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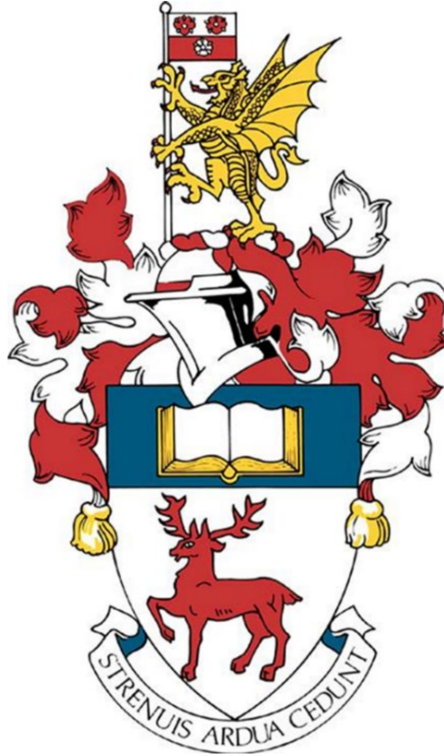

Faculty of Medicine

Clinical and Experimental Sciences Academic Unit

Cytokine Modulation of Natural Killer cells to Develop a Novel Immunotherapy

by

Rosalba Biondo

Thesis for the degree of Doctor of Philosophy

April 2024

University of Southampton

Abstract

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Clinical and Experimental Sciences

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Over many decades, numerous studies have had the common aim of improving cancer treatments, with several promising approaches proposed. Hepatocellular carcinoma (HCC) is one of the most common type of primary liver cancer, with very high death rates and increasing recurrence. However, current treatments are not successful, therefore great effort has been put into improving HCC therapies with a focus on immunotherapy. Recently, NK cells have been the centre of attention of translational medicine due to their innate cytotoxic potential and anti-tumour functions. The many challenges encountered in the *in vitro* manipulation of NK cells, though, have significantly hindered the development of NK-based therapies. This study aims to improve NK cell culture protocols, focussing on the expansion of NK cells in a feeder-free culture using soluble cytokines, to develop an improved therapy line for HCC. Here we focus on IL-21, to unveil its role in NK cell expansion and function. The results demonstrate that a short exposure to IL-21 causes a rapid and increased NK cell expansion. NK cells present a phenotype of mature and immature cells, expressing CD117, NKG2A, CD16, but lack CD57. Cells upregulate STAT3 and show high metabolic rates. Pre-activation of IL-21+IL-15, followed by IL-18+IL-2 combination causes the highest antitumour function of NK cells against HCC cell lines. However, the IL-21 effect was masked in chimeric antigen receptor (CAR)-engineered NK cells, though cytokine-primed CAR-NK showed a significant advantage in killing HCC cell lines. These findings suggest important features of NK cells, which can be exploited for the improvement of NK culturing protocols and of HCC therapies.

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   R. Biondo, S. I. Khakoo. Presentation at the **HUNTER Consortium Meeting 2020 and 2021.**

2. IL-21 augments the activation of cytokine-induced memory NK cell: a role in immunotherapy? R. Biondo, S. I. Khakoo. Poster presented at the **BSI Congress 2022**

3. CAR-engineered memory-like NK cells: a novel therapy for hepatocellular carcinoma.
   R.Biondo, I. Nayak, M. Quadflieg, S. Cramer, C. Zhang, N. Möker, J. Das, S. I. Khakoo. Poster presented at the **EASL Liver Cancer Summit 2023.**

Research Thesis: Declaration of Authorship

Print name: Rosalba Biondo

Title of thesis: **Cytokine Modulation of Natural Killer cells to Develop a Novel Immunotherapy**

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as shown in the list of accompanying materials.

Signature: Rosalba Biondo .... Date: 13/12/2023..........................

Acknowledgements

I started my PhD a few months before the emergence of the Covid-19 pandemic, which presented many difficulties and uncertainties and that aggravated the many challenges a PhD brings. Nonetheless, I made it to the finish line! The work in this thesis, though, would have not been possible without the support of many people. First and foremost, I would like to thank my supervisor Professor Salim Khakoo for guiding me throughout this journey. He taught me to think like a scientist by formulating my own ideas and hypotheses. Salim's door was always open which was incredibly helpful to resolve the many challenges faced. He created many opportunities for me to further my learning, such as the internship at Miltenyi, several collaborations, conferences, and workshops, which have helped me to become a more confident scientist and adaptable individual. For this I am very grateful!

I would like to acknowledge Miltenyi Biotec company who cofounded this work together with Cancer Research UK. I would like to specifically thank Congcong Zhang and Nina Möker from Miltenyi Biotec Cologne, Germany, for giving me the opportunity to undertake an internship in their laboratories. I am extremely grateful for the support, patience and teaching of laboratory techniques received from them and the research team. This was a fantastic experience which allowed me to further progress my scientific and personal development. I am deeply grateful for the knowledge and connections that were established during this time. I would also like to acknowledge the collaborators from the Das Laboratory from The Ohio State University, Columbus, Dr Jayajit Das, and Dr Indrani Nayak for the mathematical modelling outlined in chapter 4 and for the many discussions about the data.

I would also like to thank all the members of the Khakoo laboratory, for helping me throughout the PhD, providing great teaching in terms of laboratory techniques and analytical skills, as well as moral support. Rebecca Fulton for teaching me how to navigate the lab, especially at the start of my PhD, for ensuring the organisation and storage of materials within the lab and for always offering warm and encouraging words. Jack Fisher for showing me

experimental procedures, as well as discussions on statistical analysis of data and the frequent non-academic coffee breaks. Evie Rogers, a shared PhD between the Khakoo lab and the Utz lab (School of Chemistry, University of Southampton), for or a collaborative work outlined in chapter 4, which required carrying out experiments together, sharing data results and analysis. I would also like to acknowledge the support from Carolann McGuire from the FACS facility at the University of Southampton, whose flow cytometry teachings were integral in the success of my experiments. I would like to thank all the generous volunteers who provided blood samples for my research and all the trained peers that helped me getting samples by venepuncture of volunteers.

I am particularly thankful to several friends whose support has been fundamental over the years. Kulveer a friend and a brother, his door always open for me. He guided me to always look for the silver lining, supported me, encouraged, and spoiled me with infinite chats. I would also like to thank Cindy, Adriana, Roberta for listening to my many rants and my housemates Abi, Charlotte and Peter, fellow PhD students, who made me feel understood and less lonely as we were all going through similar hardships.

A special thanks goes to my parents, Mario and Caterina Biondo, and my siblings Francesca and Vito who could not be more supportive. Despite being miles apart, you never made me feel alone. Thank you for teaching me how to be independent, for always encouraging and listening to me, for always believing in me; thank you for the many phone calls and your warm words when I needed them the most. Last, but certainly not least, a massive thanks goes to my amazing partner Laurens, for always believing in me even when I did not, for supporting me and helping me throughout this challenging journey. Thank you for the endless conversations, adventures, and endeavours, for always making me laugh and for providing delicious meals. I could have not done half of it without you!

Definitions and Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
BaEV	Baboon Envelop
CAR	Chimeric antigen receptor
CAR-ML NK	Memory-like CAR-NK
cNK	conventional NK
CRS	Cytokine Release syndrome
GPC-3	Glypican-3
GvHD	Graft-versus-host-disease
HCC	Hepatocellular Carcinoma
HLA	Human leukocyte antigen
IFN-γ	Interferon-γ
KIR	Killer cell immunoglobulin-like receptor
lr-NK	Liver resident NK
LV	Lentiviral vector
MHC	Major Histocompatibility Complex
ml-NK	Memory like-NK
NK	Natural Killer
NK	Natural killer
PB	Peripheral Blood
PBMC	Peripheral blood mononuclear cells
TINK	Tumour infiltrating lymphocytes
TME	Tumor microenvironment
TNF-α	Tumour necrosis factor-α

Chapter 1 Introduction

1.1 Natural killer cells

Natural killer (NK) cells are lymphoid cells (ILCs), part of the innate immune system, together with dendritic cell, macrophages, neutrophils, basophils, and eosinophils. NK cells show specific receptors and cytokines expression which modulate immune responses (1,2). They are defined as $CD56^{+}CD3^{-}$ and comprise 5-15% of circulating cells in peripheral blood but are also found throughout the body in lymphoid organs, the lungs, the liver, the uterus, and the gut (3). Unlike T and B lymphocytes, which require prior sensitization, NK cells can rapidly mount an immune response against virally infected and tumour-transformed cells, making them essential effectors during first line immune defence (2). NK cells detect cues from the environment using the array of germline encoding activating and inhibiting receptors expressed on their surface, without the need to rearrange their receptor genes. Under normal circumstances, the balance of signals between activating and inhibiting receptors is tilted towards inhibition. A change in this balance causes NK cells to become active (2,4). After activation, NK cells can kill target cells in several ways. They release toxic granules such as perforin that create pores on the membrane of the target cells, and granzymes that enter and induce cell death. The expression of the receptor CD16, allows NK cells to recognise and interact with the Fc domain of immunoglobulin (Ig) G antibody which opsonise target cells, in a mechanism known as antibody dependent cytotoxicity (ADCC). NK cells also secrete cytokines such as that interferon (IFN)-γ, tumour necrosis factor (TNF)-α, granulocyte-macrophage colony stimulating factor (GM-CSF), and chemokines such as CC-chemokine ligand (CCL)-3, macrophage inflammatory protein (MIP)-1α, CCL4 (MIP-1β), CCL5 (RANTES). Both cytokines and chemokines play an essential role in coordinating the immune response to viral infections or tumour-transformed cells, by recruiting and priming other immune effector cells, including macrophages, dendric cells, T and B lymphocytes (3,5,6).

Minimal cytokine release syndrome (CRS), no neurotoxicity and a lack of graft-versus-host disease (GvHD) compared to T cells (7,8), make NK an attractive approach for novel cancer

immunotherapies. Over the years, many efforts have been focussed on growing and expanding NK cells from different sources such as peripheral and umbilical cord blood or even from induced pluripotent stem cells, following clinical grade methods, to create what can be called an "off the-shelf therapy". Promising preliminary results have been obtained, especially against blood tumours such as leukaemia, using cytokine-induced and feeder-cell based protocol either alone or in combination. However, *in vitro* expansion of NK cells has still many challenges to overcome, such as developing sufficient numbers to be used for therapy and increase their reactivity to mount a stronger anti-tumour response (9,10). Therefore, further studies need to be done to develop expansion and activation protocols that can be translated in the clinic.

1.1.1 NK cell education

NK cells express a combination of activating and inhibiting receptors that bind to ligands expressed by target cells. Balanced signals received by receptors cause NK cells to be inhibited. This is very important to prevent NK cells attacking adjacent healthy cells, and to recognise them as "self". Upon encountering target cells, the balance between inhibitory and activating signals will determine NK cell response. The threshold to overcome for cell activation is variable and depends on their education status. NK cells during development are "educated" to recognise major compatibility complex (MHC) class I molecules via NK cell inhibitory receptors such as killer-cell immunoglobulin-like receptors (KIRs) and NKG2A, maintaining tolerance to "self" (11–13). Inhibitory KIR2DL1/2/3, KIR3DL1 and NKG2A recognise different human leukocyte antigen (HLA) class I as their ligands. NK cells that express KIRs or NKG2A inhibitory receptors that have a cognate ligand show a greater cytotoxic potential than those cell that do not express inhibitory receptors or that lack the corresponding complementary ligand (11–15).

In the early 1990s Karre and Ljunggren realised that NK cells were able to detect the absence of "self" HLA ligands on target cells (16). They observed that NK cells killed lymphoma cell lines that had lost major compatibility complex (MHC) class I molecules, but no lysis was seen in the cell lines that expressed MHC class I molecules. This was addressed as the "missing self-hypothesis", which partially explains NK cells activity (16). Additionally, during development, NK cells are exposed to self-MHC molecules and the interaction between KIR and MHC

provides signals for cell maturation, contributing to the acquisition of cytotoxic ability as well as tolerance for healthy “self-cells”. This is a very important mechanism needed for the “education” or “licencing” of NK cells, which have the capacity to attack foreign or mutated cells but not healthy “self-cells” (17). NK cell response and function is determined by the number and type of MHC alleles engaged (18,19). NK cells missing inhibitory self-MHC-I receptors chronically respond to activating receptors and are rendered hyporesponsive (anergic) to stimulation (2,19). Once NK cells are fully competent, they are suppressed by ligation of self-MHC which is released if the MHC-I downregulated or altered as it occurs in tumour cells (17). This mechanism can, however, be exploited for the development of NK-based therapies by selecting donors with HLA-KIR mismatch to favour NK cells activation. This is still limited however, and the KIR-mediated inhibition can be vigorously overcome by the introduction of chimeric antigen receptors (CAR), which can readily kill autologous lymphoblastic leukemia (ALL) cells, resistant to NK cells classical killing (20).

