxPhos and maximal respiration (Figure 4.1 A-C). While TGF-β treatment did not alter the basal rate of glycolysis, it inhibited the glycolytic capacity of IL-2 stimulated NK cells (Figure 4.2 A-C).

Analysis of nutrient receptor expression revealed that TGF-β inhibited IL-2 induced expression of the CD71, the transferrin receptor, on CD56\textsuperscript{bright} NK cells but not that of CD98, a component of the L-amino acid transporter (Figure 4.3 and Figure 4.4). IL-12/15 also induced expression of both CD71 and CD98 receptors and while CD71 expression was inhibited by TGF-β over a range of IL-12/15 concentrations, there was no consistent effect of TGF-β on the expression of CD98 (Figure 4.3 and Figure 4.4). Taken together, these data indicates that TGF-β inhibits NK cell metabolic markers and NK cell metabolism.
Figure 4.1 NK cell OxPHOS is impaired by TGF-β.

NK cells from healthy blood donors were purified and stimulated for 18 h with IL-2 (500 U/ml) or left unstimulated. TGF-β (10 ng/mL) was added as indicated. Detailed metabolic analysis was performed using the Seahorse extracellular flux analyser. (A) Representative traces for the OCR for unstimulated cells or in response to IL-2 with or without TGF-β are shown. (B-C) Individual paired responses with or without TGF-β for OxPhos (B) and maximal respiration. (n = 6 donors). Samples were compared using a paired Student t-test. ns = not significant. * P ≤ 0.05.
Figure 4.2 NK cell glycolysis is impaired by TGF-β.

NK cells from healthy blood donors were purified and stimulated for 18 h with IL-2 (500 U/ml) or left unstimulated. TGF-β (10 ng/mL) was added as indicated. Detailed metabolic analysis was performed using the Seahorse extracellular flux analyser. (A) Representative traces for the ECAR in response to IL-2 with or without TGF-β are shown. Individual paired responses with or without TGF-β for Glycolysis (B) and for Glycolytic capacity (C) are shown for IL-2 stimulation (n = 6 donors). Samples were compared using a paired Student t-test. ns = not significant. *P ≤ 0.05.
Figure 4.3 TGF-β inhibits CD71 expression on human NK cells.

Freshly isolated PBMC were stimulated for 18 h with either IL-2 (500 U/ml) or various concentrations of IL-12/IL15 as indicated TGF-β was used at 10ng/ml. (A) Representative dot plots of CD71, the transferrin receptor, expressed on unstimulated NK cells or IL-2 stimulated cells in the presence or absence of TGFβ as indicated. (B) Pooled data showing the frequency of CD56bright cells positive for CD71 after stimulation with either IL-2 or IL-12 (30 ng/ml)+IL-15 (100 ng/ml), in the presence or absence of TGF-β (n = 10-12 donors). (C) Pooled data showing the frequency of CD56bright cells positive for CD71 after stimulation with either IL-12/IL15 at (10ng/ml and 30ng/ml) or (2ng/ml and 6 ng/ml) respectively, in the presence or absence of TGF-β (n = 6 donors). Bars show the average frequency and individual data points are shown. The panels on the right show paired data sets. Samples were compared using a paired Student t-test. *P ≤ 0.05, ***P ≤ 0.001.
Figure 4.4 TGF-β does not inhibit CD98 expression on human NK cell.

Freshly isolated PBMC were stimulated for 18 h with either IL-2 (500 U/ml) or various concentrations of IL12+IL15 as indicated; TGF-β was used at 10ng/ml. (A) Representative dot plots of CD98, a component of the L-amino acid transporter, expressed on unstimulated NK cells or IL-2 stimulated cells in the presence or absence of TGF-β as indicated. Freshly isolated PBMC were stimulated for 18 h with either IL-2 or IL-12 + IL-15 in different concentrations or left unstimulated. TGF-β (10 ng/ml) was added as indicated. NK cells were stratified into CD56<sup>bright</sup> and CD56<sup>dim</sup> subsets. (B) Pooled data of CD56<sup>bright</sup> (left panel) and CD56<sup>dim</sup> (right panel) NK cells subsets expressing CD98 after stimulation with IL-2 (500 U/ml) or IL-2 (100 U/ml). (C) Pooled data of CD56<sup>bright</sup> (left panel) and CD56<sup>dim</sup> (right panel) NK cells subsets expressing CD98. Cells were stimulated with either IL-12/15 at (30ng/ml and 100ng/ml), (10ng/ml and 30ng/ml) or (2ng/ml and 6 ng/ml) respectively, in the presence or absence of TGF-β. (n = 6 - 10 donors).
4.2.2 TGF-β inhibits NK cell effector function

We also confirmed that TGF-β inhibited some key NK cell functions as seen by others. NK cells activated by cytokine (either IL-2 or IL-12/15), inhibited the frequency of CD56^{bright} and CD56^{dim} cells that produced IFN_{γ}, and the amount of IFN_{γ} that NK cells expressed per cell (MFI) in response to IL-12/15 stimulation (Figure 4.5 A-C). Expression of CD69, commonly used as a marker of activation in peripheral blood NK cells, was highly expressed in both CD56^{bright} and CD56^{dim} subsets after stimulation with IL-2 and IL-12/15; this upregulation was inhibited in both NK subsets when cells were stimulated with cytokines in the presence of TGF-β (Figure 4.6 A-C). TGF-β inhibited both granzyme B (Figure 4.7 A-C) and TRAIL (Figure 4.8 A-B) expression only on CD56^{bright} NK cells, as might be expected from their induced expression on CD56^{bright} cells in response to cytokine. These findings support our knowledge that TGF-β significantly impairs NK cell effector functions and we have detailed shown that TGF-β inhibition is similar in in both CD56^{bright} and CD56^{dim} subsets.
Figure 4.5 TGF-β impairs NK cell IFNγ production and expression by human NK cells.

PBMC were stimulated for 18 h with IL-12 (30 ng/ml)+IL-15 (100 ng/ml) or left unstimulated. TGF-β (10 ng/mL) was added where indicated. (A) Representative dot plots of IFNγ production by NK cells. Frequency (B) and expression (MFI) (C) of IFN-γ producing CD56^{bright} (left) and CD56^{dim} (right) NK cells are shown for IL-12/15 stimulation in the presence of absence of TGF-β; individual paired samples are shown, n=8 donors. Samples were compared using paired Student t-test. ns = not significant. * P ≤ 0.05, ** P ≤ 0.01, **** P ≤ 0.0001.
Figure 4.6 TGF-β impairs CD69 expression on human NK cells.

PBMC were stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. TGF-β (10 ng/mL) was added where indicated. Representative dot plots of CD69 expression on NK cells (A), and individual paired frequencies of CD69 expression on NK cells stimulated with either IL-2 or IL-12/IL15, in the presence of TGF-β for CD56^bright (B) and CD56^dim (C) subsets are shown, n=11 donors. Samples were compared using paired Student t-test. ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.
Figure 4.7 TGF-β impairs granzyme B production by human NK cells.

PBMC were stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. TGF-β (10 ng/mL) was added where indicated. Representative histograms of granzyme B expression on NK cells (A), and individual paired expression (MFI) of granzyme B expression on NK cells stimulated with either IL-2 or IL-12/IL15, in the presence of TGF-β for CD56bright (C) and CD56dim (D) subsets are shown, n=8 donors. Samples were compared using paired Student t-test. ns = not significant, * P ≤ 0.05, ** P ≤ 0.01.
Figure 4.8 TGF-β treated NK cells have reduced TRAIL expression.

PBMC were stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. TGF-β (10 ng/mL) was added where indicated. Representative dot plots of TRAIL expression on NK cells (A), and individual paired frequencies of TRAIL expression on CD56 bright NK cells stimulated with either IL-2 (left panel, n=9 donors) or IL-12/IL15 (right panel, n=12 donors), in the presence or absence of TGF-β as indicated. Samples were compared using paired Student t-test. *P ≤ 0.05, **P ≤ 0.01.
4.2.3 Non-canonical TGF-β signaling pathway does not play a role in regulating short-term NK cell activation in response to cytokine

The canonical TGF-β pathway involving SMAD2/3 has previously been described to be important in NK cells\textsuperscript{101, 125}. However, a recent study in murine NK cells suggested that TGF-β represses murine NK cell metabolism and function through the inhibition of mTORC1 signalling\textsuperscript{105}. Here, we undertook experiments to investigate potential mechanisms of TGF-β modulation of human NK cell metabolism and function. Given that we have previously shown that mTORC1 is required for cytokine-induced metabolism and that mTORC1 is activated in NK cells cultured overnight in cytokine, we first considered whether TGF-β could impact on mTORC1 signalling under these conditions. To investigate, we analysed two readouts of this signalling pathway: S6 ribosomal protein phosphorylated on serine 235/6 (pS6) and levels of 4EBP1 phosphorylated on threonine 37/46 (p4EBP1), both are direct mTORC1 substrate. IL-2 stimulation increased pS6 levels in both CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cell subsets and this was inhibited by the mTORC1 inhibitor rapamycin as expected (Figure 4.9 A) and as previously reported\textsuperscript{123}. Somewhat surprisingly, TGF-β treatment did not inhibit pS6 levels in our experiments (Figure 4.9 A-E). We generated similar results for IL-12/15 stimulated cells (Figure 4.9 B-E). Additionally, both IL-2 and IL-12/15 increased levels of p4EBP1 and the mTORC1/2 inhibitor AZD8055 reduced p4EBP1 levels as expected. However, TGF-β treatment did not reduce 4EBP1 phosphorylation in IL-2 or IL-12/15 stimulated NK cells (Figure 4.10 A-E). Taken together, these data show that TGF-β does not inhibit sustained mTORC1 signalling in NK cells stimulated with cytokine for 18 hours.