The type of education received by NK cells changes their metabolism. NK cells educated via NKG2A show superior metabolic functions and resilience, are phenotypically more diverse and functionally more potent compared to NK cells educated via KIRs (21,22). As demonstrated by Horowitz et al (21), how NK cells are educated is dependent on the interplay between the NKG2A and KIRs cognate ligands. NKG2A is a much older and more conserved receptor which recognises the ligand HLA-E in a complex with a nanomer peptide, cleaved from the leader sequence of the MHC class-I members HLA-A, HLA-B or HLA-C (23,24). KIRs have evolved more recently, are highly polymorphic and recognise confirmations of HLA-A, HLA-B and HLA-C (25). Therefore, the KIR system emerged and evolved in a background of the already established NKG2A system. The peptides that form a complex with HLA-E have a methionine (M) amino acid at position 2. This corresponds to position 21 in the HLA-A, HLA-B and HLA-C leader sequence, which was fixed on these polymorphic MHC class-I members, before the emergence of the KIR family. This is the case for HLA-A and HLA-C, but not for HLA-B where in 80% of allotypes it is replaced by a threonine (T) amino acid, which prevents surface expression and recognition of HLA-E (21). Therefore, HLA-B determines the functionality of the HLA-E complex and divides the world population in M/M, M/T and T/T groups. Individuals with at least one methionine in the HLA-B alleles, present more functional NKG2A+ educated NK cells, compared to those that express a threonine amino acid.

Therefore, not only the expression of inhibitory receptors is important during education, but so is the level of cognate ligands expressed. Additionally, each education school generates NK cells with different characteristics, highlighting the importance in the specific signalling pathway undertaken during education (21,22).

1.1.2 Clonal expansion of NK cell is subdivided according to receptor expression

During their expansion, NK cells express different receptors according to their maturation stage (**Figure 1**). Stimuli from the surrounding environment, such as signals from cytokines, contribute to determining the expression of specific receptor types. Therefore, NK cells are characterized by high plasticity and adaptations to different circumstances. Their plasticity and receptor expression have been studied extensively, and while a set and static phenotype might not be attributed to a specific maturation stage, a detailed understanding of each receptor is needed to unravel the function of NK cells in health and diseases.

1.1.3 NK cell maturation determined by receptor expression

NK originate from the common lymphoid progenitor in the bone marrow, traffic to secondary lymphoid tissues and once they reach maturation, they exit in the circulation (2,26). NK cells are defined as $CD56^{+}$ and $CD3^{-}$. **Figure 1** elucidates the developmental stages of human NK cells. The expression of CD56 indicates the transition of immature NK cells to mature NK cells, marking the rise of $CD56^{bright}$ and subsequently of $CD56^{dim}$, both important for their effector functions. The initial and less mature $CD56^{bright}$ NK are characterized by the absence of CD16 ($CD56^{bright}CD16^{-}$) and are mostly present in secondary lymphoid tissues; the latter and more mature population of $CD56^{dim}$, is characterized by the presence of CD16 ($CD56^{dim}CD16^{+}$) and it is prevalent in peripheral blood (~90%) (2,27). $CD56^{bright}CD16^{-}$ NK cells are not cytotoxic and produce large quantities of potent inflammatory cytokines; on the other hand, $CD56^{dim}CD16^{+}$ are highly cytotoxic and mediate vital anti-tumour features (2). Peripheral $CD56^{bright}$ NK cells have been shown to mature into $CD56^{dim}$, losing the expression of receptors such as NKG2A, NKp30, NKp46 and acquiring CD57 and KIR (28–32), with CD57 expression marking an advanced and mature stage. During their transition, some $CD56^{dim}$ NK cells might also express NKG2A ($NKG2A^{+}CD94^{+}CD57^{-}KIR^{-}$), some might be $CD57^{+}$ ($CD57^{+}KIR^{+}NKG2A^{-}CD94^{-}$) and others might be $CD57^{-}$ (28,32–35), highlighting the fluidity of receptor expression at any one

point of NK cell lifetime. However, NK cells behave differently in tissues. It has been hypothesised that tissue-infiltrating NK cells might resemble tissue-resident NK cells, perhaps because of the environment influence. This is specifically important in tumours, when the tumour microenvironment (TME) is well known to have an influence on immune cells. Often this is negative, and it correlates with downmodulation of immune cells and disease progression, as it has been seen in hepatocellular carcinoma (HCC).

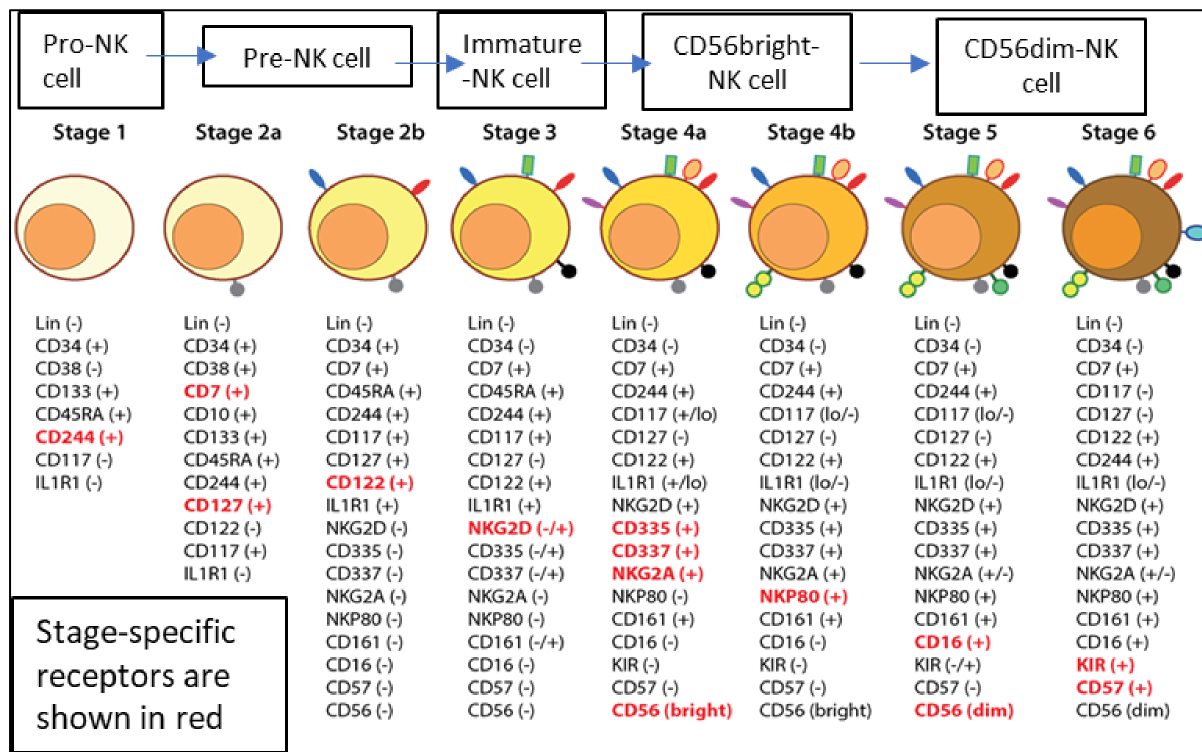


Figure 1. Developmental stages of human NK cells.

(Adapted from Abel et al (2)).

In addition to the classical CD56 bright/dim classification, NK cells can also be divided into subgroups according to the expression of the receptors CD27 and CD11b (36). It was observed in mice, that the density expression of these receptors divides the NK cells into subgroups, each representing a different level of maturation and cytotoxicity (37). The same receptors with the same subgroup functions were also seen in human NK cells and might explain the heterogeneity of the $CD56^{+}$ $CD3^{-}$ NK cells. Additionally, these markers can be important for understanding the role of NK development in different tissues, as well as in pathologies where NK dysfunction might contribute to the disease.

According to the CD11b and CD27 classification, NK cells can be divided into four different subsets: $CD11b^- CD27^-$ (DN), $CD11b^- CD27^+$ ($CD27^+$ SP), $CD11b^+ CD27^+$ (DP) and $CD11b^+ CD27^-$ ($CD11b^+$ SP) (36,38,39). The study by *Fu et al* (38), analysed NK cells from peripheral blood, cord blood and decidua and observed that different subgroups were expressed in each tissue. Most importantly they saw that the DN subgroup, generally seen in the decidua, had an immature phenotype, with high expression of inhibiting receptors such as NKG2A, low expression of activating receptors such as NKG2C and NKG2D and very low levels of mature phenotype markers such as CD11c, CD7 and CD2. It is not surprising that, DN NK cells also showed low levels of degranulation and cytotoxicity (38). The study also showed that $CD56^{bright}$ NK cells were mostly made up of DN NK cells as well as some $CD27^+$ SP (38).

1.1.4 Activating receptors

NK cells express an array of activating receptors that mediates their intrinsic cytotoxic ability. Some of the major activating receptors are shown in **Figure 2**.

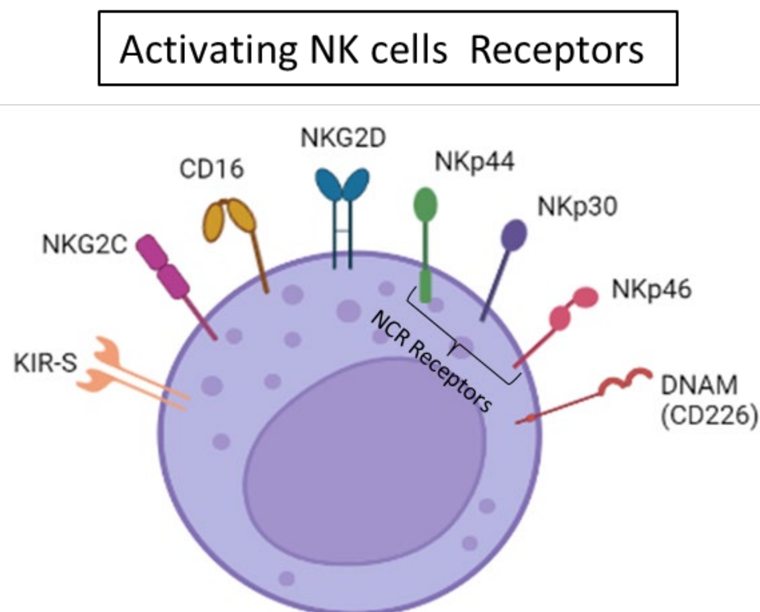


Figure 2. **Major activator receptors expressed on NK cells.**

Cartoon representation of NK cell characterized as CD56+ CD3-. NK cells express a range of activating receptors to enable their innate cytotoxic function. KIR receptors are divided in activators and inhibitors; KIR-S stands for stimulator KIR. Image generated with BioRender.

The natural killer group 2, member D (NKG2D) is an activating receptor that plays a key role for the function of NK cells, generating an immediate immune response. Also expressed on T cells and $CD56^{+}$T cells, NKG2D is very important against tumour repression, especially during the early stages of the disease (40–42). NKG2D binds to a variety of ligands such as MICA, MICB, ULBP, which are upregulated in stressed cells. Multiple stimuli can upregulate NKG2D, including DNA damage response, viral infections, and tumour, however, healthy cells normally do not express NKG2D ligands (42,43). *Guerra et al* showed that NKG2D deficient mice fail to control tumour progression, compared to NKG2D competent mice, both in a prostate and B-cell lymphoma models. Tumour killing by $NKG2D^{+}$ NK cells was depended on the number of ligands expressed by the tumour cells, indicating an NKG2D-dependent immunoediting response by the tumour (41).