In the report describing TGF-β inhibition of mTORC1 in murine NK cells, pS6 signal was measured after 1 hour stimulation with high dose IL-15\textsuperscript{105}. Given that TGF-β could potentially inhibit early signalling events with effects on downstream effector functions, we wanted to address whether mTORC1 signalling in human NK cells was sensitive to TGF-β at these early time points. We therefore measured pS6 signal by flow cytometry after 30 minutes and 1 hour of stimulation, over a range of IL-2 or IL-12/15 concentrations, in the presence or absence of TGF-β. While there was donor variation in terms of the magnitude of responses, both cytokine combinations potently activated mTORC1. However, TGF-β had no effect of pS6 expression under
these experimental conditions (Figure 4.11 A-B and Figure 4.12 A-B). As the study by Viel et al. showed comparable effects of TGF-β and rapamycin on human NK cell functional outputs after 36 hours IL-2-stimulation we considered whether TGF-β inhibits mTORC1 in NK cells stimulated with IL-2 for extended time periods. We therefore stimulated human NK cells in IL-2 for 5 days in the presence and absence of TGF-β. After this time, mTORC1 signalling was activated with increased levels of pS6 and p4EBP1 in human NK cells. However, and in contrast to the shorter stimulation periods (30 mins, 1 hour, 18 hours), TGF-β did inhibit the increased mTORC1 activation (Figure 4.13 A-C). These data suggest that the kinetics of mTORC1 activation and inhibition are important factors and while TGF-β has the potential to inhibit mTORC1 induced activity at extended time points, it was unlikely to be the key mechanism for the TGF-β-mediated inhibition of NK cell metabolism in this current study.
Figure 4.9 TGF-β does not inhibit sustained mTORC1 activity in cytokine stimulated NK cells.

PBMC were stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. TGF-β (10 ng/mL) was added where indicated. Cells were then analysed for pS6 by intracellular flow cytometry staining. (A) Representative dot plots of mTORC1 activity. (B-C) pS6 expression on (B) CD56^{bright} and (C) CD56^{dim} NK cells are shown for IL-2 and IL-12/15 stimulation, in the presence or absence of TGF-β. (D-E) Cells were analyzed for pS6 MFI levels by intracellular flow cytometry staining. Individual data points are shown for CD56^{bright} (D) and CD56^{dim} (E) NK cells subsets. (n = 9 - 11 donors). Samples were compared using paired Student t-test. ns = not significant.
Figure 4.10 TGF-β treated NK cells have no changes in phosphorylation of 4EBP1 protein.

PBMC were stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. TGF-β (10 ng/mL) was added where indicated. Cells were then analysed for p4EBP1 by intracellular flow cytometry staining. (A) Representative dot plots of mTORC1 activity. (B-C) p4EBP1 expression on (B) CD56<sup>bright</sup> and (C) CD56<sup>dim</sup> NK cells are shown for IL-2 and IL-12/IL-15 stimulation, in the presence or absence of TGF-β. (D-E) Individual data points are shown for CD56<sup>bright</sup> (D) and CD56<sup>dim</sup> (E) NK cells subsets for p4EBP1 MFI expression. (n = 9 - 11 donors). Samples were compared using paired Student t-test. ns = not significant.
Figure 4.11 TGF-β does not inhibit short-term cytokine induced mTORC1 activity in human NK cells.

PBMC (n=7 donors) were stimulated for (A) 30 minutes or (B) 1 hour with either IL-2 (500 U/ml) or IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. TGF-β (10 ng/ml) was added where indicated. Cells were then stratified into CD56\textsuperscript{bright} cells (left panel) and CD56\textsuperscript{dim} cells (right panel) and analyzed for pS6 levels by flow cytometry. Bars show the average value ±SEM; individual sample points are also shown. Samples were compared using one-way ANOVA followed by a Kruskal–Wallis post hoc test. ns = not significant.
Figure 4.12 TGF-β does not inhibit the expression or MFI of pS6 in short-term cytokine stimulated human NK cells.

PBMCs were stimulated for 1 hour with either IL-2 (100 U/mL) or with either IL-12+IL-15 at (10ng/ml and 30ng/ml) or (2ng/ml and 6 ng/ml) respectively. TGF-β (10 ng/ml) was added as indicated. Cells were analyzed for pS6 expression of CD56bright (left panel) and CD56dim (right panel) NK cells subsets (A) and MFI (B) by intracellular flow cytometry staining. (n = 8 donors).
Figure 4.13 TGF-β inhibits cytokine induced mTORC1 activity in human NK cells at longer time points.

PBMC were cultured for 5 days in IL-2 (100U/ml) in the presence or absence of TGF-β (10ng/ml). Representative dot plots of pS6 expression in NK cells are shown in (A) while (B) shows the frequency (left panel) and MFI (right panel) of pS6 for paired samples of NK cells cultured in IL-2 with or without TGF-β, n=6 donors. (C-D) Representative histogram of p4EBP1 expression in NK cells is shown in (C) while (D) shows the frequency (left panel) and MFI (right panel) of p4EBP1 for paired samples of NK cells cultured in IL-2 with or without TGF-β, n=6 donors. Samples were compared using a paired Student t-test. ns, not significant. * P≤0.05.
4.2.4 Canonical TGF-β signaling pathways repress NK cell metabolism and function

We turned to the canonical TGF-β signal transduction pathway to investigate its potential contribution to the TGF-β modulation of NK cell metabolism and function that we observed. To address this, an inhibitor of the TGF-β receptor 1 subunit, SB431542, was utilised which inhibits the TGF-β type I receptor activating receptor-like kinase ALK5, ALK4 and ALK7 but not BMP, ERK, JNK or p38 MAP kinase pathways126. Purified NK cells were stimulated with IL-2 and TGF-β in the presence or absence of SB431542 and effector functions investigated. SB431542 mediated inhibition of TGF-βR1 resulted in enhanced IFNγ production (Figure 4.14 A-B), CD71 (Figure 4.15 A-B), CD69 (Figure 4.16 A-C) and TRAIL (Figure 4.17 A-B) expression in cytokine activated NK cells treated with TGF-β.

In terms of metabolism, SB431542 treatment restored mitochondrial metabolism in IL-2+TGF-β stimulated NK cells with complete restauration of OxPHOS (Figure 4.18 A-C) and the maximal respiratory capacity (Figure 4.18 D-E). The effect of TGF-β on the glycolytic capacity in this set of experiments was less consistent but nevertheless there was a trend towards increased glycolytic capacity in SB431542 treated NK cells (Figure 4.19 A-E). Therefore, SB431542 restored most of the metabolic and functional responses of NK cells that were inhibited by TGF-β, suggesting that the canonical TGF-β signalling pathway is the predominant pathway in human NK cells.
Figure 4.14 Impaired IFN-γ production by TGF-β is restored with TGF-βRI inhibitor.

PBMC were stimulated for 18 h with IL-12 (30 ng/ml) + IL-15 (100 ng/ml) or left unstimulated for functional analysis. TGF-β (10 ng/mL) and the TGF-βRI inhibitor SB431542 (5µM) was added as indicated. (A) Representative dot plots of IFN-γ production by NK cells. (B) Frequency of IFN-γ producing CD56^{bright} (left) and CD56^{dim} (right) NK cells are shown for IL12/15+ TGF-β stimulated cells in the presence or absence of SB431542, n=7 donors. Samples were compared using paired Student t-test. ns = not significant. * P ≤ 0.05.
Figure 4.15 Impaired CD71 expression by TGF-β is restored with TGF-βRI inhibitor.

PBMC were stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml) +IL-15 (100 ng/ml), or left unstimulated for CD71 expression. TGF-β (10 ng/mL) and the TGFβRII inhibitor SB431542 (5µM) was added as indicated. (A) Representative dot plots of CD71 expression by NK cells. (B) Frequency of CD56<sup>bright</sup> NK cells positive for CD71 after stimulation with either IL-2 (left panel) or IL-12/15 (right panel) in the presence or absence of SB431542. (n=7 donors). Samples were compared using paired Student t-test. *P ≤ 0.05.
Figure 4.16 Impaired CD69 expression by TGF-β is restored with TGF-βRI inhibitor.

PBMC were stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml) + IL-15 (100 ng/ml), or left unstimulated for functional analysis. TGF-β (10 ng/mL) and the TGFβRI inhibitor SB431542 (5µM) was added as indicated. (A) Representative dot plots of CD69 expression by NK cells. (B-C) Frequency of CD56\textsuperscript{bright} (B) and CD56\textsuperscript{dim} (C) NK cells positive for CD69 after stimulation with either IL-2 (left panel) or IL-12/15 (right panel) in the presence or absence of SB431542. (n=7 donors). Samples were compared using paired Student t-test. ns = not significant. *P ≤ 0.05.
Figure 4.17 Impaired TRAIL expression by TGF-β is restored with TGF-βRI inhibitor.

PBMC were stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml) + IL-15 (100 ng/ml), or left unstimulated for functional analysis. TGF-β (10 ng/mL) and the TGFβRI inhibitor SB431542 (5µM) was added as indicated. (A) Representative dot plots of TRAIL expression by NK cells. (B) Frequency of CD56<sup>bright</sup> NK cells positive for TRAIL after stimulation with either IL-2 (left panel) or IL-12/15 (right panel) in the presence or absence of SB431542. (n=7 donors). Samples were compared using paired Student t-test. ns = not significant. *P ≤ 0.05.
Figure 4.18 Impaired OxPHOS in cytokine stimulated NK cells is restored with TGFβRI inhibitor.

NK cells were purified and stimulated for 18 h with IL-2 (500 U/ml) or left unstimulated. TGFβ (10 ng/mL) and the TGFβRI inhibitor SB431542 (5µM) was added as indicated. Detailed metabolic analysis was performed using the Seahorse extracellular flux analyzer. (A) Representative traces for the OCR in response to IL-2 with or without TGFβ and SB431542 are shown. Pooled data (B) and individual paired responses (C) for Oxphos in response to IL-2 with or without TGFβ and SB431542 as indicated. Pooled data (D) and individual paired responses (E) for maximum respiration in response to IL-2 with or without TGFβ and SB431542. n=8 donors. Samples were compared using paired Student t-test. ns = not significant. *P ≤ 0.05, **P ≤ 0.01.
Figure 4.19 Glycolysis and glycolytic capacity do not change in the presence TGF-β with TGFβRI inhibitor.

NK cells were purified and stimulated for 18 h with IL-2 (500 U/ml) or left unstimulated. TGF-β (10 ng/mL) and the TGFβRI inhibitor SB431542 (5µM) was added as indicated. Detailed metabolic analysis was performed using the Seahorse extracellular flux analyzer. (A) Representative traces for the ECAR in response to IL-2 with or without TGF-β and SB431542 are shown. (B-C) Pooled data (B) and individual paired responses (C) for glycolysis in response to IL-2 with or without TGF-β and SB431542. (D-E) Pooled data (D) and individual paired responses (E) for glycolytic capacity in response to IL-2 with or without TGF-β and SB431542. n=8 donors. Samples were compared using paired Student t-test. ns = not significant. *P ≤ 0.05, **P ≤ 0.01.
4.3 Discussion

We have shown that cytokines cause metabolic changes in NK cells and these changes are important for optimal NK cell effector functions. However, little is known about how these processes are controlled and regulated. Here, we define that TGF-β, which is known to inhibit many NK cell functions also inhibits many aspects of human NK cell metabolism. IL-2 cytokine induced metabolic reprogramming in human NK cells towards increased glycolysis and OxPHOS during 18 hours as previously reported, and TGF-β inhibited many of these metabolic changes. We have shown that the negative effect of TGF-β on NK cell metabolism and effector function was regulated through the canonical TGF-β pathway, supporting the importance of this pathway in human NK cell regulation.