Another set of activating receptors expressed on NK cell is the NCR family, comprising NKp30, 44 and 46, which contribute to degranulation and cytotoxicity upon activation (44). NKp46 is encoded by the gene *NCR1,* and it is expressed by $CD56^{bright}$ and $CD56^{dim}$(45). It has been shown to be important against tumours, causing cellular polarization at the immune synapse (46), favouring the release of IFN-γ and TNF-α (45). Additionally, NKp46 can be upregulated via IL-15, regulating TRAIL surface expression on NK cells, and allowing the cells to mediate anti-tumour activity also via TRAIL pathway (45).

NKp44 is encoded by the gene *NCR2*. Unlike NKp30 and NKp46, that are constitutively expressed by resting CD56bright and dim, NKp44 is only expressed by NK cells activated by cytokines such as IL-2, IL-15 or IL-1β and mostly present on $CD56^{bright}$ (45). Showing strong anti-tumour activity (47) , NKp44 has three different splice variants NKp44-1 is inhibitory, and it is upregulated by in vitro culture with IL-2 and IL-15, and NKp44-2 and NKp44-3 are both activating (48). Expression of specific isoform is determined by the cytokine milieu (45,49,50).

The *NCR3* gene codes for NKp30 and it is transcribed in three forms: NKp30a, b and c. Both NKp30a and b are immunostimulatory and induce cytotoxicity; however, NKp30c, is inhibitory

and high levels of its mRNA are associated with negative prognosis of cancer, favouring disease progression (44,51). Engagement of NKp30c, causes inhibition of NK cell function by secretion of IL-10 (44,52). The splice variant expressed is dictated by cytokines present in the environment (52,53). NCR receptors have been shown to cooperate with each other, mediating strong NK cell cytotoxicity and anti-tumour effect (45).

CD160 is another activating receptor that plays a role in NK function. It is expressed in the $CD56^{dim}$ $CD16^{+}$ NK cells, which constitute a large subpopulation in peripheral blood NK cells. Peripheral blood $CD160^{+}$ NK cells produce high levels of cytokines as well as IFN-γ; however, CD160 works independently and does not need co-receptor to be activated (54). Given the strong cytotoxicity that this receptor gives to NK cells, CD160 seems to be an important marker to determine NK function. However, not a lot is known about its behaviour, ligand binding and specific role in healthy and disease tissues. A recent study showed that CD160 expression positively correlates with IFN-γ production, as well as high levels of gene and protein expression of the activating receptors NKp30, NKp44, NKp46, NKG2D, CD244. In addition, inhibitory receptors such as NKG2A, TIM3, CD96, LAG-3, PD-1, are downregulated in $CD160^{+}$ NK cells (55). CD160 is important marker and has an active role as signalling molecule in NK cells, modulating their function (56), however, further research needs to be carried out in order to better highlight the role of $CD160^{+}$ NK cells, especially in the HCC context.

KIR are a family of receptors expressed on NK cell membrane, essential in mediating anti-viral and anti-tumour activity. They are highly polymorphic and regulate NK cell function, activation, education, and maturation (30,32). KIRs are divided into activating and inhibitory receptors and recognise MHC molecules expressed on the target cells. However, due to high similarities to the inhibitory counterpart, it has been challenging to discern the specific role as well as the ligands of activating KIRs. In this regard extensive research has been carried out, to exploit the anti-tumour activity of activating KIRs (57).

1.1.5 Inhibiting receptors

The cytotoxic ability of NK cells is modulated and counteracted by a series of inhibitory receptors; the major ones are illustrated in **Figure 3**. NKG2A form a heterodimeric complex with CD94 (CD94/NKG2A). It is one of the most prominent inhibitory receptors, a crucial immune checkpoint that controls NK cells activation. Indeed, high expression on NK cells is associated with lower NK function (58). It binds to the nonclassical HLA-E ligand, transducing the signal through immune-receptor tyrosine-based inhibition motifs (ITIMS), suppressing cytotoxicity and cytokine secretion (58,59).

CD96, T-cell immunoglobulin and ITIM domain (TIGIT), as well as CD226 all bind to the common ligand CD155, with CD96 and TIGIT engagement to send inhibitory stimuli, while CD226 sends activating stimuli (32). They bind to CD155 with different affinities, with CD96 showing intermediate affinity between TIGIT and CD226, CD226 has the highest affinity and TIGIT the lowest (60). Upon binding with its ligand, CD96 transduce the signal via an ITIM domain, causing inhibition of cytokine release and dampening NK cells response (61). Given the limited knowledge about additional role and function of CD96 in NK cell, further studies are needed.

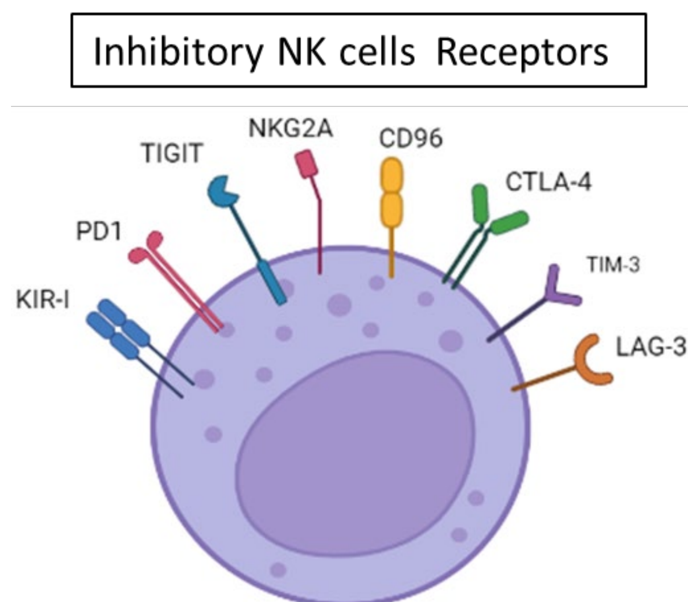


Figure 3. Major inhibitory receptors expressed on NK cells.

Cartoon representation of NK cell characterized as CD56+ CD3-. NK cells express a range of inhibiting receptors, to prevent continuous NK cell activation. KIR receptors are divided in activators and inhibitors; KIR-I stands for inhibitory KIR. Image generated with BioRender.

1.1.6 NK cell activation, signalling and response to stimuli

NK cells are influenced by the environment they reside in, responding to stimuli that control and hence influence their expansion as well as their activation. Key players in this are cytokines such as interleukins, which are secreted either by other immune cells or by cells within tissues. Cytokines that greatly influence NK cell maturation, expansion and activations are interleukin (IL)-2, IL-15, IL-12, IL-18, and IL-21 (**Table 1**) (10). Understanding how signals are integrated by NK cells can be of extreme benefit into the clinic. To this end, some studies have investigated the effect of using these cytokines to culture NK cells *ex vivo*, indicating encouraging results for NK expansion as well as increased anti-tumour response (35,62–68). However, *ex vivo* expansion of peripheral blood (PB)-derived NK cells, is very challenging. While substantial research has been done on T cells and cord blood-derived NK cells, limited studies have been done on PB-NK cells. These offer a safe and very accessible recourse which can be used to produce “off-the-shelf” treatment. Understanding the *ex vivo* manipulation of PB-NK cells by using cytokines, can thus be very important for the development of NK-based cancer immunotherapies.

Part of the common gamma (γ_c) chain group, IL-2, IL-15, and IL-21 display some shared features that are essential for NK function. These cytokines bind to a shared γ_c receptor subunit, which assembles with other different subunits to from a functional receptor complex, to which each cytokine binds with high affinity, resulting in optimal signalling (69). IL-2 and IL-15 share similar roles in NK cell biology, as they share the γ_c receptor as well as another receptor subunit (IL-2Rβ) and have common signalling pathways. They are both involved in the stimulation of NK cell proliferation, survival, and functional activities. However, there are differences between the two cytokines, as they are needed for specific cellular function, linked with their third receptor subunits that completes the IL-2 and IL-15 receptors (IL-2Rα and IL-15Rα respectively) (70). Although both are important for NK cell proliferation, IL-2 has been shows to not be essential for NK development whereas IL-15 might be. Additionally, IL-15 is essential for generation and maintenance of NK cells (71). Both IL-2 and IL-15 can rescue the cytolytic potential of poorly functional NK cells to induce enhanced antitumour activity (71), however it has been shown that continuous high-dose

treatment with IL-15 leads NK cells to exhaustion which might not be seen with IL-2, however this needs further research (72). IL-2 modulates the signalling and effect of other cytokines such as IL-12, as it upregulates the transcription factor STAT4 which mediates IL-12 signalling. This increases NK cells cytotoxic potential (73,74). NK cells exposed to irradiated feeder cells expressing membrane bound IL-15 (mbIL15) showed high expansion (75,76), however, continued proliferation was hindered by shortening of telomers that promotes senescence (77). As IL-15 also controls the balance between pro- and anti-apoptotic molecules, it is possible that reduced proliferation observed could be a consequence of a reduction of the important anti-apoptotic BCL-2 levels in rapidly cycling NK cells during their expansion phase (78,79).

IL-21 is a pleiotropic cytokine that influences the maturation and function of NK cells, such as cytotoxicity. However, conflicting results (80) have been reported regarding its effect on cell proliferation, with several studies suggesting that IL-21 induces apoptosis and shorter lifespan, while other research support its role in cell expansion. It has recently been indicated that boosting NK culture by adding IL-21 for short intervals increased cytotoxicity and proliferation of NK cells, making them more aggressive and reactive against cancer cells (63,67). This indicates that IL-21 effect is dependent on the NK exposure time to the cytokine (81). IL-21 modulates receptor expression by NK cells, and it has been suggested that IL-21 is able to reverse senescent Tim-3^+ PD-1^+ NK cells, restoring their anti-cancer function (82). IL-21 anti-tumour activity is dependent on NKG2D, which plays an important role in NK cells function as it is one of the main activating receptors (83). Interestingly, feeder cells expressing membrane bound IL-21 are thought to prevent NK cell senescence by preventing telomere shortening (77), which supported cell expansion and proliferation greater than what seen with mbIL-15 (77,84). A later study showed that IL-21 supports NK expansion by activating the transcription factor STAT3 which induces c-Myc, essential for the regulation of cellular processes such as glycolysis and cell cycle (84), therefore sustaining cell expansion. Therefore, understanding the right concentration to use as well as the exposure time to IL-21 is critical to see the benefits of this cytokine in *ex vivo* cultures (10).

Cytokine	Role in NK development	Receptor	Signalling
IL-2	Upregulates proliferation and function (2,71)	Binds to heterotrimeric receptor complex with high affinity. IL-2Rα + IL-2Rβ + γc (2,71)	JAK-1 + JAK-3 → STAT-1, STAT-3, STAT-5 (2,71)
IL-15 **Common γ-chain cytokines**	Similar role to IL-2. It is also essential for NK generation and survival. It mediates NK development, activation, and maturation (2,71)	Binds to heterotrimeric receptor complex with high affinity. IL-15Rα + IL-2Rβ + γc (2,71)	JAK-1 + JAK-3 → STAT-3, STAT-5 (2,71)
IL-21	Supports NK proliferations and increases NK cell cytotoxicity, upregulating CD16 expression (increasing ADCC) and secretion of IFN-γ, granzyme and perforins (2,71)	IL-21Rα + γc (2,71)	JAK-1 + JAK-3 → STAT-1, STAT-3, STAT-5 (2,71)
IL-18	Stimulates NK function synergizing with IL-2, IL-15, and IL-12, increasing IFN-γ production. IL-18 alone is not enough to induce IFN-γ (2,71) (2,71)	IL-18R (2,71)	STAT-4 (2,71)
IL-12	Induces production of IFN-γ and other pro-inflammatory cytokines, causing strong cytolytic activity and tumour suppression. It synergizes with IL-2, IL-15, IL-18 (2,71)	Binds to IL-12Rβ1 + IL-12Rβ2. Expression of both subunits required for high affinity binding (2,71)	STAT-4(2,71)

Table 1. Summary of cytokines role and classification

Part of different cytokine families, IL-12 and IL-18 are both pro-inflammatory cytokines which prime NK cells and upregulate their cytotoxicity by increasing IFN-γ secretion (2). IL-12 is a heterodimeric pro-inflammatory cytokine part of the IL-12 cytokine family, secreted by the antigen presenting myeloid cells during pathogen infection (85), which also shows potent antitumour response (86,87). NK cells sensitivity to IL-12 is increased in the presence of IL-2, IL-15, and IL-18, which synergise to increase IL-12 receptor expression, increase signalling via the transcription factor STAT4 and sustain survival (74,88–91). Not only IL-2 is needed to increase IL-12 sensitivity, but these two cytokines act in a positive feedback loop as IL-12 stimulation causes CD25 upregulation, consequently increasing NK cells response to IL-2, sustaining a robust NK cell expansion and proliferation, which also has been seen to support NK cells persistence in cancer patients (64,92–94). Furthermore, IL-12 has been shows to be essential for the generation of memory-like NK cells, together with IL-15 and IL-18, albeit only for a short exposure time (66,94,95). After IL-2 or IL-15 priming, IL-12 alone was able to sustain NK cells survival and activation but was not able to support proliferation (96). However, prolonged IL-12 stimulation, together with IL-2, IL-15, and IL-18 has limited effect on proliferation and might induce apoptosis (88,97–99).

Member of the IL-1 cytokine family, IL-18 shares biological functions and secretory cells with IL-12, however, the molecular mechanism leading to the increased expression of IFN-γ differ (100,101). IL-18 alone is not able to elicit IFN- γ production and it synergises with IL-12, which induces IL-18 receptor expression, and together mediate NK cells stimulation and activation (96,102). IL-18 has been described as a costimulatory cytokine that functions together with other cytokines, specifically IL-12, to increase NK cells production of IFN-γ and IL-15 to increase NK cell expansion (102–104).

The orchestration of cytokine action on NK cell proliferation, activation and maturations is mediated by an intricate web of intracellular signalling mediated by several transcription factors required for signal transduction and subsequent gene expression.

1.1.6.1 JAK/STAT pathways and activation by cytokines

Most of the cytokines determining NK cell functions signal via the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. Therefore, it is essential to understand their molecular mechanisms to better manipulate NK cells. Some of the cytokines that signal through the JAK-STAT pathway are IL-2, IL-12, IL-15, IL-21 (**Table 1**). As per the canonical signalling cascade, upon cytokine binding to its specific receptor, JAK kinases phosphorylate each other creating docking sites for STATs molecules, which become themselves phosphorylated and hence activated. Subsequently, STATs form homo- or heterodimers which translocate in the nucleus, bind to DNA and initiate target gene transcription. There are four JAK kinases, JAK1, JAK2, JAK3 and Tyk2, and seven STATs, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6 (2,69,105).

IL-2 and IL-15 stimulate the same pathway: cytokine binding to the specific receptor leads to activation of JAK1 and JAK3, which then activated STAT1, STAT3 and STAT5. Out of the three STATs activated, IL-2 and IL-15 heavily upregulate STAT5 and to a lesser extent STAT1 and STAT3 (69,106). Analysis of epigenetic changes by Wiedemann *et al* (88) showed that IL-2 and IL-15, in combination or alone, promote heavy chromatin changes to allow gene transcription, mediated by STAT5. However, under special circumstances such as hypoxia, IL-15 effect in NK cells is mediated via STAT3 which, however, interfered with IL-12/IL-18 mediated IFN-γ production, but did not impede degranulation, even in normoxic conditions (107). Interestingly, IL-2 is able to enhance expression of STAT4, which is a critical STAT involved in the signalling of IL-12. This leased to increased response of NK cells in terms of cell-mediated cytotoxicity and IFN-γ production (74). In line with other studies, IL-2 also leads to the activation of STAT4, in addition to STAT1, STAT3 and STAT5 activation (108,109).

IL-21 utilises JAK1 and JAK3 which allows the recruitment and activation of STAT1, STAT3 and STAT5, mediating its action predominantly via STAT3 (69,80). NK cell proliferation is increased via STAT3 mediated signalling (77,84). STAT3 activates human telomerase reverse transcriptase (hTERT), which leads to NK cells with longer telomeres, prevents senescence

and hence leads to greater cell expansion. Increased levels of hTERT in the presence of IL-21 might also coincide with the increase of other survival mechanisms, warranting the study of IL-21 implications in apoptosis (77). Culturing NK cells with either IL-21 or IL-15 alone, showed that each cytokine independently activated the binding of STAT1, STAT3, STAT4, and that IL-15 but not IL-21 induced STAT5 binding to regulatory elements of *IFN-γ* gene which initiate its mRNA synthesis and IFN-γ production. However, IL-15 strongly activates STAT5, and IL-21 primarily signals through STAT3 and the other STATs they activate are to a significantly lower level (110).

The pro-inflammatory cytokines IL-12 and IL-18 are produced early during infection by macrophages and dendritic cells, strongly stimulate NK cell leading to IFN-γ release. However, they induce a different intracellular signalling cascade. IL-12 induces phosphorylation of JAK1 and Tyk2, which lead to the activation of STAT1, STAT2, STAT3, STAT4, STAT5. STAT4 is the chief cytokine that mediates IL-12 signalling. IL-18 signalling activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), a different mechanism from the JAK-STAT pathways (69,106). However, in the absence of the kinase Tyk2, IFN-γ mediated by IL-18 is reduced. Additionally, the synergistic effect of IL-12 and IL-18 is abolished in the absence of Tyk2, suggesting crosstalk between the JAK-STAT and the NF-kB signalling mechanisms (111). The idea of a crosstalk between the two signalling mechanisms is further supported by the work carried out by Wiedemann *et al* (88). STAT4 via IL-12 and IL-18 promotes chromatin changes to allow gene transcription. However, the most significant changes identified was in a domain associated with the NF-kB pathway. Analysis of cells cultured with either cytokine alone, showed that IL-12 induced enrichment for STAT4 whereas IL-18 induced enrichment for NF-kB (88). IL-12 is responsible for the cytomegalovirus infected NK cells expansion and generation of memory NK cells. This is mediated via STAT4, and further sequencing analysis showed that STAT4 targets genes Runx1 and Runx3, which promote adaptive behaviour of NK cells and clonal expansion (112,113). IL-12 is included in the combination used to generate cytokine-induced memory-like NK cells (35), and it can be hypothesised that it induces memory-formation through STAT4-mediated chromatin remodelling. Furthermore, it has recently been shown that IL-12 sustains viability and proliferation of IL-12/IL-15 primed NK cells via STAT5 signalling (96).

It is clear to see that, although each cytokine signals through a preferred JAK-STAT pathway, there are significant intersections and overlaps, indicating that NK cell regulation is a complex process. Because of the impact that cytokine have on NK cell proliferation and cytotoxicity, there are in place negative feedback loops that attenuate the JAK-STAT signalling cascade. This is achieved by a group of proteins named as suppressor of cytokine signalling (SOCS) which prevent signal transduction by suppressing JAK kinase activity, by competing with STATs proteins and/or or by proteasomal degradation of proteins and are induced by the cytokines themselves (69).

The JAK-STAT pathway is a highly conserved system which regulates gene transcription in NK cells. With such a complex interplay the JAK-STAT pathway is an attractive target for drug development. As such, inhibition of the checkpoint CIS, a crucial negative regulator of IL-15 signalling, coupled with chimeric antigen receptor armed NK cells, has the potential to unleash an incredible antitumour response (114). This shows how the metabolic fitness of NK cells can be manipulated in vitro, and how important it is to understand the molecular mechanisms of signalling pathways.

1.1.6.2 NF-kB pathway and activation by cytokines

Important for both innate and adaptive immune cells, the transcription factor NF-kB is heavily involved in regulating inflammatory responses, by inducing expression of various chemokines and cytokines, such as IFN-γ and TNF-α. Additionally, NF-kB regulates several factors involved in apoptosis and cell proliferation, regulating cell viability and expansion. The NF-kB transcription factor family is composed of five structurally related members, which are NF-κB1 (also named p50), NF-κB2 (also named p52), RelA (also named p65), RelB and c-Rel, which are involved in a signalling cascade. The NF-kB pathway is divided into canonical and non-canonical pathway (115). These proteins are retained in the cytoplasm by a family of inhibitory proteins, including the IκB family of which IκBα is the best investigated member. Mimicking the role of IκB proteins, the proteins p105 and p100, precursors of NF-κB1 and NF-κB2 respectively, are also inhibitors of NF-κB. The NF-κB canonical pathway is activated by the degradation of the protein IκBα through site-specific phosphorylation. This is carried out by a multi-subunit IκB kinase complex, named IKK which is activated by a series of stimuli including

cytokines, growth factors, microbes, stress elements. Activated IKK phosphorylates IκBα, triggering ubiquitin-dependent degradation through the proteasome. This leads the formation of the dimers NF-κB1/RelA and NF-κB1/c-Rel, which rapidly and transiently translocate in the nucleus and bind to target genes (115). Conversely, the non-canonical pathway does not rely on the degradation of IκBα, and it is activated by specific stimuli. To start the non-canonical pathway, the NF-kB-inducing kinase (NIK) activates and cooperates with IKKα, a subunit of the protein IKK. Activated IKKα phosphorylates p100 which is ubiquitinated and degraded via the proteasome. As a result, mature NF-κB2 forms a dimer with RelB and both translocate in the nucleus to initiate transcription of target genes (115). The canonical pathway is involved in almost all aspects of the immune system, and the non-canonical pathway seems to have evolved as a complementary pathway to assist only in specific functions of adaptive immunity (115).

IL-18 has been shown to induce activation of the NF-kB1 protein, part of the canonical pathway, mediating IFN- γ production (100,116). As outlines above, IL-18 shares its biological function with IL-12, despite different signalling pathways, and its key in mediating IFN-γ secretion via NF-kB (88,110,111,116). Since NF-kB is involved in various cell processes, it offers attractive approaches for therapies development.

1.1.7 NK cell Immunometabolism

NK cells are important effector lymphocytes that mediate strong antiviral and anti-cancer activities. These immune responses involve rapid and extensive changes within the immune cells, and it has become clear that great metabolic remodelling is needed in order to support changes in energy demand and biosynthesis. Metabolic reprogramming is needed for IFN-γ (117) synthesis, expression of nutrients transporters, and increased cell metabolic pathways reconfiguration. In certain pathologies such as obesity or cancer, NK cells are found to be dysfunctional because of altered metabolic activities. Therefore, immunometabolism is fast emerging as a key regulator of NK cell effector function and ultimately responsible for NK cell-based immunotherapies outcome. To better understand NK cells capability and how to

modulate them, metabolic profiling can provide key information (118). Resting NK cells have low basal metabolic rates, with low levels of glycolysis and oxidative phosphorylation (OxPhos), which is enough to mediate acute IFN-γ production and hence NK cell effector function response. Interestingly, a short stimulation of 4-6 hours with cytokines IL-15 or IL-12 + IL-15 or IL-12 + IL-18 does not increase glycolysis or OxPhos rates (99,119). NK cell stimulation for a sustained period, such as overnight, results in an increase of both glycolysis and OxPhos (99,117,120,121). Metabolic differences are also seen in the two NK cell subsets $CD56^{bright}$ and $CD56^{dim}$. After IL-2 or IL-12 + IL-15 stimulation, $CD56^{bright}$ show upregulation of the glucose transporter GLUT1, amino acid transporters SLC7A5 and SLC3A2 and transferrin receptor CD71 higher than in $CD56^{dim}$ NK cells. This matches the $CD56^{bright}$-specific function of increased IFN-γ production (117,122). Cytokine modulation of NK cells' metabolism is crucial and needs to be carefully studied, as long-term exposure of NK cells to cytokines such as IL-15 *in vitro* causes a reduction in metabolism (72).

Activated NK cells show increased glucose uptake, increased glycolysis, and a subsequent increase in the expression of glycolytic enzymes and nutrient transporters. This great increase in glycolysis, and subsequently in OxPhos, comes with an increase in mitochondrial mass. The increased metabolic changes are necessary to facilitate NK cell effector functions and indicate the importance of NK cells in prolonged immune responses (118). Inhibition of glycolysis does not prevent NK cells to maintain homeostasis, however they cannot support proliferation or mediate cytotoxic effector functions to lower IFN-γ and granzyme B expression (120). This suggests that glycolytic metabolism is vital for NK cells effector functions. Glycolysis upregulation replicates the typical metabolic profile observed in tumour cells: the Warburg effect, characterized by increased glycolysis and anabolic metabolism in the absence of oxygen to allow tumour cells' survival in the hypoxic and starved TME. Essential for this IL-21 signalling through STAT3 seen in NK cells expanded with IL-21-expressing feeder cells (123). Therefore, these Warburg-like NK cells might be more metabolically suitable to survive within the harsh tumour microenvironment and constitute an attractive therapeutic target. Additionally, because of the increased glycolysis, measuring glucose uptake and lactic acid release as a by-product provide a good quantitative indication of NK cells effector functions.