TGF-β functions are important physiologically to preserve balance within the organism. Tissue differentiation, tissue regeneration, cell proliferation and immune regulation are tightly controlled by TGF-β signaling (section 1.4). If TGF-β signaling is abnormal, recruitment of excessive cells can lead to different diseases like tissue fibrosis or suppression of immune cells effector functions which facilitate the advance of cancerous cells. Nevertheless, NK cells have endogenous TGF-β possibly to maintain cell homeostasis which is important to avoid excessive inflammation in the body.

Stimulation of freshly isolated human NK cells with TGF-β for 18 hours affected CD56bright cells and CD56dim cells in a similar fashion. CD71, transferrin receptor, is a general marker for increased cell metabolism. We have shown that CD71 is mainly expressed in CD56bright and here we demonstrated that TGF-β significantly impairs CD71 expression on these NK cells. CD69 is highly expressed in both NK cell subsets and was significantly reduced by TGF-β. While CD56bright cells are known to produce large quantities of IFNγ, CD56dim cells are generally considered to be more cytotoxic but also able to produce IFNγ in short periods of time (4 hours) after receptor activation. Therefore, it is not surprising that TGF-β strongly inhibited IFNγ production in both subsets. TRAIL is mainly expressed in CD56bright cells and its expression was also significantly impaired by TGF-β suggesting that NK cell killing potential is impaired. It is known that CD56dim cells have a higher basal expression of granzyme B then CD56bright cells. After stimulation with cytokines, CD56dim
cells increase slightly granzyme B expression, on the other hand, striking differences can be seen in granzyme B expression by CD56^{bright} cells. TGF-β did not interfere in the basal expression of granzyme B in CD56^{dim} cells but significantly reduced the expression of granzyme B by CD56^{bright} cells. We have shown that many cytokine induced NK cell effector functions are impaired in cells exposed to TGF-β and others have shown that TGF-β reduces direct NK cell killing^{68, 105}. Therefore it would be informative to investigate NK cell killing in future studies.

We have previously shown that inhibition of glycolysis impaired IFN_{γ} production by NK cells while inhibition of OxPHOS inhibited a broader range of effector responses including IFN_{γ} production and NK cell degranulation, which is important for cytotoxicity. As TGF-β had no effect on basal rates of glycolysis, inhibition of IFN_{γ} production by TGF-β was independent of this mechanism of regulation. Our earlier study used pharmacological inhibition of OxPHOS with low dose oligomycin that limits the rate of ATP production. Here, we provide evidence that TGF-β also inhibits OxPHOS. We previously observed a stronger inhibition of NK cell functions by oligomycin in the CD56^{bright} subset of NK cells, and when IL-2, rather than IL-12/15, was used as a stimulus. These trends strongly correlate with the TGF-β data obtained here. Together, the data suggest that downregulation of mitochondrial metabolism by TGF-β may represent a physiological mechanism to regulate NK cell functions.

Most data in the literature supports that the canonical pathway of TGF-β signalling is important in NK cells^{70, 101}. However, it has recently been suggested that TGF-β may also potentially signal through an alternative pathway that attenuates mTORC1 signalling^{105}. Our data support that TGF-β effects on early metabolic and functional changes in NK cells were predominantly mediated through the canonical TGF-β signalling pathway and not through inhibition of mTORC1. Direct comparison of mTORC1 activity in NK cells from the study of Viel et al and our data is shown in Figure 4.20. It can be seen that inhibition of mTORC1 activity by TGF-β in Viel et al are modest and our data set for similar time points show similar modest decreases for some donors. However, other samples had either no change or increased slightly mTORC1 activity (Figure 4.20). This suggests that the data observed were fairly similar but differed in the conclusion based on statistics. Analysis at longer time
points in our experiments found that mTORC1 signaling is inhibited after prolonged TGF-β signaling. This is consistent with the functional data of Viel et al (36 hours) and somewhat reconcile discrepancies; however, they did not directly measure mTORC1 signaling at this time point\textsuperscript{105}.

In our experiments, data suggests that TGF-β inhibition of NK cells metabolism and function was through the canonical pathway as we observed that TGF-β did not inhibit mTORC1 signalling in cytokine stimulated CD56\textsuperscript{dim} or CD56\textsuperscript{bright} NK cells. Supporting these findings, the inhibitor SB431542 reversed most of the TGF-β inhibitory effects back to baseline levels. Also, the fact that TGF-β and rapamycin have distinct inhibitory effects on NK cells was further evidence that TGF-β and mTORC1 were functioning independently at these early time points. Specifically, CD69 expression on NK cells was inhibited by TGF-β but not by rapamycin and conversely, the expression of CD98 was strongly inhibited by rapamycin but did not change in response to TGF-β. In terms of glucose metabolism, TGF-β had no effect on IL-2-induced glycolysis while we have previously shown that rapamycin strongly inhibited glycolytic metabolism in IL-2-stimulated NK cells\textsuperscript{31, 123}. It is worth noting that some different effects between rapamycin and TGF-β were also reported in the paper by Viel et al. For instance, after mTORC1 deletion in murine NK cells generation of different NK cell receptors like NKG2D and IL-12 receptor was inhibited, however constitutive TGF-β signaling did not interfere in these receptors.

Here we have shown that TGF-β signaling has a strong impact on human NK cell metabolism and effector functions which impairs NK cells when potent activation is needed, for example, in the context of a tumour. TGF-β levels in the tumor microenvironment may be high and oxygen levels low and our data suggest that the OxPHOS pathway may be preferentially affected by this environment and might be a better therapeutic target. Indeed, targeting TGF-β signalling may be a valid way to promote OxPHOS in NK cells to boost their function. While TGF-β did not affect basal glycolysis of NK cells above cytokine alone, it did inhibit their glycolytic capacity, a measure of the potential for glycolysis if required. Boosting glycolytic capacity \textit{ex vivo} has the potential to improve function and longevity of T cells\textsuperscript{127}. We suggest that this approach also has the potential to boost NK cell functions in various immunotherapy settings.
Figure 4.20 mTORC1 activity in IL-2 stimulated NK cells.

Differences of mTORC1 activity in NK cells stimulated for 1 hour in the presence or absence of cytokine and TGF-β adapted from Viel et al.\textsuperscript{105} publication (left panel) and our data (right panel). Data was compared by Student’s $t$-test. ns = not significant. **$P \leq 0.01$. 

Viel et al. IL-15 for 1 hour

Our data IL-2 for 1 hour
Chapter 5
Investigating the impact of prolonged metabolic disruption on IL-2 induced metabolism and function in human NK cells
5.1 Introduction

Immune cells isolated from blood of patients with chronic diseases like cancer often have impaired function, explained by the immunosuppressive cancer microenvironment\textsuperscript{71, 128, 129}. Cancer cells secrete many chemokines and cytokines able to suppress cytotoxic function of cells, this includes the immunoregulatory protein TGF-β which strongly inhibits production of IFNγ by NK cells and T cells\textsuperscript{65, 130, 131}. Crucial metabolic changes occur when immune cells are activated, providing energy and biomolecules necessary for optimal immune cell response and function. Disrupted metabolism in effector lymphocytes provides a reasonable explanation for why immune cells from patients are impaired and potentially contributes to the limited success of many therapies against cancer\textsuperscript{73, 132}. Indeed, it has been shown by others that metabolic competition between tumor cells and immune cells favors cancer progression while restraining effector cells function\textsuperscript{129}. Glycolytic tumors take up large quantities of nutrients and glucose, reducing the availability of carbon sources for immune cells\textsuperscript{129, 131}. Furthermore, oxygen availability is reduced in the tumor microenvironment and this hypoxic environment activate mechanisms to inhibit immune cells function through inhibition of metabolic pathways\textsuperscript{133}.

At Chapter 3 we revealed the importance of metabolism for sustaining optimal NK cell functions and IFNγ production. Acute stimulation of NK cells for 18 hours with IL-2 revealed that mTORC1, a key master regulator of metabolism, is important for upregulation of glycolysis and IFNγ production\textsuperscript{123}. IL-2 cytokine is produced by T cells and known to provide a link between adaptive and innate immunity, allowing NK cells to work alongside adaptive immune cells during chronic diseases like cancer and viral infection\textsuperscript{110}. It is becoming accepted that cancer can subject lymphocytes to conditions that restrict cellular metabolism in immune cells\textsuperscript{71}. Therefore, we considered whether in the context of the chronic inflammation associated with cancer, or other chronic diseases, restriction of NK cell metabolism would lead to defective NK cell effector responses. Therefore, the goal of this chapter was to replicate the inflammatory environment \textit{in vitro} to investigate whether metabolic restriction in this context leads to NK cell dysfunction, similar to what seen in patients with chronic inflammatory disease.
5.2 Results

5.2.1 mTORC1 is required for IL-2 upregulation of NK cell metabolism

We have previously observed that short-term (18 hours) stimulation of human NK cells with cytokines increased their metabolism and expression of metabolic markers compared to unstimulated NK cells, and these increases were linked to enhanced effector functions (chapter 3). We first determined the metabolic changes that NK cells undergo in response to stimulation for 5 days with the pro-inflammatory cytokine IL-2. As a control, we maintained NK cells in low dose IL-15 that supported NK cell survival but did not induce activation (hereafter called control cells). IL-2 stimulated NK cells had increased OxPHOS and glycolysis compared to control cells (Figure 5.1 A-B). Along with these observations, CD71 expression, the transferrin receptor, and uptake of the fluorescent glucose analogue 2-NBDG were lower in the control cells than in the IL-2 stimulated cells (Figure 5.1 C-D). These findings show that IL-2 stimulated NK cells are activated and undergo significant metabolic reprogramming compared to control cells.

Our first approach to investigate if restriction of NK cell metabolism led to defective NK cell effector responses was to culture NK cells for 5 days in IL-2, in the presence or absence of rapamycin. Rapamycin is an mTORC1 inhibitor that restricts IL-2-induced NK cell glycolysis and glycolytic capacity in 18 hour stimulated NK cells (chapter 3)\(^1\)\(^2\)\(^3\). Indeed, the elevated levels of CD71 expression and 2-NBDG uptake observed in IL-2 stimulated NK cells, were significantly decreased in cells cultured in IL-2 plus rapamycin (Figure 5.2 A-C).