The mammalian target of rapamycin complex 1 (mTORC1) is a key regulator of metabolic functions in several cell types including NK cells, highly regulated by cytokine stimulation. NK cells rely on mTORC1 signalling for metabolism reprogramming needed to sustain the energetic demands during activation and development (120). As already mentioned, resting NK cells have a low metabolic rate, which is increased after IL-2 and IL-12 stimulation as a results of a robust activation of mTORC1 signalling (120). This leads to increased upregulation of nutrient transporters and glycolytic enzymes, which induce increased glucose uptake and increased mitochondrial mass to produce effector molecules such as IFN-γ and granzyme B (118,124). Consistent with their function of increased IFN-γ production, mTORC1 is upregulated in CD56^{bright} NK cells (117,122). Interestingly though, the requirement for mTORC1 signalling is modulated by the presence of specific cytokines. For example, mTORC1 is required by IL-2 stimulated NK cells for increased glycolysis, however, this was not observed when cells were stimulated with IL-12 + IL-15 (117). The role of the other mTOR complex (mTORC2) in NK cells is less known. Analysis of underlying mechanisms have shown that mTORC1 and mTORC2 promote NK cell maturation in a cooperative manner, however they regulate NK cell cytolytic functions in opposite ways. mTORC1 maintains CD122-mediated IL-15 signalling which sustains mTORC2 activity, however, mTORC2 suppresses mTORC1 effector function by inhibiting STAT-5-mediated expression of the amino acid transporter SLC7A5 (125). The crosstalk and interplay between different signalling systems (STATs and mTOR) indicate NK cells' ability to regulate their effector functions, providing the opportunity for attractive drug targets.

Addition of rapamycin, a strong mTORC1 inhibitor, to NK cells stimulated with IL-2 + IL-12 or IL-15 only leads to reduced proliferation, IFN-γ production and cytotoxicity causing greater tumour burden. This shows the importance of mTORC1 for NK cell metabolic reprogramming and hence effector functions. Interestingly, IL-18 can rescue glycolysis in rapamycin-treated NK cells as it does not depend on mTORC1 for the upregulation of nutrient transporters (124). Another inhibitor of mTOR is TGF-β, one of the strongest immunosuppressive cytokine. Its target is mTORC1, strongly blocking IL-15 induced activation of mTOR pathway and its associated metabolic and cytotoxic changes. Therefore TGF-β inhibition rescues mTOR

signalling and NK cell function, providing a strong rationale for TGF- β targeted drugs to be used together with NK cell-based cancer immunotherapies (121).

1.1.8 Summary

Based on their cytotoxic potential NK cells are an attractive tool for clinical applications such as cancer therapies. Hence, understanding how NK cells repertoires are formed and maintained and how they can be manipulated can be of great value for future treatments development. As such, it is critical to fully explore the vast array of fundamental mechanisms that drive intrinsic NK cells roles and functions at basal levels as well after cytokine stimulation. Additionally, it would be beneficial to understand how to isolate and enhance specific cytotoxic NK cells subpopulations, how to ensure their expansion *ex vivo,* and how NK cells can be genetically engineered to augment their features even further, avoiding exhaustion. Lastly, essential to the development of a successful therapy targeted to cancers is the understanding of how the surroundings affect NK cells. The harsh TME will shape NK cells responses and cause alterations in their biology, therefore finding ways to circumvent that will ensure more positive outcomes.

Given the controversies gathered around IL-21, studying its role in *ex vivo* feeder cell-free culture in detail helps to elucidate its role in NK cell biology. Furthermore, it is worth exploring the importance of adding IL-21 at specific point during cell culture and what the implication of this are. Therefore, pursuing research involving IL-21 in NK cell culture is necessary as the results might be important for improving NK-based cancer immunotherapies.

1.1.9 Trained immunological memory of NK cells

As mentioned before, unlike B and T cells, NK cells do not rearrange their receptor genes. NK cells rely on a limited number of germline encoded activating and inhibitory receptors, to detect signals for environmental cues, such as cytokines. Furthermore, B and T cells show antigen specificity and increased response upon later antigen exposure. However, NK cells with a limited receptor repertoire, provide limited antigen recognition, and are thought to show similar responses upon a repeated stimulation (95,126,127). This, however, was contradicted when NK cells were shown to mediate hapten-specific adaptive immunity in mice lacking B and T cells. This indicated that NK cells can develop immunological trained memory and hence show memory-like characteristics, similar to the adaptive system (128).

Over the last decades a large volume of work has shown that NK cells show memory and memory-like responses, and these are well established after hapten exposure, viral infection, or cytokine stimulation (129,130). Advances in the understanding of NK cells' adaptive feature have been fuelled by the opportunity to translate memory-like NK cells into the clinic as cancer immunotherapies. To this end, several investigations have shown interesting and promising results which warrant further analysis.

1.1.9.1 Hapten-induced memory NK cells

Hapten-induced contact hypersensitivity (CHS) is a classic example of adaptive immunity. This occurs when the epithelium is exposed to certain molecules that have the ability of chemically modify proteins. These molecules are then recognised as foreign antigens and induce formation of hapten-specific immune memory, mediated by memory T cells. However, mice lacking T and B cells were able to exhibit hapten-induced CHS and to discriminate between different haptens (129,130). This was mediated by NK cells, and it persisted for at least four weeks. More specifically, adoptive transfer of hepatic NK cells in naïve mice, but not splenic NK cells, showed CHS responses in the recipients upon exposure to the same hapten (128). Later studies were then able to demonstrate that the happen-specific CHS response mediated by hepatic NK cells was dependent on the expression of the chemokine receptor CXCR6, responsible for NK homing to the liver (131). This was the first study which showed adaptive responses from NK cells.

1.1.9.2 Virus-induced memory NK cells

It was later showed that murine NK cells can exhibit adaptive responses after mouse cytomegalovirus (MCMV) infection (132). This was also demonstrated in humans, where infection with human CMV (HCMV) resulted in a specific NK cell subset which expressed high levels of the receptor NKG2C. NKG2C+ cells in hematopoietic stem cell transplants (HCT) demonstrated increased adaptive responses upon a second HCMV infection. The NKG2C+ NK cell population expanded well, had enhanced cytokine secretion, persisted long after human CMV infection was cleared, expressed high levels of CD57 (a marker of NK cell maturity and terminal differentiation) and lacked expression of the inhibitory receptor NKG2A (133). Analysis of memory NK cells in HCMV positive individuals showed that these cells have increased glycolysis and oxidative phosphorylation compared to conventional NK cells from CMV negative individuals (134). Additionally adaptive NK cells from CMV+ individuals expressed high levels of CD16, indicating that memory NK cells can be paired with therapeutics antibodies and applied in clinical settings (135). However, 4% of all humans do not express NKG2C due to a homozygous deletion in the gene. Interestingly, NKG2C- individuals also showed adaptive NK cell responses, suggesting that NKG2C might not be essential to induce adaptive NK cells and that other cellular mechanisms are involved, such as antibody-dependent cell mediated cytotoxicity (136).

1.1.9.3 Cytokine-induced memory-like NK cells

Recently, studies on NK cell cultures have revealed a critical role for IL-12, IL-15 and IL-18 used in combination to generate cytokine-induced memory-like (CIML) NK cells (35,64–66,94). CIML NK cells are more reactive than NK cells cultured with each cytokine alone or compared to IL-2+IL-15 only, with enhanced longevity, proliferation and IFN-γ production. CIML NK cells arise after a short incubation period (~16 hours) with IL-12+15+18, followed by a rest phase with low dose IL-15 only. A later cytokine stimulation causes an even higher IFN-γ production and persistence of memory-like characteristics in the NK offspring allows CIML NK to survive for several months in patients (35,66,95). These results encourage further studies on CIML NK cells, exploring their function in a variety of tumours and their use in the clinic for immunotherapy.

Detailed phenotypical analysis of CIML NK cells *in vitro*, revealed increased CD94, NKG2A, NKG2D, CD25, NKp30, NKp44, NKp46, and CD56 indicating the presence of a large proportion of CD56^{dim} CIML NK cells, compared to control cells (not CIML NK). Furthermore, CIML NK cells showed higher expression of granzyme B, perforin and TRAIL. Regarding graft-versus-host disease caused in hematopoietic cell transplant (HCT), CIML NK suppressed GvHD, however the mechanism for this needs further evaluation (66). Mechanisms involved in the formation of CIML NK cells features are not fully understood. Downregulation of TGF-β and its signalling pathway by the cytokines IL-12, IL-15, IL-1, might contribute to the enhanced antitumour response seen (137). A study by Ewen et al, showed that these cytokines can also downregulate inhibitory KIRs, reducing CIML NK sensitivity to cognate HLA-I ligands (138). Some epigenetic changes are also observed in CIML NK which coincide with that is observed in virus-induced memory-like NK cells. As outlined in section **1.1.6**, IL-12 is responsible for the clonal expansion of cytomegalovirus infected NK cells and generation of memory NK cells, mediated via STAT4, (112,113). Thus, it can be hypothesised that IL-12 induces memory-formation through STAT4-mediate chromatin remodelling in CIML NK. CIML differentiation might include alterations similar to those seen in memory T cells, especially in NKG2C+ CIML NK cells, however, further studies in the specific CIML setting need to be done (139). A recent study showed increases expression of transferrin receptor, amino acid transporters and glucose transporters in CIML NK cells. The increased expression of amino acid transporter was also seen during the resting phase, after 16 hours pre-activation with IL-12+15+18. CIML NK cells upregulated glycolysis and its high activity persisted even during the resting phase. The study also showed that IFN-γ production was affected by glycolysis inhibition in CIML NK cells, whereas other functions such as TNF-α production and degranulation where not affected, suggesting that CIML NK could maintain higher antitumour activity in the glycolysis restrictive conditions found in the TME (140). However, different tumours present different TME make-up therefore it is important to explore CIML NK ability to overcome metabolic suppressions in different environments, as well as understanding the contribution of other metabolic pathways.

It is no surprise that CIML NK cells are attractive tools to use in adoptive cell transfer therapies, due to their beneficial characteristics. CIML NK maintained memory-like phenotype in a

human phase I clinical trial against acute myeloid leukemia (AML). None of the patients involved in the trial showed any severe cytokine release syndrome (CRS) toxicity or GvHD. CIML NK showed increased proliferation, expansion, and sustained AML cells after adoptive transfer (66,129). Evaluation of CIML NK cells against ovarian cancer cells, showed increased cytokine production and tumour cells killing compared to base-line NK cells. Additionally, CIML NK cells maintained enhanced and potent antitumour functions in the ovarian tumour immunosuppressive TME (141). Multidimensional analysis of AML patients treated with CIML NK, shows phenotypically distinct marker in CIML NK compared to baseline NK cells. These where the same as those explored *in vitro* by Fehninger et al (66), however, adoptively transferred CIML NK did not express CD25 (IL-2Rα). Correlation analysis showed that NKG2A upregulation in CIML NK was associated with treatment failures in these AML patients. The frequency of donor's CD8 effector T also negatively correlated with CIML NK therapy success. (142). Therefore, this multidimensional analysis reveals important factors that might lead to therapy failure, which can be used as new venues to improve NK cell-based treatments, for example by using a combinational therapy of CIML and NKG2A inhibitors.

As many cancers are not recognised by NK cells receptors, and are thus resistant to treatments, CIML-NK have been enhanced by the addition of chimeric antigen receptors (CAR) targeting a specific receptor on tumour cells. Recent analysis on NK-resistant B-lymphoma malignancy, showed increased CAR-CIML NK tumour burden control *in vitro*, *in vivo*, and improved survival in human xenograft models, compared to controls of conventional CAR-NK or CIML only NK (143). The same group also developed CAR-CIML NK cells targeting AML tumour with the NPM1 mutation. CAR-CIML NK targeting NPM1 showed increased anti-tumour function and specific killing. The group that conducted the proof-of-concept study, is in the process of gathering enough supporting pre-clinical data in order to start a phase I clinical trial for CAR-CIML NK cells in patients with relapsed or refractory NPM1-AML (129,144).

All these positive outcomes, warrant further work on NK cell-based therapies on a range of different tumours, especially solid tumours which present a much more complex TME than myeloid tumours.