To investigate changes in metabolism, we undertook detailed analysis of NK cell metabolism using the extracellular flux analyzer. IL-2 cultured NK cells had high rates of OxPHOS, maximum respiration, glycolysis and glycolytic capacity that were significantly reduced in rapamycin treated NK cells to levels comparable with control NK cells (Figure 5.3 and Figure 5.4). To further explore the observed differences in OxPHOS in NK cells treated with rapamycin a number of mitochondrial parameters were measured; mitochondrial mass, the expression of the ATP synthase subunit ATP5B and mitochondrial membrane potential. Rapamycin treatment prevented IL-2 dependent increases in these mitochondrial...
parameters, consistent with the OxPHOS rates observed in these NK cells (Figure 5.5 A-D and Figure 5.6 A-B). We also measured mitochondrial ROS production and found this to be unchanged with rapamycin treatment (Figure 5.6 C).
Figure 5.1 NK cells stimulated with IL-2 cytokine for 5 days undergo metabolic reprogramming.

NK cells were purified from PBMC and stimulated for 5 days with low IL-15 (1ng/ml), reported as control and IL-2 (100U/ml). Detailed metabolic analysis was performed using Seahorse extracellular flux analyzer. Representative traces for (A) OCR and (B) ECAR in response to low IL-15 and IL-2 stimulation. Representative traces of 2 repeats. PBMC from freshly isolated blood of healthy donors were stimulated for 5 days with low IL-15 or IL-2. (C) Pooled data of NK cells expressing CD71, the transferrin receptor (n=10 donors). (D) Pooled data of fluorescent glucose analogue 2-NBDG uptake (n=5 donors). Samples were compared using a parametric Student t-test. *P ≤ 0.05.
Figure 5.2 mTORC1 regulates CD71 expression and nutrient uptake on human NK cells.

PBMC from freshly isolated blood of healthy donors were stimulated for 5 days with low IL-15 (1ng/mL), IL-2 (100U/mL) +/- Rapamycin (20nM). (A-B) Representative dot plots (A) and pooled data (B) of NK cells expressing CD71, the transferrin receptor (n = 9 donors). (C-D) Representative histogram (C) and pooled data (D) of fluorescent glucose analogue 2-NBDG uptake (n = 7 donors). Samples were compared using a parametric Student t-test. *P ≤ 0.05, **P ≤ 0.01.
Figure 5.3 mTORC1 is required for sustained metabolic respiration of human NK cells.

NK cells were purified from PBMC and stimulated for 5 days with low IL-15 (1ng/mL) reported to as control, IL-2 (100U/mL) +/- Rapamycin (20nM). Detailed metabolic analysis was performed using Seahorse extracellular flux analyzer. (A) Representative traces for OCR in response to low IL-15 and IL-2, with or without rapamycin. (B) Individual paired responses for OXPHOS for IL-2 stimulation with or without rapamycin (n = 6 donors). (C) Individual paired responses for maximal respiration for IL-2 stimulation with or without rapamycin (n = 6 donors). Samples were compared using a parametric Student t-test. * P ≤ 0.05.
Figure 5.4 mTORC1 is required for sustained glycolysis in human NK cells.

NK cells were purified from PBMC and stimulated for 5 days with low IL-15 (1ng/mL) referred to as control, IL-2 (100U/mL) +/- Rapamycin (20nM). Detailed metabolic analysis was performed using Seahorse extracellular flux analyzer. (A) Representative traces for ECAR in response to low IL-15 and IL-2 with or without rapamycin. (B) Individual paired responses for glycolysis for IL-2 stimulation with or without rapamycin (n = 6 donors). (C) Individual paired responses for glycolytic capacity for IL-2 stimulation with or without rapamycin (n = 6 donors). Samples were compared using a parametric Student t-test. *P ≤ 0.05.
Figure 5.5 mTORC1 is required for sustained increase in mitochondrial mass and ATP5B expression in cytokine activated NK cells.

PBMC from freshly isolated blood of healthy donors were stimulated for 5 days with low IL-15 (1ng/mL), IL-2 (100U/mL +/- Rapamycin (20nM). Cells were analyzed for mitochondrial function by flow cytometry staining. (A-B) Representative histogram (A) and pooled MFI (B) of MitoTracker in response to IL-2 with or without rapamycin (n = 7 donors). (C-D) Representative histogram of ATP5B (C) and (D) in response to IL-2 with or without rapamycin (n = 6 donors). Samples were compared using a parametric Student t-test and fold changes in MFI caused by rapamycin were compared with a one-sample t-test against a theoretical mean set to 1.00. ns = not significant. * P ≤ 0.05.
Figure 5.6 mTORC1 is required for sustained increase in mitochondrial polarization in cytokine activated NK cells.

PBMC from freshly isolated blood of healthy donors were stimulated for 5 days with low IL-15 (1 ng/mL) or IL-2 (100 U/mL) +/- Rapamycin (20 nM). Cells were analyzed for mitochondrial function by flow cytometry staining. (A-B) Representative histogram (A) and frequency (B) for TMRM MFI in response to IL-2 with or without rapamycin (n = 7 donors). (C) Mitochondrial superoxide levels was determined by MitoSox staining. Pooled data in response to IL-2 with or without rapamycin is shown (n = 4-6 donors). Samples were compared using a parametric Student t-test. ns = not significant. * P ≤ 0.05.
5.2.2 mTORC1 is required for long-term IL-2-induced effector functions in human NK cells

NK cells stimulated for 5 days in the presence of IL-2 and rapamycin had lower rates of OxPHOS and glycolysis. We therefore investigated if NK cell effector functions were also altered in these cells. Rapamycin treated NK cells had lower expression of NKp44 (Figure 5.7 A). However, TRAIL, Granzyme B and CD69 were equivalent in the presence or absence of rapamycin (Figure 5.7 C-D).

Neither control NK cells nor IL-2 stimulated NK cells in the presence or absence of rapamycin spontaneously produced IFNγ (Figure 5.8 top panel). However, when these cells were further stimulated for 18 hours with IL-12 (30ng/mL) + IL-15 (100ng/mL) clear differences in IFNγ production emerged. IL-12/15 stimulation significantly induced IFNγ production in both control NK cells and IL-2 cultures, but this was not the case in IL-2 + rapamycin cultures (Figure 5.8 lower panel). NK cells treated with IL-2 + rapamycin had substantially reduced IFNγ production following IL-12/15 stimulation in terms of the percentage of NK cells making IFNγ. Furthermore, re-stimulated NK cells in IL-2 + rapamycin had reduced levels of the amount of IFNγ (MFI) produced per cell compared to cells stimulated with IL-2 alone (Figure 5.9 A-B).
Figure 5.7 Cytokine-induced effector functions of human NK cells at 5 days are dependent of mTORC1.

PBMC were stimulated for 5 days with low IL-15 (1ng/mL), IL-2 (100U/mL) or IL-2 (100U/mL) + Rapamycin (20nM). (A) Frequency of expression of NKp44 on NK cells are shown for low IL-15 and IL-2 with or without rapamycin (n = 11 donors) (B) Frequency of TRAIL expression on NK cells are shown for low IL-15 and IL-2 with or without rapamycin (n = 17 donors). (C-D) Pooled data of GranzB expression (MFI) (C) and CD69 expression (D) in response to low IL-15 and IL-2 +/- rapamycin. (n = 7 donors). Samples were compared using a Student t-test. Fold changes in GranzB MFI caused by rapamycin was compared with a one-sample t-test against a theoretical mean set to 1.00. ns = not significant. *P≤0.05.
Figure 5.8 mTORC1 is important for NK cell IFNγ production.

PBMC were cultured for 5 days in low IL-15 (1ng/mL), referred as control or IL-2 (100U/mL) with or without rapamycin (20nM). Cells were further stimulated for 18 hours with IL-12 (30ng/mL) + IL15 (100ng/mL) where indicated. IFNγ production was analyzed by flow cytometry. Representative dot plots of IFNγ production by NK cells is shown.
Figure 5.9 mTORC1 is important for NK cell IFNγ production and expression.

PBMC were cultured for 5 days in low IL-15 (1ng/mL) or IL-2 (100U/mL) with or without rapamycin (20nM). Cells were further stimulated for 18 hours with IL-12 (30ng/mL) + IL15 (100ng/mL) where indicated. IFNγ production was analyzed by flow cytometry. (A) Pooled data of frequency of IFNγ producing NK cells is shown. (B) Individual paired responses of IFNγ expression (MFI) by NK cells is shown (n = 18 donors). Samples were compared using either a one-way ANOVA followed by a Kruskal-Wallis post hoc test or a Student t-test. ns = not significant. *P ≤ 0.05, **P ≤ 0.01.
5.2.3 mTORC1 is required for optimal NK cell IFNγ production

We next considered explanations for IFNγ defects seen with IL-2 + rapamycin cultured cells. We have previously reported that IFNγ production does not require mTORC1 activity and is insensitive to rapamycin treatment in primary NK cells stimulated with IL-12/15 for 18 hours (chapter 3). Several possible explanations exist that could explain the failure of IL-12/15 to stimulate IFNγ production in NK cells after culture in IL-2+rapamycin for 5 days. One possibility could be that 5 days of culture in IL-2 altered NK cells such that IL-12/15 induced IFNγ production became dependent on mTORC1. We have observed this phenotype before where particular effector functions that are mTORC1 independent in NK cells stimulated overnight with cytokines become mTORC1 dependent upon prolonged IL-2 stimulation e.g. NKp44 expression (see chapter 3 and Figure 5.7). We addressed this possibility experimentally by repeating the 5 day IL-2 experiment and including rapamycin during the final IL-12/15 re-stimulation only. The result showed that rapamycin did not inhibit IFNγ production (Figure 5.10 A), supporting that IL-12/15 induced IFNγ production during re-stimulation did not require mTORC1.

We also considered a caveat of the original experiment as rapamycin was present throughout the full cell culture (during 5 day culture with IL-2 and during the 18h re-stimulation with IL-12/15). Under these conditions, the effect of rapamycin on inhibiting IFNγ production could potentially be either during the 5 day IL-2 culture or isolated to the final re-stimulation (or potentially in both). In addition to the experiment described above (adding rapamycin during re-stimulation only), we addressed the question by repeating the 5 day IL-2+rapamycin culture and removing rapamycin during the re-stimulation with IL-12/15. Allowing mTORC1 signalling during the IL-12/15 re-stimulation did not restore IFNγ production (Figure 5.10 B) suggesting that the deficit was not a temporal mTORC1 dependent signalling requirement after culture in IL-2. Together, these experiments suggested that inhibition of mTORC1 signalling during the 5 day culture in IL-2 was likely responsible for the impaired capacity of NK cells to produce IFNγ.

The lost capacity to make IFNγ was associated with clear metabolic alterations in the NK cells. As described above, 5 day IL-2+rapamycin cultured NK cells had low levels of OxPHOS and glycolysis and they did not engage a convincing metabolic
response after IL-12/15 re-stimulation, maintaining low levels of OxPHOS and glycolysis compared to cells in IL-2 for 5 days and re-stimulated with IL-12/15 (Figure 5.11). CD71 expression and mitochondrial polarisation were both shown to be impaired in NK cells stimulated with IL-2 for 5 days in the presence of rapamycin (Figure 5.2 A-B and Figure 5.6 A-B shown above). These defects persisted in NK cells following IL-12/15 re-stimulation even when rapamycin was withdrawn for the last 18 hours (Figure 5.12 A-B). Together, these data showed that subjecting NK cells to a prolonged inflammatory stimulus along with an insult to restrict cellular metabolism, resulted in a persistent metabolic and functional defect.

We next considered whether there was an intrinsic defect in the NK cells (due to altered metabolism) or whether the defect was context specific. To this end, we investigated if PMA+ionomycin signalling could stimulate IFNγ production in NK cells after 5 days in IL-2+rapamycin. Indeed, as can be seen in Figure 5.12 C, this was the case, indicating that the IFNγ locus was available for transcription i.e. reduced IFNγ production was not a result of epigenetic changes. This result suggests that not only reduced metabolism but a signalling defect might be also a contributory factor for observed IFNγ production defects.
PBMC were cultured for 5 days in IL-2 (100U/mL) with or without rapamycin (20nM) or kept in low IL-15 as control. Cells were further stimulated for 18 hours with IL-12 (30ng/mL) + IL15 (100ng/mL) where indicated. Cells were analyzed by flow cytometry for IFNγ production (A) Pooled data of IFNγ production of cells stimulated with low IL-15 or IL-2 for 5 days and re-stimulated for 18 hours with IL-12/15 with or without rapamycin (n = 12 donors). (B) Pooled data of IFNγ production of cells cultured in IL-2 +/- rapamycin for 5 days. Rapamycin was washed away from IL-2 + Rapamycin cultures prior to re-stimulation with IL-12/15 where indicated (n = 6-8 donors). Samples were compared using one-way ANOVA followed by a Kruskal-Wallis post hoc test. ns = not significant.
Figure 5.11 mTORC1 is required for sustained metabolic activation of NK cells.

NK cells were purified from PBMC and stimulated for 5 days with IL-2 (100U/mL) +/- Rapamycin (20nM). Cells were further stimulated for 18 hours with IL-12 (30ng/mL) + IL15 (100ng/mL) where indicated. Detailed metabolic analysis was performed using Seahorse extracellular flux analyzer. (A-B) Representative traces for OCR (A) and ECAR (B) in response to stimulation. Representative traces of 3 donors.
Reduced metabolism is related to impaired NK cell IFNγ production.

PBMC were cultured for 5 days in IL-2 (100U/mL) with or without rapamycin (20 nM). Cells were further stimulated for 18 hours with IL-12 (30ng/mL) + IL15 (100ng/mL) where indicated. Cells were analyzed by flow cytometry. (A) Pooled data of CD71 expression on NK cells cultured for 5 days in IL-2 +/- rapamycin and further stimulated for 18 hours with IL-12/15. Rapamycin was washed out of the culture for the last 18h (n = 6 donors). (B) Pooled data for TMRM staining (MFI) of cells stimulated with IL-2 +/- rapamycin and re-stimulated with IL-12/15. Rapamycin was washed out of the culture for the last 18h (n = 7 donors). (C) Frequency IFNγ-producing NK cells is shown for samples cultured for 5 days in IL-2 + rapamycin and re-stimulated with either IL-12/15 for 18 hours or PMA/ION for 4 hours (n= 3 donors). Samples were compared using a Student t-test. *P ≤ 0.05.
5.2.4 TGF-β inhibits cell metabolism and mitochondria function

High concentrations of TGF-β can be detected in the serum of blood from patients with chronic diseases e.g. cancer; however, the effects of TGF-β on NK cell metabolism and function are not well understood. Having shown that TGF-β inhibits NK cell OxPHOS in short-term stimulated NK cells (chapter 4) we investigated changes in metabolism in long-term stimulated NK cells. We cultured PBMC in low IL-15 (1ng/mL) and IL-2 (100U/mL) in the presence or absence of TGF-β (10ng/mL) for a period of 5 days and NK cells were analyzed for metabolic and function properties.

CD71 expression and uptake of the fluorescent glucose analogue 2-NBDG were significantly reduced in NK cells treated with TGF-β (Figure 5.13 A-D). Reduced CD71 expression and 2-NBDG uptake correlated to the data shown in acutely stimulated (18 hours) NK cells (Figure 4.3). As expected, detailed metabolic analysis revealed TGF-β treatment significantly inhibited OxPHOS and glycolysis rates in 5 day IL-2 stimulated NK cells Figure 5.14 A-C and Figure 5.15 A-C).

Having seen defects on OxPHOS in cells stimulated with IL-2+TGF-β for 5 days we further investigated mitochondrial alterations in these cells. Mitochondrial mass, expression of ATP synthase subunit ATP5B and mitochondrial polarization were substantially decreased in IL-2 stimulated cells in the presence of TGF-β (Figure 5.16 A-D and Figure 5.17 A-B). There was no significant changes in mitochondrial ROS (Figure 5.17 C). Taken together, these data revealed that TGF-β inhibits NK cell metabolism and mitochondria activity in human NK cells.
Figure 5.13 TGF-β regulates CD71 expression and 2-NBDG uptake on human NK cells.

PBMC from freshly isolated blood of healthy donors were stimulated for 5 days with low IL-15 (1 ng/ml) referred as control or IL-2 (100 U/ml) +/- TGF-β (10 ng/ml). (A-B) Representative dot plots (A) and (B) pooled data of NK cells expressing CD71, the transferrin receptor (n = 6 donors). (C-D) Representative histogram (C) and pooled data (D) of glucose fluorescent analogue 2-NBDG uptake (n = 6 donors). Samples were compared using a Student t-test. * P ≤ 0.05.
Figure 5.14 TGF-β inhibits NK cell OxPHOS.

NK cells were purified from blood of healthy donors and stimulated for 5 days with low IL-15 (1ng/mL) referred as control or IL-2 (100U/ml) +/- TGF-β (10ng/ml). Detailed metabolic analysis was performed using Seahorse extracellular flux analyzer. (A) Representative traces for OCR in response to low IL-15 and IL-2 with or without TGF-β. (B) Individual paired responses for OxPHOS in response to L-2 with or without TGF-β (n = 6 donors). (C) Individual paired responses for maximal respiration in response to IL-2 with or without TGF-β (n = 6 donors). Samples were compared using a Student t-test. *P ≤ 0.05.
Figure 5.15 TGF-β inhibits NK cell glycolysis.

NK cells were purified from blood of healthy donors and stimulated for 5 days with low IL-15 (1ng/mL) referred as control or IL-2 (100U/mL) +/- TGF-β (10ng/mL). Detailed metabolic analysis was performed using Seahorse extracellular flux analyzer. (A) Representative traces for ECAR in response to low IL-15 and IL-2 with or without TGF-β. (B) Individual paired responses for glycolysis in response to IL-2 with or without TGF-β. (n = 6 donors). (C) Individual paired responses for glycolytic capacity in response to IL-2 with or without TGF-β. (n = 6 donors). Samples were compared using a Student t-test. *P ≤ 0.05.
Figure 5.16 TGF-β impairs mitochondrial mass upregulation and ATP5B expression.

PBMCs were stimulated for 5 days with low IL-15 (1ng/mL), IL-2 (100U/mL) +/- TGF-β (10ng/mL). NK cells were then analyzed for mitochondria mass and ATP5B expression by flow cytometry staining. (A-B) Representative histogram (A) and frequency (B) of mitochondria mass in response to IL-2 with or without TGF-β (n = 7 donors). (C-D) Representative histogram (C) and pooled data (D) for TMRM staining in response to IL-2 with or without TGF-β (n = 7 donors). Samples were compared using a Student t-test and fold changes in MFI caused by TGF-β were compared with a one-sample t-test against a theoretical mean set to 1.00. ns = not significant. *P ≤ 0.05.
Figure 5.17 TGF-β reduces mitochondrial polarization of human NK cells.

PBMCs were stimulated for 5 days with low IL-15 (1ng/mL), IL-2 (100U/mL) +/- TGF-β (10ng/mL). NK cells were then analyzed for mitochondrial polarization and ROS production by flow cytometry staining. (A-B) Representative histogram (A) and pooled data (B) of TMRM in response to IL-2 with or without TGF-β (n = 6 donors). (C) Mitochondrial superoxide levels was determined by MitoSOX staining. Pooled data of ROS production in response to IL-2 with or without TGF-β is shown (n = 6 donors). Samples were compared using a Student t-test. ns = not significant. *P ≤ 0.05.
5.2.5 TGF-β inhibits NK cell effector function in 5 day cultured cells

NK cell metabolism is important for the effector function of both murine NK cells and human NK cells. We have shown previously (chapter 4) that the presence of TGF-β for 18 hours inhibits OxPHOS and glycolytic capacity of human NK cells and this metabolic inhibition was correlated to impaired NK cell effector functions. Presence of TGF-β for 5 days shown broader negative effects on NK cell metabolism, significantly inhibiting both OxPHOS and glycolysis, therefore we further investigated the effect of failed metabolism and dysfunctional mitochondria on effector functions of NK cells.

Presence of TGF-β for 5 days significantly impaired expression of TRAIL, NKp44 and CD69 on NK cell surface (Figure 5.18 A-C). No significant differences in granzyme B production was observed between cultures in IL-2 with or without TGF-β (Figure 5.18 D).