1.1.9.4 Memory-like NK cells summary

Once thought as simple immune cells belonging to the primitive innate immune system, NK cells have demonstrated remarkable complexity, playing an essential role in mediating a robust and effective immune response. With much knowledge gathered on NK cells, we now know that they are capable of spectacular anti-tumour activities, which can be further enhanced via cytokine stimulation. NK cells also show adaptive immunity, which greatly increases their functions and cytotoxic potential. Crucial in this has been the analysis of cytokine stimulation and the realization that the specific cocktail of IL-12, IL-15, IL-18 is needed in order to achieve cytokine-induced memory-like NK cells. This has been incredibly advantageous in the generation of CIML from healthy donors, without the need of having previous HCMV infection or exposition to potentially poisonous haptens. The increased cytotoxicity of CIML NK cells has been shown to be particularly useful against blood tumours such as leukemia, showing complete remission in AML patients treated with CIML NK therapies. The enrichment of CIML NK cells by the engineered addition of CARs increased their antitumour activity and their application to different tumours. Solid tumours, however, still require an abundance of research and understanding to fully utilize CIML NK cell potential in this harsh environment.

Quick cell response and long-lasting memory features are a huge asset to developing an NK-based cancer immunotherapy. As memory like NK cells are generated using cytokines, it would be interesting to assess whether the introduction of another cytokine to the defined IL-12, IL-15, IL-18 combination might show increased cytokine effect on NK cells. Understanding the addition of IL-21 in this context would be very interesting, given its proven benefit on increased NK cell expansion and cytotoxicity (77,84).

1.2 NK cells and the liver

1.2.1 Hepatic environment

The liver presents a unique environment as it is very immunosuppressive, due to the countless antigens to which hepatic cells are exposed, that can elicit an unnecessary immune response. As a result, the liver contains a range of immunomodulatory cells that secrete anti-inflammatory and immunosuppressive cytokines such as TGF and IL-10, as well as expressing inhibitory molecules such as indoleamine 2,3-dioxygenade (IDO), to prevent NK and T cells cytotoxic activity. However, the strong tolerogenic nature of the liver can allow development of chronic diseases such as tumours. HCC thrives in the immunosuppressed liver environment as cancer cells can escape immunosurveillance. Impaired or a lack of functional NK cells contributes to disease progression, which is amplified by the liver own immunosuppressive environment and the immunosuppressive TME (27,145). Not only does this contribute to tumour burden but impairs HCC treatment with immunotherapy, and for this reason detailed analysis of TME, NK cell metabolism and further therapy developments are vital for HCC treatment improvement and management.

1.2.2 Development of Hepatocellular Carcinoma

The burden of liver cancer on global health constitutes a persistent and growing challenge. With its incidence increasing worldwide, it is the leading cause of cancer related mortality. HCC is the most prevalent type of liver cancer, which accounts for over 90% of cases (146–149). HCC typically develops in the context of chronic liver disease which proceeds to liver inflammation, known as hepatitis, and causes liver cirrhosis. This can have both viral and non-viral origins (146). The main risk factors for HCC include non-alcoholic steatohepatitis (NASH), caused by excess accumulation of fat in hepatocytes, non-alcoholic fatty liver disease (NAFLD), chronic alcohol consumption, and infection by hepatitis B virus (HBV) or hepatitis C virus (HCV) (147). HBV infections occur in endemic areas and account for 60% of HCC cases in Asia and Africa, but only for 20% of HCC cases in western countries. HBV is a DNA virus which integrates in the host genome and leads to oncogenes activation. HCV, on the other hand, is an RNA virus that does not integrate with the host genome, but HCV chronic infection leads to cirrhosis with the potential of developing into HCC. HCV infection is the most common

underlying liver disease among patients with HCC in North America, Europe, and Japan (146,147).

While prevalence of viral-related HCC has declined due to vaccines development and distribution, the incidence of NAFLD and NASH-related HCC has increased (146,150). Patients with diabetes mellitus or obesity can develop NASH, which increases the risk of HCC. Because of increased prevalence of obesity worldwide, NASH has become the most common cause of cirrhosis, however, incidence of HCC is lower in NASH-related cirrhosis compared to virally induced cirrhosis (147). NASH and NAFLD promote HCC via similar mechanisms which involve metabolic stress, altered immune function, pathological inflammatory responses, and altered signalling. Specific NASH or NAFLD associated risk factors have not yet been discovered (147,150), therefore it is challenging to discern between NASH and NAFLD-based liver disease.

Excessive alcohol intake sets the stage for alcohol liver disease, cirrhosis development and eventually HCC. The prevalence of alcohol-related cirrhosis is rising, and accounts for 15-30% of HCC cases, varying across different geographical regions (147). Chronic alcohol abuse also increases risk of HCC development from other aetiologies, for examples individuals with HBV who consumed alcohol have an increased risk of HCC (151). Furthermore, several sociodemographic factors influence HCC development, particularly in a cirrhosis setting. HCC is mostly reported in individuals >70 years old, with a strong predominance in males. Additionally, racial, or ethnic minorities tend to have a higher incidence of HCC, likely linked to different gene variants expression across ethnicities. Lastly, smoking has also been associated with increased risk of HCC development (147).

Although well-defined risk factors of HCC are available, it is still challenging to detect HCC formation. Patients usually get diagnosed in the later stages of the cancer, making treatment ineffective (147). However, the underlying aetiology of HCC plays a role in how patients respond to therapy (152–154) which should be taken into consideration when developing novel immunotherapies for optimal efficacy.

1.2.3 Liver NK cells

NK distribution in human tissues differs from peripheral blood and these populations are phenotypically and functionally diverse. One of the major immunological tissues in the body is the human liver, which has a specific microenvironment, being one of the first line of defence from pathogens, with a high exposure to enormous amounts of antigens (155,156). Within the liver, the NK population consists of conventional NK (cNK), and CXCR6^{+} liver-residents NK (lr-NK) cells (27). Unlike peripheral blood NK cells, the intrahepatic NK cell population is made up of 50% CD56^{bright} and 50% CD56^{dim} NK cells (27,157). However, during liver damage, such as hepatocellular carcinoma (HCC), CD56^{dim} NK cells are significantly reduced both in the peripheral blood and in the tumour, regions compared to healthy liver tissue (158). Conventional NK cells in the liver might be derived from the CD56^{dim} NK cells present in the peripheral blood, which circulate via the liver blood system. However, lr-NK cells are significantly different from cNK cells (27). Liver resident NK cells resemble the immature conventional NK cells (CD56^{bright}), with a higher production of proinflammatory cytokines and low cytotoxicity (159). Indeed, some studies have compared liver-resident NK cells to innate lymphoid cells group 1 (ILC1), with similar functions but diverse phenotype. Due to the many differences, lr-NK cells are thought to originate and develop differently than cNK cells (160).

Liver-resident NK cells show high expression of tissue specific receptors, such as CD69+, CCR5+ and CXCR6+ and are located within hepatic sinusoids (161). CXCR6^{+} liver resident NK cells are Tbet^{low} Eomes^{high}, have an immature phenotype and are highly abundant in healthy and diseased liver compared to blood (162). High frequencies of CXCR6^{+} CD69^{+} NK cells are also found in HCC tissues, compared to relatively lower frequencies present in the blood (163). Furthermore, it has been discovered a separate subset of liver-resident NK cells that express CD49a^{+}, defined as CD49a^{+} CD69^{+} CD56^{bright} (164). Their transcriptional profile is similar to CD16^{+} cNK, characterized by the expression of Tbet^{+} and Eomes^{-}. CD49a^{+} lr-NK cells represent only ~3% of the total NK population (27,164), lack the CXCR6 receptor (162,164), activating KIR and CD16, but present high levels of the activating receptors NKG2C^{+}, NKp46, NKp30, NKG2D (164). The CD49a^{+} NK cells are found in the liver (162,164) and are absent from

peripheral blood. Mainly present in liver parenchyma than liver vasculature (162), but at lower frequency than CXCR6$^+$ NK cells (162), these cells are seen both in the liver of healthy patients and in the tumour-free regions of patients with liver cancer (164). CD49a$^+$ NK cells accumulate in the liver during HCC, correlating with NK cells downregulation and inhibition as well as worse disease progression (165). Expression of the activating receptor NKG2D is significantly lower in HCC (163); however, the expression of TRAIL receptor, partially mediates NK cytotoxicity (27). During HCC, other NK cell receptors show an altered expression compared to circulating- and liver-NK cells in healthy conditions. Some of these alterations are discussed below and summarized in **Table 2**.

NK Marker	Presence of marker in liver NK cells within HCC tissues
$CD56^{bright}$/tot NK cells	~50%
$CD56^{dim}$/tot NK cells	~50%, but significantly lower in HCC CHANGE
CD3	-
CD16	-
CD69	+
CD49a	+ (in some lr-NK cells)
CCR5	+
CXCR6	+
NKG2D	Low
NKp30a, b, c	-,-,+
NKG2A	+ (in tumour tissue)
CD96	+ (high in tumour tissue)
Tim-3	+ (in tumour tissue)
PD-1	Expressed on tumour NK cells and in T cells
KIR • **KIR2DL2/L3/S2** • **KIR2DL1/S1/S3/S5** • **KIR3DL1/S1/L2**	+ (in $CXCR6^-$ NK cells) + (in $CD49a^+$ NK cells)
CD57	-
CD11b	- (in tumour tissue)
CD27	- (in tumour tissue)
CD160	- (low in intratumoural tissue)
TRAIL	Expressed on some lr-NK

Table 2. NK markers specific for hepatic NK cells.

Using a combination of antibodies allows the detection of NK cells in HCC tissues via immunohistochemical staining of liver sections. In the left-hand side of the table is shown the expected staining for each marker.

NKG2D

NKG2D is upregulated on $CXCR6^+$ lr-NK cells compared to their $CXCR6^{-}$ cNK liver-infiltrating counterparts, both in HCC and normal liver tissues. However, comparison of HCC liver with healthy liver showed an overall downregulation of NKG2D in HCC (163). This overall downregulation was attributed as to the expression of NKG2D ligand by the liver and subsequent excessive interaction with the receptor (41,163,166,167). Additionally, NK cells with low NKG2D levels have lower cytotoxic activity against liver tumour cells. This is in part due to internalization of NKG2D after exposure to HCC cells (163) Interestingly, a recent study in a mouse model of HCC, indicated that NKG2D expression might encourage tumour development, due to continuous release of pro-inflammatory components and increased liver damage (168). However, the effect might be driven by $NKG2D^+CD8^+$T cells rather than $NKG2D^+$ NK cells (168), thus it is important to make a distinction between T and NK cells, as well as other cell-cell interactions that might alter normal function.

NKG2A

Elevated expression of this receptor in peripheral blood NK cell has been shown, but less is known about hepatic NK cells in healthy and HCC liver. It has recently been suggested that NKG2A is also expressed in NK cells residing in the liver, especially in $CD56^{dim}$ NK in intratumour tissue, whereas expression on $CD56^{bright}$ was similar between intratumour and peritumour regions (169).

NKp30

The NCR3 gene codes for NKp30 and it is transcribed into three forms: NKp30a, b and c. Both NKp30a and b are immunostimulatory and induce cytotoxicity; however, NKp30c, is inhibitory and high levels of its mRNA are associated with negative prognosis of cancer, further allowing disease progression (170,171). It has been suggested that peripheral blood and tumour-infiltrating NK (TINK) cells have an exhausted phenotype, with low cytotoxicity and high expression of the immunosuppressive NKp30c isoform (51). NKp30 mediated cytotoxicity was also reduced in the liver with different NKp30 levels in tumour compared to healthy corresponding liver tissue (51). NKp30c isoform is also seen in the liver and responsible for lower cytotoxicity despite high levels of the receptors NKp30 in NK (51). However, further studies need to be carried out to further outline the role of NKp30 in the context of HCC.

CD11b and CD27

The study conducted by *Zhang et al*, investigated whether the CD27 and CD11b have an influence in shaping HCC (39). The authors found that when they compared HCC and healthy tissues, there was a high level of tumour-infiltrating NK (TINK) cell accumulated in tumour tissue (39). However, a high percentage of TINK were DN NK cells, with an inactive and immature phenotype. Indeed, DN NK cells had a low expression of activating receptors (including NKG2D, NKp30 and CD226) and downregulation of markers expressed on highly mature NK cell, such as CD2, CD7, CD57, CD11c. Not surprisingly, tumour infiltrating DN NK cells had a high expression of inhibiting receptors (NKG2A, KIR2DL2/DL3 and KIR3DL1/DS1) and upregulation of markers expressed on immature NK cells (CD117 and CD127) (39). Additionally, DN NK showed low degranulation and cytotoxicity, contributing to the dysfunction of NK cells seen in HCC. Furthermore, the presence of DN NK in tumour tissue is associated with HCC progression and tumour size and negatively correlates with patient survival (39). In addition to HCC, the immature $CD11b^{-}$ $CD27^{-}$ subtype was also seen in the TINK cells of other tumours, such as breast and lung cancer, associated with the progression of the disease (172,173).

CD96

Sun et al (2019) (174) investigated the interplay of the immune checkpoints CD96, TIGIT and CD226, in NK in the context of HCC. The study by *Sun et al* revealed that in HCC there is an unbalanced expression of these receptors, due to elevated $CD96^+$ NK cell expression in intratumour HCC tissue, compared to peritumour tissue and healthy liver (174). Additionally, increased expression of $CD96^+$ NK cells is seen in the blood of HCC patients. $CD96^+$ NK cells are functionally exhausted, with upregulation of genes related to inhibitory NK receptors, such as TIGIT, PD-1, NKG2A and LAG-3 and downregulation of genes related to activating NK receptors, such as CD69, NKp30, NKp40, KLRG1 (174). Not surprisingly, $CD96^+$ NK cells have impaired cytokine production. HCC patients with high intratumoural levels of $CD96^+$ NK cells have poor prognosis, short disease-free survival, and more aggressive disease. Poorer prognosis is seen when the patients present high levels of CD155 (174). Interestingly, in $CD49a^+$ NK cells CD96 is upregulated as well as other inhibitory receptors such as NKG2A, LAG3 or PD-1 (165). As previously mentioned, $CD49a^+$ NK cells have an exhausted phenotype and are abundant in intratumoural liver tissues (165), further contributing to the impaired function of NK cells in HCC as well as correlating with a worse prognosis.

Tim-3

Very little is known about liver resident NK cells regarding Tim-3 expression both in healthy individuals and patients with HCC. *Ju et al* (175) showed an increased Tim-3 expression both in PBMC and hepatic NK cells in patients with hepatitis B (HBV^+). High levels of Tim-3 correlated with inhibited NK function and impaired cytotoxicity, which was reversed using an anti-Tim-3 antibody (175). A recent study reported that lr-NK and cNK cells are reduced in HCC tissue, compared to healthy tissue (176). Additionally, lr-NK and liver-infiltrating cNK cells had a very high expression of Tim-3, TIGIT and PD-1, with Tim-3 being the most elevated one which correlated negatively with HCC patient survival. Tim-3 expression was significantly higher in tumour tissues compared to peritumour tissue. Blocking Tim-3 with an antibody restored the NK cell degranulation and IFN-γ production, and this correlated with death of tumour cells. This was also seen *in vivo* (176). High levels of Tim-3 inhibited peripheral blood NK cell functions and contributed to worse disease progression in lung adenocarcinoma (177), advanced melanoma (178), gastric cancer (179), oesophageal cancer (180). Upregulation of

Tim-3 correlates with impaired NK cell function, modulating immune anti-tumoural activity. However, further research needs to be done for HCC to better understand the expression of function of Tim-3 in lr-NK and cNK, both in the presence or absence of viral infections (HBV/HCV).

KIR

In tissues NK cells might behave differently. It has been hypothesised that tumour-infiltrating NK cells might resemble tissue-resident NK cells, perhaps because of the environment influence. This is specifically important during tumours, when the tumour microenvironment is well known to have an influence on immune cells. Often this is negative, which correlates with the downmodulation of immune cells and contributes to disease progression, as it has been seen in HCC. KIR expressing NK cells are protective for hepatitis (HCV and HBV) infections (181–185), one of the major causes of HCC development. Two different studies done on lr-NK showed that the immature $CD56^{bright}$ $CXCR6^{+}$ NK cells have very low expression of KIR, however this was a lot higher on liver $CXCR6^{-}$ NK cells which have similar expression level to peripheral blood NK cells (186,187). *Hydes et al* (186) showed that $CD49a^{+}$ NK cells had high levels of KIR receptors, specifically KIR2DL1/S1/S3/S5 and KIR2DL2/L3. Upon analysis of gene expression, they saw that $CXCR6^{+}$ NK cells had a reduced expression of KIR and CD57 genes. Interestingly, they found two more NK subsets, $CD49a^{+}$ $CXCR6^{+}$ and $CD49a^{-}$ $CXCR6^{-}$, and that the $CD49a^{+}$ $CXCR6^{+}$ subset was $CD69^{+}$ $CD16^{low}CD57^{low}$, with high frequency of KIR (186). *Lunemann et al* showed that $CXCR6^{-}$ NK cells expressed KIR2DL1/L2/L3, KIR2DS1, KIR3DL1/L2 (187). It is not surprising to see a low expression of KIR as well as CD57 in liver resident NK cells, as these receptors are usually expressed in advanced maturations stages. However, most of the lr-NK cells in HCC are immature. A study done on healthy livers where $CD56^{bright}$ and $CD56^{dim}$ NK cells exist in equal proportion, indicated that liver $CD56^{bright}$ have lower expression of NKG2A, whereas liver $CD56^{dim}$ have lower expression of CD57 compared to peripheral blood NK cells. Additionally, they showed that the levels of KIR remained the same in liver and peripheral blood $CD56^{dim}$ NK cells (188). More detailed studies on the specific KIR genotyping of lr-NK compared to cNK, both in healthy liver and HCC, can further aid the understanding of liver NK cells and their role in HCC.

1.3 Natural Killer cells immunotherapy

In recent years, NK cells have gained increasing attention for their use in cancer immunotherapy. This was particularly seen after the FDA approval of chimeric antigen receptor (CAR)-T cells targeting CD19 in patients with relapsed leukaemia (189–192). The therapy came with several disadvantages and the initial positive results were soon followed by severe side effects, which demanded further understanding of CAR-T therapy and perhaps propose a substitute immune-based therapy line. T-cell immunotherapy is very expensive, and the manufacturing process is very lengthy. Additionally, there is a requirement for autologous T cell to be used, to prevent graft-versus-host-disease (GvHD). Because the process needs to be patient-specific, it can be challenging to produce the specific amount of cells required for the final therapy; allogeneic T cells might be used, but this requires a meticulous tissue matching process, which can still result in adverse effect. Furthermore, one of the most dangerous side effects of T cell therapy is cytokine-release syndrome (CRS), which can be lethal for the patient; it can also cause off-tumour targeting and neurotoxicity. Lastly, CAR-T cell activation is restricted by TCR recognition of complementary receptor on the tumour cell, which will be impaired if malignant cells miss the MHC complex (19,193–196).

As an alternative to T cells, NK cells provide a safer source for cell therapy, favouring the use of allogeneic cells with little toxicity and limited CRS. Key feature that makes NK cells suitable for cell therapy, is their array of inhibitory and activating receptors. These receive cues from the surrounding environment and rely on signals sent to the activating receptors from targets cells, usually virally infected or malignant cells with downregulation of HLA complexes. This activates NK cells which mediate immediate killing of target cells, as they do not require prior priming and exposure to antigen, unlike T cells (3,197). Additionally, NK cells are short lived *in vivo*, with a lifespan of about 2 weeks, limiting the risks of long-term toxicity (197,198). More importantly, the detrimental effects of using allogenic T cells, are not seen with allogenic NK cells, which induce a much stronger tumour response than autologous NK cells (7,195,199,200). Therefore, it seems reasonable to consider NK cells as an option for cancer immunotherapy. Despite the promising results seen using NK cell therapy in patients with blood tumours such as leukaemia, patients with solid tumours do not have the same benefits

(196,201–203). Thus, more studies need to be done on this end. Furthermore, focussing future studies on improving NK cell's inherent ability to kill malignant cells, by understanding how to expand and activate them *ex vivo*, can overcome limits encountered by previous immunotherapy lines.

Several studies about *ex vivo* culture of NK cells use a feeder system, and despite eliciting an exponential growth of NK cells, they come with serious side effects. When the feeder-cell-cultured-NK cells are injected into the patients, it is possible to develop GvHD due to some feeder cells or their fragments being carried over. Additionally, ensuring that the feeder cells are "clinical grade" and safe to use with patients is a long and expensive process (10), limited to only restricted institutions. Therefore, using a combination of cytokines and/or specific growth factors may be a better option for *ex vivo* NK cell expansion.

1.3.1 NK cell immunotherapy for HCC

Dysfunctional NK cells are typical of hepatocellular carcinoma and largely contribute to disease progression. High levels of healthy NK cells expressing activating receptors, show better outcome and increased overall survival of patients with HCC (27). Thus, rescuing NK cell functions within the tumour, offers several immunotherapies approaches. Recently, it has been shown that NK cells from patients with HCC have metabolism and cell motility defects that could be restored by using TGF-β blockade, stopping the inhibitory effects of TGF-β on NK cells (204). *Easom et al*, suggested that adding IL-15 reverses the detrimental effect of HCC on liver resident NK cells indicating its role in immunotherapy (163). Additionally, *Zhuang et al* indicated that using a combination of cytokines, such as IL-12+15+18 to prime NK cells derived from HCC-patients, enables HCC-NK cells to have similar levels of activity against HCC cells as healthy-donor NK cells (65). These studies would suggest that it is possible to reverse the effect of HCC on NK cells which can then be used for the development of autologous therapies. Furthermore, NK cells are governed by a plethora of signals and receptors therefore targeting only one of these might not give the desired outcome. Several studies have been carried out, focussing on NK cells as immunotherapy, and encouraging results have also prompted many clinical trials evaluating the effect of NK cells as a monotherapy or in association with other treatments (205).

NK cell activity can be enhanced by blocking inhibitory receptors, which work as checkpoints that regulate NK cells ligand recognition and function. Checkpoint receptors including NKG2A, TIGIT, LAG-3, PD-1, TIM-3, CD96, normally highly expressed in NK cells during HCC, have been targeted in other tumours with successful outcomes and the results can also be applied in HCC (206,207).

Additionally, other potential therapies have also been explored to better understand their application in HCC. These involve engineered immune cells, with the scope to potentiate their target-recognition and killing of tumour cells.

1.3.2 Chimeric antigen receptor (CAR)-NK

The idea of CAR-engineered immune cells has been around for over three decades (208–210), initially developed for T cells and recently also applied to NK cells, with ongoing evolution and improvement of the CAR construct. Lately CAR-engineered NK cells have gained increasing interest over T cells, as mentioned above (section **1.3**). CARs are formed by single-chain variable fragment (scFv), linked to an intracellular signalling domain. The scFv is complementary to a target antigen on cancer cells, signalling the immune cell to attack the cancerous cells, upon binding (7). Co-stimulatory signalling domains have been added to newer generation of CAR-engineered immune cells to induce better responses. This has led to the formation of different generation of CARs (211).

Although there are no authorized CAR-NK treatments yet, several clinical and pre-clinical studies have been done, based on structures and knowledge used in CAR-T. In HCC, the antigen MUC1 was used as a target for a clinical trial (NCT02839954). In a preclinical study on HCC, the antigen glypican (GPC)-3 was used as a target, inducing CAR-GPC3-NK cells to kill GPC-3^{+} tumour cells more effectively, compared to unmodified NK cells. (212). The study also demonstrated that CAR-GPC3-NK were not effective against HCC cells that did not express the antigen GPC-3, indicating low toxicity profile of this potential treatment approach (212).

Because NKG2D is an important receptor for the activation and function of NK cells, some studies have focussed on creating NKG2D signalling-based chimeric receptors. *Chang et al*. constructed a chimeric receptor that contained NKG2D-DAP10 and intracellular signalling molecule CD3ζ (213). This greatly increased the cytotoxicity of transfected NK cell against tumour cells expressing NKG2D ligands (NKG2DL). However, there was little or no cytotoxicity against cell lines that had a low or absent expression of NKG2D ligands (213,214). Later studies, concentrated on modifying the signalling system of the chimeric receptor, to make the CAR-NK more responsive and cytotoxic (215,216). Other studies are being done to improve the signalling cascade of NKG2D-based CARs and the NK activation. NKG2D-based CAR-NK recognise NKG2DL expressed by immunosuppressive cells found in the tumour microenvironment (TME), such as fibroblasts. This triggers the killing of these cells and contribute to disease regression, which is especially important in more advanced disease (205,217). It is also possible to have on-target, off-tumour action due to NKG2DL expression on different tumours as well as other cell types. However, the low toxicity observed upon infusion and the advances in CAR signalling mechanisms, make NKG2D-based CAR-NK a potentially and effective immunotherapeutic approach for HCC.