As previously shown, control cells or cells stimulated with IL-2 for 5 days did not produce IFNγ, however, upon further re-stimulation for 18 hours with IL-12/15, NK cells were able to produce large quantities of IFNγ at similar levels. Cells cultured in IL-2 + TGF-β for 5 days did not produce IFNγ as expected. However, upon re-stimulation with IL-12/15 for 18 hours, cells in IL-2 + TGF-β did not produce IFNγ (Figure 5.19). This data is similar to the data obtained from cells cultured in IL-2 + rapamycin, which also revealed a strong defect on IFNγ production after re-stimulation (Figure 5.8). Additional repeats are required to obtain significance (Figure 5.20 A), however we can observe significant decrease in IFNγ production after a direct comparison of re-stimulated cells cultured in IL-2 with or without TGF-β for 5 days. There was a modest inhibition of level of IFNγ production per cell (MFI) in the presence of TGF-β (Figure 5.20 C-B). Furthermore, when TGF-β was removed from the culture for the last 18 hours and re-stimulated with IL-12/15, NK cells were still unable to produce IFNγ (Figure 5.21 A). Nonetheless, similar to what we have previously shown in our short-term cultured cells with TGF-β and in contrast to the rapamycin data, adding TGF-β only during the re-stimulation with IL-12/15 (after 5 day in IL-2) also impaired IFNγ production (Figure 5.21 B). Taken together, these data supports the knowledge that TGF-β is a potent NK cell inhibitor and also
suggests that short-term presence of TGF-β is enough to downregulate NK cell effector functions.
Figure 5.18 TGF-β significantly reduces TRAIL, NKp44 and CD69 expression on human NK cells.

PBMC were cultured for 5 days in low IL-15 (1ng/mL) or IL-2 (100U/mL) with or without TGF-β (10ng/mL). Cells were analyzed by flow cytometry. (A) Pooled data of TRAIL expression on NK cells surface stimulated with low IL-15, IL-2 or IL2 + TGF-β for 5 days (n = 10). (B) Pooled data of NKp44 expression on NK cells surface stimulated with low IL-15, IL-2 or IL2 + TGF-β for 5 days (n = 6 donors). (C) Pooled data of CD69 expression on NK cells surface stimulated with low IL-15, IL-2 or IL2 + TGF-β for 5 days (n = 7 donors). (D) Pooled data of Granzyme B expression (MFI) on NK cells stimulated with low IL-15, IL-2 or IL2 + TGF-β for 5 days (n = 10 donors). Samples were compared using either one-way ANOVA followed by a Kruskal-Wallis post hoc test. Fold changes in GranzB MFI caused by TGF-β were compared with a one-sample t-test against a theoretical mean set to 1.00 and a Student t-test as appropriate. ns = not significant. *P ≤ 0.05, **P ≤ 0.01.
Figure 5.19 TGF-β impairs IFNγ production.

PBMC were cultured for 5 days in low IL-15 (1ng/mL), referred as control or IL-2 (100U/mL) with or without TGF-β (10ng/mL). Cells were further stimulated for 18 hours with IL-12 (30ng/mL) + IL-15 (100ng/mL) where indicated. Cells were analyzed by flow cytometry for IFNγ production. Representative dot plots of IFNγ production by NK cells is shown.
Figure 5.20 TGF-β treated NK cells have less IFNγ production and expression.

PBMC were cultured for 5 days in low IL-15 (1ng/mL) or IL-2 (100U/mL) with or without TGF-β (10ng/mL). Cells were further stimulated for 18 hours with IL-12 (30ng/mL) + IL-15 (100ng/mL) where indicated. Cells were analyzed by flow cytometry for IFNγ production. (A) Pooled data of frequency of IFNγ producing NK cells for cells cultured in 5 days in low IL-15, IL-2 +/- TGF-β re-stimulated or not with IL-12/15 stimulation is shown. (B) Individual paired responses of cells stimulated for 5 days in IL-2 +/- TGF-β after re-stimulation with IL-12/15 is shown. (C) Individual paired responses of IFNγ producing NK cells (MFI) for cells stimulated for 5 days in IL-2 +/- TGF-β after re-stimulation with IL-12/15 is shown (n = 10 donors). Samples were compared using a Student t-test as appropriate. **P ≤ 0.01.
Figure 5.21 Acute or chronic presence of TGF-β inhibits NK cells IFNγ production.

PBMC were cultured for 5 days in IL-2 (100U/mL) with or without TGF-β (10ng/mL). Cells were further stimulated for 18 hours with IL-12 (30ng/mL) + IL-15 (100ng/mL). Cells were analyzed by flow cytometry for IFNγ production. (A) Pooled data of IFNγ production of cells stimulated with IL-2 or IL-2 + TGF-β for 5 days and re-stimulated for further 18 hours with IL-12/15. TGF-β was washed away from IL-2 + TGF-β cultures prior to re-stimulation where indicated (n = 6 donors). (B) Pooled data of IFNγ production of cells stimulated with IL-2 for 5 days and re-stimulated with IL-12/15 with or without TGF-β for the last 18 hours where indicated (n = 7 donors). Samples were compared either using one-way ANOVA followed by a Kruskal-Wallis post hoc test or a Student t-test as appropriate. ns = not significant. *P ≤ 0.05.
5.2.6 Canonical TGF-β signaling pathway inhibits NK cell effector functions

TGF-β signaling pathway can be divided into canonical (SMAD-dependent) and non-canonical (SMAD independent) pathways as previously described (section 1.4). We have shown that the canonical TGF-β signaling pathway is important during short-term inhibitory effects on NK cells. Given that TGF-β have broader and more profound effects after prolonged culture e.g. glycolysis is now inhibited, we investigated the contribution of the canonical TGF-β signaling pathway in this phenotype.

In terms of general metabolism, CD71 expression and 2-NBDG uptake, mitochondrial mass and ATP5B expression were all profoundly inhibited by the presence of TGF-β in the 5 day IL-2 cultures (Figure 5.13, Figure 5.16 C-D and Figure 5.17 A-B). The TGF-βRI inhibitor, SB431542 resulted in a significant increase in CD71 expression, 2-NBDG uptake and ATP5B expression, though not of mitochondrial mass (Figure 5.22 A-D). mTORC1 is a key regulator of cell metabolism and we have shown that at early time points TGF-β does not inhibit mTORC1 activity (Figure 4.9). However, after 5 days, cells stimulated with IL-2 in the presence of TGF-β had impaired mTORC1 activity through measuring pS6 and p4EBP1 expression (Figure 4.13 shown above). Inhibition of TGF-βRI with SB431542 resulted in partial increase of mTORC1 signalling (Figure 5.23 A-B).

In terms of function, CD69 and TRAIL expression were inhibited by TGF-β in 5 day IL-2 cultures (Figure 5.18 A,C) and their expression was fully restored in the presence of SB431542 (Figure 5.24 A-B). Analysis of IFNγ production (upon re-stimulation with IL-12/15) showed that similar to short-term cultures, a longer exposure (5 days) to TGF-β trends to inhibition of the frequency of NK cells producing IFNγ however, more repeats should be performed to obtain significance (Figure 5.25 A). This defect on IFNγ production could be fully restored by inhibition of the canonical TGF-β signaling pathway when analysis of IL-2 + TGF-β with or without SB431542 was performed (Figure 5.25 B). Inclusion of SB431542 had no effect on IFNγ MFI expression (data not shown). Together these data indicated that both canonical and non-canonical pathway account for the functional defects observed in NK cells stimulated for 5 days in IL-2 + TGF-β.
Figure 5.22 Impaired NK cell metabolism is restored with TGF-βRI inhibitor.

PBMC were cultured for 5 days in low IL-15 (1ng/mL) or IL-2 (100U/mL) with or without TGF-β (10ng/mL) and IL-2 + TGF-β with or without the TGF-βRI inhibitor SB431542 (5µM). Cells were analyzed by flow cytometry staining. (A) Pooled data of NK cells expressing CD71, the transferrin receptor on NK cells stimulated with IL-2 +/- TGF-β and SB431542 or left unstimulated (n = 7 donors). (B) Pooled data of fluorescent glucose analogue 2-NBDG uptake by NK cells stimulated with IL-2 +/- TGF-β and SB431542 or left unstimulated (n = 4 donors). (C) Pooled data of ATP5B expression in response to low IL-15 and IL-2 with or without TGF-β and SB43154 (n = 5 donors). (D) Pooled data of mitochondrial mass in response to low IL-15 and IL-2 with or without TGF-β and SB431542 (n = 6 donors). Samples were compared using one-way ANOVA followed by a Kruskal-Wallis post hoc test. ns = not significant. *P ≤ 0.05.
Figure 5.23 Non-Canonical TGF-β signaling pathway is restored when canonical TGF-β pathway is inhibited.

PBMC were cultured for 5 days in low IL-15 (1ng/mL), IL-2 (100U/mL) with or without TGF-β (10ng/mL) and IL-2 + TGF-β with the TGF-βRI inhibitor SB431542 (5µM). Cells were analyzed for pS6 and p4EBP1 levels by intracellular flow cytometry staining. (A-B) Representative (A) dot plots and (B) individual paired responses showing the frequency of NK cells positive for pS6 in IL-2 cultured cells +/- TGF-β and SB431542 (n = 6 donors). (C) Representative histogram of p4EBP1 expression in IL-2 +/- TGF-β and SB431542 (representative of 2 donors). Samples were compared using a Student t-test. *P ≤ 0.05.
Figure 5.24 Expression of CD69 and TRAIL are restored with TGF-βRI inhibitor.

PBMC were cultured for 5 days in low IL-15 (1ng/mL), IL-2 (100U/mL) with or without TGF-β (10ng/mL) and IL-2 + TGF-β with the TGF-βRI inhibitor SB431542 (5µM). Cells were analyzed by flow cytometry staining. (A) CD69 expression on NK cells stimulated with IL-2 +/- TGF-β and SB431542 or left unstimulated (n=6). (B) Frequency of TRAIL expression on NK cells is shown for IL-2 +/- TGF-β and SB431542 or unstimulated cells (n = 7 donors). Samples were compared using one-way ANOVA followed by a Kruskal-Wallis post hoc test. *P ≤ 0.05.
Figure 5.25 Impaired NK cell IFNγ expression is restored with TGF-βRI inhibitor.

PBMC were cultured for 5 days in low IL-15 (1ng/mL), IL-2 (100U/mL) +/- TGF-β (10ng/mL) and IL-2 + TGF-β + TGF-βRI inhibitor SB431542 (5µM). Cells were analyzed by flow cytometry staining for IFNγ production. (A-B) Pooled data (A) and individual paired responses (B) of IFNγ expression (n = 7 donors). Samples were compared using a Student t-test. ns = not significant. *P ≤ 0.05.
5.3 Discussion

Poor prognosis of patients with chronic inflammatory diseases like cancer is linked to defective NK cell function. Recent research on the tumor microenvironment has shown that cancer cells can reduce immune cell function in different ways e.g. limiting nutrients availability or releasing immunoregulatory cytokines. However, how these can potentially regulate NK cell function is not well understood. Here we established a long term in vitro NK cell culture that allowed us to investigate changes on NK cell effector function and metabolism after stimulation with the pro-inflammatory cytokine IL-2. In the present study, we propose that chronic inhibition of the metabolic regulator mTORC1 is linked to defective NK cells effector functions. Furthermore, our data support that TGF-β, an immunosuppressive cytokine present in high concentrations in serum of patients with chronic diseases, strongly inhibits NK cell function and metabolism. Most of the observed defects on NK cells caused by TGF-β were restored by inhibition of the TGF-β canonical signaling pathway.

We stimulated human NK cells for 5 days with IL-2 with or without rapamycin (mTORC1 inhibitor) to investigate the importance of mTORC1 for activation and function of NK cells at later time points of cell activation. Our data revealed that metabolism and effector functions of NK cells stimulated for long periods of time become more dependent on mTORC1 signaling than short-term stimulated NK cells (chapter 3). When mTORC1 was inhibited for 5 days, glycolysis, glycolytic capacity, OxPHOS and maximal respiration were all strongly inhibited. In contrast, NK cells stimulated for 18 hours with IL-2 + rapamycin, only glycolysis and glycolytic capacity were inhibited (chapter 3). Along with OxPHOS suppression, rapamycin prevented NK cell mitochondrial polarization, reduced expression of the ATP synthase component ATP5B and decreased mitochondrial mass. Indeed, mTORC1 has been directly linked to mitochondrial biogenesis and activity in NK cells and other cell types. Corresponding to the decreased metabolism, cells in rapamycin showed reduced expression of metabolic markers CD71 and CD98 and decreased 2-NBDG uptake, similar to what we have observed in 18 hours IL-2 + rapamycin stimulated NK cells (chapter 3). TRAIL and NKp44 expression are independent of mTORC1 signaling (chapter 3); however, in 5 day IL-2 cultures their
expression significantly rely on mTORC1 expression. No changes in Granzyme B and CD69 were observed either in long-term or short-term stimulation. To investigate possible IFNγ production defects, after 5 days stimulation with IL-2, NK cells were further re-stimulated for 18 hours with a cytokine combination (IL-12/15) to give a boost in IFNγ production. We observed high IFNγ production from re-stimulated cells in IL-2. However, clear inhibition of IFNγ production after cytokine boost was detected in cells stimulated with IL-2 + rapamycin. Furthermore, we observed that cells in IL-2 + rapamycin were unable to upregulate their metabolic machinery after stimulation, indicating a metabolic defect.

One known physiological inhibitor of mTORC1 in NK cells is the immunosuppressive cytokine TGF-β as shown by ourselves (Figure 4.13) and others\(^\text{105}\). Detection of high concentrations of TGF-β in serum is linked to poor prognosis as TGF-β promotes growth of cancer cells and inhibit immune cells functions\(^\text{90}\). With this in mind, we decided to investigate the effect of TGF-β on NK cell effector functions and metabolism in the context of chronic disease by culturing NK cells in vitro for 5 days in IL-2 in the presence of absence of TGF-β. We have previously shown that TGF-β strongly inhibits NK cell OxPHOS, glycolytic capacity and effector functions at 18 hour time point through activation of the canonical pathway (chapter 4)\(^\text{65}\). There are many explanations on how TGF-β could inhibit mTORC1 signaling. TGF-β has been shown to increase cell ROS which can regulate mTORC1 activity through direct inhibition of phosphorylation of pS6 and 4EBP1 in different cell lines\(^\text{137}\). This does not seems to be case in our cells as ROS production did not change over the period of 5 days. One promising explanation is that TGF-β has been shown to bind to FKBP12 protein, inhibiting mTORC1 signalling in a similar way as rapamycin yet, this has to be investigated\(^\text{138}\). Moreover, investigation of SMAD target genes would give us a better understanding of TGF-β signaling in NK cells. We have shown that TGF-β strongly inhibited mitochondrial mass, mitochondrial polarization and ATP synthase component ATP5B. Indeed, TGF-β has been linked by others as a possible candidate to inhibit NK cells mitochondria function through inhibition of PGC-1α expression (a master regulator of mitochondrial biogenesis) in NK cell\(^\text{139}\). NK cells cultured in the presence of TGF-β for 5 days are unable to produce IFNγ after IL-12/15 re-stimulation in a similar way observed in NK cells cultured in rapamycin. When TGF-β was removed during the
period of re-stimulation, NK cells were still unable to increase their IFNγ production. The addition of TGF-β to the last 18 hours re-stimulation of IL-2 cultured cells significantly inhibited IFNγ production suggesting that TGF-β signaling pathway is immediate in controlling NK cell function. Inhibition of mTORC1 (with rapamycin) for the last 18 hours re-stimulation does not inhibit NK cell IFNγ production however, when mTORC1 is inhibited for the period of 5 days, cells are completely impaired suggesting that direct inhibition of mTORC1 requires prolonged periods of time to impair NK cell functions.

We have previously shown that the canonical TGF-β signaling is the main pathway that regulates NK cell metabolism and effector functions in 18 hour stimulated NK cell (chapter 4). By inhibiting the canonical pathway through the TGF-β receptor I inhibitor SB431542 in 5 day IL-2 stimulated NK cells with TGF-β we support the knowledge that the canonical pathway is the main pathway by which TGF-β inhibits NK cell metabolism and effector functions. Interestingly, mTORC1 activity was partially recovered with SB431542 as we observed recovery of phosphorylation of pS6 and 4EBP1. Mitochondrial function was not significantly restored but there is a trend towards mitochondrial function recovering to IL-2 levels. Inhibition of the canonical TGF-β pathway restored production of IFNγ and expression of CD71, CD69 and TRAIL. Because of time constraints, we did not investigate the recovery of OxPHOS and glycolysis in our cells but we predict that the results would be similar to the ones observed at 18 hour time point (chapter 4), where metabolism recovered. SB431542 and other inhibitors of TGF-β receptor signaling are a promising therapeutic drug as they have been shown to suppress progression of many tumors in vivo and in vitro; here in our study, we show that SB431542 can potentiate NK cell effector functions and metabolism126, 140. Indeed, there are other publications showing that culture of NK cells with different canonical TGF-β pathway inhibitors increase NK cell function and improve killing of tumor cells68, 141. Rodon et al established a safe therapeutic dosage for the LY2157299, a TGF-βRI kinase inhibitor, to be used in humans with advanced cancer and gliomas. Because no major cytotoxicity was observed, this molecule is now in phase II clinical investigation142. Taken together, our data supports previous findings that targeting TGF-β signaling in human NK cells can improve metabolism and function.
We have previously discussed the main differences observed between cells stimulated for 18 hours in rapamycin and TGF-β (chapter 4). Cells stimulated with IL-2 in the presence of rapamycin or TGF-β for 5 days had impaired metabolism, mitochondria function and IFNγ production. Upregulation of mitochondrial mass and changes in morphology are key to mitochondrial function; however, we did not investigate mitochondrial morphology in our studies. It would be valuable to explore if rapamycin and TGF-β actually promote defects on mitochondria fission and fusion necessary to maintain mitochondria activity\textsuperscript{143}. The main difference observed between rapamycin and TGF-β treated cells was the expression of the activation receptor CD69. Direct inhibition of mTORC1 does not affect CD69 expression while TGF-β significantly inhibits CD69 expression at short-term or long-term stimulated NK cells. These data support our argument that mechanisms by how rapamycin and TGF-β impairs NK cell effector functions are not mainly though inhibition of mTORC1 signaling pathway although the final outcome is similar.

Our studies lead us to conclude that mTORC1 signaling is important during prolonged stimulation of NK cells and that not only mTORC1 control of metabolism (discussed at chapter 3) is necessary but other aspects of mTORC1 signaling are also involved in NK cell optimal activation. Repressive epigenetic modifications could have taken place during prolonged inhibition of mTORC1, obstructing accessibility to the IFNG locus by transcription factors like T-bet. T-bet is required for optimal NK cell IFNγ production\textsuperscript{144} furthermore, mTORC1 signaling has been linked to T-bet phosphorylation in CD4+ T cells\textsuperscript{145}. However, NK cells with impaired mTORC1 expression and metabolism were able to produce IFNγ at similar levels to control cells after stimulation with PMA/Ionomycin. Activation of cells with PMA/Ionomycin essentially bypasses the normal signaling pathways to promote IFNγ expression arguing that the IFNγ locus is open and that the inability of NK cells to produce IFNγ in response to IL-12/15 is likely due to a signaling defect. It would be worth investigating IL-12 or IL-15 receptor expression levels, and downstream signaling events such as phosphorylation of STAT4 or STAT5 to identify the signaling defect. Indeed, mTORC1 activity could be linked to receptor expression and would be valuable to address this in our cells. Interestingly, in T cells IL-2 signalling is important for the expression of IL-12Rβ2 subunit and mTORC1 is
known to be a key aspect of IL-2 induced signaling in these cells\textsuperscript{146}. Another explanation could be that the observed defects in IFNγ production in rapamycin treated cells is that the reduced metabolism in these cells results in low level of ATP and the activation of the energy sensor AMPK. AMPK has been shown to directly limit the rate of IFNγ translation\textsuperscript{72,147}. A key activation in our cultures would be the investigation of reduced phosphorylation of acetyl-CoA carboxylase. mTORC1 can also coordinate cell fate by making T cells, monocytes and macrophages more pro- or anti-inflammatory and this could be the case in our NK cells; however, we did not explore this in the present study\textsuperscript{147}. For instance, TGF-β has been shown to shape NK cells into a more naïve phenotype. Cortez et al shown that NK cells with defect on TGF-β canonical pathway became ILC1-like cells with reduced cytotoxicity and limited IFNγ production\textsuperscript{148}. It would be interesting to investigate if this is also the case in our experiments by measuring the expression of receptors highly expressed in ILC1 cells.

Taken together, our data argue that early impairment of mTORC1 mechanisms lead to complete abrogation of NK cell function and metabolism at later time points, which could be detrimental in disease. Investigation of physiological mTORC1 inhibitors or methods to restore mTORC1 function in NK cells could be used as new strategies for treatment of disease and drug development. Our data also supports previous findings indicating that targeting TGF-β signaling pathway is a promising way to improve immune cell function. Therefore, understanding mechanisms of microenvironmental control are important for development of new drugs to either increase NK cell effector functions for elimination of tumor cells or limit NK cell activation to avoid excessive immunopathology.
Chapter 6

Final discussion
NK cells are cytotoxic lymphocytes critical for the immune system. These cells have important effector functions, killing virally infected cells and tumors without prior activation. Moreover, NK cells have been shown to persist for long periods of time in the blood stream, releasing cytokines to shape the innate and adaptive immune response against infection\textsuperscript{149}. NK cells have great potential for treatment of cancer and viral diseases; however, progress has been hampered due to our incomplete understanding of the basic biology, function and activation of NK cells\textsuperscript{150}. Therefore, understanding cellular and molecular mechanisms that regulate NK cell effector functions is important to allow advancement of therapeutic interventions. Our group and others have shown that cytokines cause metabolic changes in NK cells\textsuperscript{31, 85}. However, little was known about how these processes are controlled and regulated in human NK cells (section 1.2). In the present thesis we investigated the impact of cell metabolism on human NK cell effector functions in response to cytokines. We showed for the first time that human NK cells undergo metabolic reprogramming after activation with cytokines and that CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells have different metabolic profiles, matching their effector functions (chapter 3)\textsuperscript{123}. The effects of the inhibitory cytokine TGF-\(\beta\) on NK cell was also investigated. We have shown that the TGF-\(\beta\) canonical pathway is important in controlling NK cell metabolism and function (chapter 4)\textsuperscript{65}. Finally, we investigated the importance of metabolism on NK cells stimulated with cytokine for longer periods of time. We showed that prolonged inhibition of the metabolic regulator mTORC1 leads to dysfunctional NK cells (chapter 5).

Immune cells traffic through different tissues, each with different availability of nutrient and oxygen. Therefore, cells must adapt to survive and exert their functions\textsuperscript{108}. In many diseases like cancer\textsuperscript{151}, metabolic syndrome, sepsis and heart diseases\textsuperscript{71} the availability of nutrients are different from healthy conditions. Recent studies have shown that alteration in nutrient viability impairs immune cell function through inhibition of cell metabolism\textsuperscript{131}. Metabolism is now known to be a key regulator of cell function by providing energy and building blocks necessary for a cell to effectively fulfill their specialized functions\textsuperscript{71}. Therefore, understanding metabolic pathways may provide attractive targets for disease therapy\textsuperscript{152}. The two main metabolic pathways for ATP generation from metabolic fuels are OxPHOS and aerobic glycolysis, and glucose can fuel both pathways. Some cells, mostly naïve cells,
rely on OxPHOS for their energy and survival, have a longer life span inside the body and exhibit low levels of cellular biosynthesis. When activated, some cells go through a metabolic switch and rely mainly on aerobic glycolysis. Aerobic glycolysis is not as effective as OxPHOS in generating energy but it increases cell glycolytic flux, which promotes cell growth, cell proliferation and synthesis of intermediates important for biosynthetic processes like lipid and nucleotides synthesis which are necessary for effector functions (section 1.2)\textsuperscript{71}.

Among lymphocytes, much progress has been made in understanding T cell metabolism. For instance, upon stimulation naïve CD8\textsuperscript{+}T cells undergo metabolic reprogramming becoming more glycolytic allowing these cells to differentiate into a functional CTL\textsuperscript{31}. Memory T cells predominantly engage OxPHOS and are dependent on a metabolic switch to glycolysis to differentiate and responds to infection (section 1.2)\textsuperscript{153}. Murine NK cells metabolism resembles CTL in term of increased glycolysis and OxPHOS in response to cytokine stimulation\textsuperscript{31}. Our laboratory and others have shown that metabolism changes are important for murine NK cell IFN-γ production, a key regulator of immune function\textsuperscript{31, 85}. When this project started, little was known about NK cell metabolism, particularly of human NK cells. During my project, our laboratory published new data on aspects of NK cell metabolism (Figure 6.1), and the importance of other metabolic mechanisms are still under active investigation. My work has helped particularly in understanding the basic biology and metabolic profile of human NK cells isolated from healthy donors. NK cells isolated from patients with chronic diseases like breast cancer and HIV have impaired function\textsuperscript{102, 154} and their metabolic profile are currently under investigation in our lab.

For our metabolic and functional studies we stimulated cells with IL-2, which potentially drives sustained NK cell activation, allowing NK cells to function in parallel to the adaptive immune response over long periods of time\textsuperscript{149}. IL-2 is predominantly produced by T cells and enhances NK cell responses by increasing IFNγ production, modulating IL-12 receptor expression and increase in NK cell killing\textsuperscript{154}. We also used a combination of cytokines more commonly found as part of the innate immune response: IL-12 plus IL-15, which we have shown to upregulate NK cell functions and metabolism for immediate and potent responses (chapter 3)\textsuperscript{14}.  

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After incubation for 18 hours, we found that cytokines robustly stimulated NK cells and increased their metabolism. Despite the fact that mTORC1 is emerging as a key molecule for the control of different cells metabolism and function, the role of this molecule in the metabolic regulation and its physiological importance in human NK cells was not well defined (section 1.3). Our laboratory has shown that mTORC1 signalling is important for NK cell effector functions in expanded murine NK cells. In the present study I have shown that human NK cells stimulated with IL-2 or IL-12/15 increase mTORC1 activity. An interesting finding was that IL-2, but not IL-12/15 stimulated NK cells for 18 hours require mTORC1 signaling for glycolysis and IFNγ production (chapter 3).

We have previously discussed that IL-2 potentially drives a more sustained NK cell activation then IL-12/15 (chapter 3). This leads to the hypothesis that during prolonged cell stimulation, similar to what is observed in an inflammatory context, NK cells may manipulate their metabolic machinery to suit their needs. Therefore, we hypothesized that chronic inhibition of NK cell metabolism might be a possible explanation for why NK cells isolated from patients with chronic inflammatory diseases, like cancer, have impaired activity. We found that chronic inhibition of mTORC1 signalling completely abrogates the ability of NK cells to make IFNγ. Furthermore, we pointed out that mTORC1 signalling is important not only for regulating NK cell metabolism, but also in regulation of other aspects of NK cell activation (chapter 5).

In this thesis, we carried out a range of experiments to show that CD56^{bright} cells and CD56^{dim} cells also differ in their metabolic profile (chapter 3). These findings could be useful in a disease context. For example, CD56^{bright} cells from patients with systemic lupus erythematosus (SLE) produce larger quantities of IFNγ compared to CD56^{bright} cells from healthy donors. Unveiling metabolic pathways in CD56^{bright} cells could help the development of new drugs aimed at reducing of NK cell activation. Based on our data, a possible strategy to limit NK cell IFNγ production is by inhibiting mTORC1 activity or limiting glucose uptake by CD56^{bright} cells (chapter 3). As mentioned previously, different NK cells subsets can be found in distinct tissues and diseases therefore, it would be valuable to investigate the metabolic profile of these subsets. Indeed, there is some data starting to emerge in this
area. For instance, Salzberger et al recently published the differences of metabolic markers (CD71, CD98 and Glut1 expression) on NK cells isolated from tissue and peripheral blood\textsuperscript{157}. These observed changes in metabolism support our data, which indicates that NK cells subsets have different metabolic profile according to their function.

TGF-β is an immunoregulatory cytokine present in many chronic inflammatory diseases (section 1.4)\textsuperscript{93}. TGF-β is a potent inhibitor of T cells and NK cells effector functions therefore, it is not surprising that this cytokine is of significant interest (section 1.4)\textsuperscript{93}. In this thesis, we have shown that TGF-β inhibits NK cell metabolism and effector functions mainly through the TGF-β canonical pathway (chapter 4 and chapter 5). This data is valuable in the context of developing new therapies. For example, deciphering specific mechanism by how TGF-β pathways inhibits metabolism could be useful to restore NK cell function. Indeed, TGF-β has been shown to activate metabolic enzymes like fructose-1,6-bisphosphatase (FBP1) which inhibits glycolysis. However, the specific mechanism by which TGF-β activates FBP1 was not investigated\textsuperscript{158}. Furthermore, TGF-β inhibitors have been tested alongside with immune check point antibodies for cancer therapy\textsuperscript{159}. We suggest that using TGF-β inhibitors with antibodies blocking e.g. TIGIT receptor on NK cell surface could prove to be beneficial in cancer treatment\textsuperscript{160}.

Immunological memory is a feature of adaptive immune cells however, evidence that NK cells can also develop antigen-specific memory has emerged and is referred to “NK cell memory” or “NK cell training”\textsuperscript{66}. Understanding what induces NK cells training and how the scientific field could use this property to improve NK cells response \textit{in vivo} must be further investigated\textsuperscript{161}. During my PhD I performed a series of experiments with metabolic inhibitors and different cytokines concentration to investigate the importance of metabolism for NK cell training. Preliminary data (not shown in the thesis) indicated that NK cells that were trained for 18 hours with IL-12/15/18 cytokines then kept in low dose IL-15 for 7 days, had higher cytokine responses upon re-stimulation with cytokines than cells that were not trained for the initial 18 hour time point. When metabolism was inhibited during the first 18 hours with rapamycin or BMS303141 (ATP-citrate lyase inhibitor), NK cells produced less IFNγ then control cells during the re-stimulation at day 7 (data not
shown). Our data suggests that metabolism is important for NK cell training and further research should be carried out to underpin which metabolic mechanisms are the most important. Recently, increase in OxPHOS and mitochondrial mass have been linked to NK cell training. Cichocki et al shown that increased metabolism is required for trained NK cells to increase OxPHOS and produce IFNγ upon re-stimulation\textsuperscript{162}.

In conclusion, this study identifies the importance of metabolism for human NK cells effector functions. With this knowledge, we must further investigate new molecules implicated in the metabolic shift of NK cells. These findings will help in the development of new ways to manipulate NK cell function to handle different conditions like inflammation, virus infection or cancer. Previous studies have shown that lymphocytes become ineffective after long term fighting against infection or inflammation. This lack of response may be directly linked to reduced metabolism (chapter 5) and may represent a new therapeutic target arising out of this research\textsuperscript{152}. 
Figure 6.1 NK cell metabolic reprogramming is essential for NK cell effector functions.

Summary of our laboratory findings on NK cells metabolism. Upon cytokine stimulation NK cells increase glycolysis and OxPHOS rates. mTORC1 is robustly stimulated in cytokine activated NK cells and is required for NK cell metabolic reprogramming and IFNγ production\textsuperscript{31,123}. The transcription factors SREBP\textsuperscript{163} and cMYC\textsuperscript{164} are essential for NK cell metabolic and functional responses. NK cell metabolism can be inhibited by reduction of nutrients (glutamine, glucose) or by the immunoregulatory cytokine TGF-β\textsuperscript{65}.
Bibliography


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