Exciting innovations regarding genetically engineered cells, make CARs a new therapy line with many positive outcomes; this novel approach coupled with NK cell innate ability to kill could revolutionise treatment for HCC and dramatically improve patients' lives. However, this area still requires significant an effort in research.

1.3.3 Conclusion

HCC is a particularly heterogenous disease, presenting a complex pathophysiology and an increasing incidence especially in Western countries (147,148). HCC is remarkably difficult to treat with poor management of the disease. Treatments currently rely on resection, transplantation and local ablation for early-stage patients, intermediate stages patients are candidates for transarterial chemoembolization (TACE) and those with advanced stages will receive systemic therapies such as sorafenib, a multiple tyrosine kinase inhibitor (TKI) (147). These have improved over the years, but results are still modest, to this end more efforts

need to be made and NK-cell based immunotherapy should play a central role. Particularly, CAR-engineered NK cells would be a very interesting option to be analysed. As such, CIML CAR-NK cells' success in disease such as AML would be the perfect candidate and the exciting results obtained with CIML CAR-NK cells warrant further analysis. Unfortunately, this is a multifaceted analysis which does not come with many great challenges. As HCC is a solid tumour with a very harsh TME, development of a new HCC treatment should include analysis of the TME and how it influences treatment efficacy.

1.4 Hypothesis

My hypothesis is that cytokine activated NK cells can be engineered to express a chimeric antigen receptor (CAR). Cytokine priming and CAR engineering synergistically combine to augment NK cell antitumour activity which can be used as therapeutics in HCC.

1.5 Study aims

CAR-engineered NK cells are an attractive anti-tumour immunotherapy. To be effective, immunotherapies require a high number of immune cells to be administered to the patient. Additionally, cells need to be highly active and functional. Unfortunately, both represent big bottlenecks in the development of NK cell-based immunotherapies. This project aims to develop a protocol for in *vitro* culture of NK cells, to ensure high proliferation and activation for increased anti-tumour activity against liver cancer cell lines. This will be done as follows:

1. **Identify optimal cytokine cocktail for NK culture**

   Ex-vivo culture of NK cells will be done in a feeder-free NK cell culture system, using the cytokines IL-2, IL-12, IL-15, IL-18, IL-21 in different combinations to achieve high cell proliferation, high expression of activating receptors, and NK cells that are not terminally mature or exhausted.

2. **Identify the influence of cytokines on *ex vivo* expanded NK cells**

   Maturation and metabolism of cytokine expanded NK cells will be examined with the aim of establishing mechanisms for NK cell expansion and function. To this end, the role of transcription factors downstream of cytokine signalling, will be investigated.

3. **Generate CAR-NK against HCC and test it with liver cancer cell lines**

   Cytokine-activated NK cells will be used to generate CAR-NK cells targeting the antigen glypican-3 expressed by HCC. The aim is to determine whether cytokine-induced CAR-NK cells present enhanced antitumour functions, compared to NK cells not expressing CAR, and to establish their killing potential of liver tumour cells.

Chapter 2 Material and methods

2.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC) and Natural Killer cells purification

Peripheral blood mononuclear cells (PBMC) were isolated from buffy cone of healthy donors by density gradient centrifugation, using Ficoll-Hypaque (Fisher Scientific UK). Blood was diluted at a 1:10 ratio in PBS. 35ml of diluted blood was layered on top of 15ml Ficoll-Hypaque and centrifuged at 2000rpm for 30 minutes (brake/acceleration 0/0). The PBMC layer was harvested, and cells were washed three times with PBS and counted. PBMCs required for immediate NK cells isolation were handled accordingly. The remainder were resuspended, at a density of $30x10^6$/ml, in FBS supplemented with 10% DMSO and stored at -80˚C for 24 hours then moved to the liquid nitrogen until needed again.
CD56+CD3- NK cells were purified from PBMC, by negative selection using the Miltenyi NK isolation kit with LS columns (Miltenyi Biotec, Surrey, UK). PBMC cell pellet was resuspend in 40µL of sterile MACS buffer 10µL of NK cell biotin-antibody cocktail per 10^7 total cells, and incubated for 5 minutes at 4°C. Subsequently, 30 µL of MACS buffer 20 µL of NK cell micro bead cocktail per 10^7 total cells were added. Cells were mix well and incubate for 10 minutes at 4°C. During the incubation time, the LS column was placed on the magnetic MACS separator and rinsed with 3ml of MACS buffer. The PBMC cell suspension was applied on the column and the unlabelled enriched NK cells were collected in the flow-through. Isolated NK cells were counted and resuspended in NK MACS medium (Miltenyi Biotec, Surrey, UK) at a density of $1x10^6$ NK cells/ml, supplemented with specific cytokine combinations, and stored at 37°C and 5% CO_2 in an incubator.

2.2 Natural killer cells cytokine stimulation

After purification, NK cells were maintained in culture in NK MACS medium (130-114-429, Miltenyi, UK), supplemented with 1% NK supplements (Miltenyi, UK), 1% Penicillin-Streptomycin (0378016, Life technologies, UK); 5% human AB serum (H6914, Sigma-Aldrich,

UK). NK cells were expanded with cytokines IL-2, IL-12, IL-15, IL-18, IL-21 (R&D System, UK), for 16 days. Different concentrations of cytokines were used and outlined as necessary in chapter 3 and chapter 4. Fresh media and cytokines were added every two-three days, unless specified; and the cells were counted using TrypanBlue (Sigma-Aldrich, UK) every two-three days to monitor their expansion rate.

2.3 Hepatocellular carcinoma cell lines

Hepatocellular carcinoma (HCC) cell lines, HepG2, Huh7, PLC were cultured in complete DMEM, 10% fetal bovine albumin (F9665, Sigma-Aldrich, UK), and 5% PenStrep (10378016, Life technologies, UK); the liver cancer cell line SNU475 was maintained culture in complete RPMI, supplied with 10% fetal bovine albumin and 5% PenStrep. Cells were kept in a humidified incubator at 37°C with 5% CO_2 and routinely tested for mycoplasma.

2.4 NK cell surface staining

NK Cells were analysed for receptor expression on day 0 (straight after isolation), day 10 and day 16 of *in vitro* culture. NK cells were added to V-bottom 96 well plate at $1x10^6$ cells per well and washed with 100µl FACS wash for 5 minutes (PBS with 1% bovine serum albumin (BSA) (Sigma) and 0.1 % sodium azide (Sigma)). Cells were then resuspended in in 100 µl blocking buffer (10% human serum (HS) (Sigma) in FACS washing buffer) for 30 minutes at 4°C. Cells were washed in FACS wash again and incubated with antibody mixed in 50µl FACS wash, for 30 mins at 4°C. NK cells were analysed for their phenotype and maturation, using antibodies panels shown in **Table 3.** Cells were fixed using 1% paraformaldehyde (PFA (Sigma)) and analysed using a three laser FACS Aria flow cytometer (BD Bioscience, Oxford, UK). Data were analysed using FlowJo v.10.9.0. Compensation beads were used to set compensation and correct for fluorescence spill-over from the different antibodies, ensuring comparable results.

Antibody	Fluorochrome	Company	Clone	Conc.
NK cell identification				
CD56	PE/Cy7	Biolegend	HCD56	1:25
CD3	BV510	Biolegend	UCHT1	1:25
NK cell phenotype				
NKp30	PerCp/Cy5.5	Biolegend	P30-15	1:10
NKG2A	APC	Miltenyi	REA110	1:100
NKG2C	PE	Miltenyi	REA205	1:25
NKG2D	FITC	Biolegend	1D11	1:25
CD96	BV421	Biolegend	NK92.39	1:10
NKp44	PerCp/Cy5.5	Biolegend	P44-8	1:25
NKp46	APC	Biolegend	9E 2	1:10
CD16	BV421	Biolegend	3GB	1:25
NK cell maturation				
CD57	PE-CF594	BD Bioscience	NK-1	1:1000
CD117	PE	Biolegend	104D2	1:20
CD94	FITC	Biolegend	DX22	1:50
CD122	PerCp/Cy5.5	Biolegend	TU27	1:25

Table 3. Antibody used for NK cells surface staining.

2.5 Functional analysis of NK cells

2.5.1 Degranulation (LAMP-1) assay

CD107a degranulation assay was performed to assess the cytotoxicity of the NK cells under the influence of the different cytokine combination, on day10 of culture. NK cells were cultured with liver cell lines (HepG2, PLC, Huh7, SNU475) in complete RPMI medium, with anti-human CD107a-eFluor660 (clone H4A3, Invitrogen, UK) at an effector:target (E:T) ratio of 1:1 for a total of 4 hrs at 37 ˚C. After 1hr of incubation, GolgiStop Protein Transport Inhibitor (BD Bioscence, UK) was added, and cells were incubated for a further three hours. Cells were then stained with antibodies CD56 and CD3 and analysed using a three laser FACS Aria flow cytometer (BD Bioscience, Oxford, UK). Antibody panel for NK cell staining is shown in **Table 4**. Data were analysed using FlowJo v.10.9.0.

Antibody	Fluorochrome	Company	Clone	Conc.
NK cell identification and surface staining				
CD56	PE/Cy7	Biolegend	HCD56	1:25
CD3	BV510	Biolegend	UCHT1	1:25
CD107a	eFluor660	Bioscience	H4A3	1:175

Table 4. Antibody panel used for NK cells staining during CD107a assay.

2.5.2 NK intracellular staining for interferon-gamma (IFN-γ) and tumour necrosis-alpha (TNF-α)

NK cells were co-cultured with HCC cell lines, at an E:T ratio of 1:1, to examine IFN-γ and TNF-α expression. NK cells were plated in U-bottom 96 well plate with target cells or cytokines, at $1x10^6$ per well and incubated at 37 °C for 1 hour. GolgiStop Protein Transport Inhibitor (BD Bioscence, UK) (4µl/6ml culture medium) and GolgiPlug Protein Transport Inhibitor (BD Bioscence, UK) (1ul/1ml cell culture) were then added, and cells were incubated for a further three hours. Unstimulated control was included. NK cells were then transferred to V-bottom 96 well plate and stained for surface markers (CD56 and CD3), as outlined in section 2.5.1. For

intracellular staining, cells were fixed by incubation with 100µl Fixation/Permeabilisation solution (BD Cytofix/Cytoperm Plus Fixation / Permeabilisation Kit, BD Biosciences) per well for 20 minutes at 4°C. This was followed by a permeabilization step, where the cells were washed with 200µl 1x BD Perm/Wash Buffer (BD Cytofix/Cytoperm Plus Fixation/Permeabilisation Kit, BD Biosciences). Intracellular staining was done by incubation with antibody mixed in 50µl 1x BD Perm/Wash Buffer, for 30 mins at 4°C. Antibody panel for surface and intracellular staining is shown in **Table 5**. Cells were fixed using 1% paraformaldehyde (PFA (Sigma)) and analysed using a three laser FACS Aria flow cytometer (BD Bioscience, Oxford, UK). Data were analysed using FlowJo v.10.9.0. Compensation beads were used to set compensation and correct for fluorescence spill-over from the different antibodies, ensuring comparable results.

Antibody	Fluorochrome	Company	Clone	Conc.
NK cell identification and surface staining				
CD56	PE/Cy7	Biolegend	HCD56	1:25
CD3	BV510	Biolegend	UCHT1	1:25
NK cell intracellular staining				
IFN-γ	PE	Biolegend	4S.B3	1:25
TNF-α	FITC	Biolegend	Mab11	1:25

Table 5. Antibody used for intracellular staining.

2.5.3 Antibody-dependent cellular cytotoxicity (ADCC)

Hepatocellular carcinoma cell lines, HepG2 and PLC, were incubated with the anti-EGFR antibody cetuximab or with isotype control (10µg/ml) for 20 minutes at 37˚C, prior to co-culture with NK cells. After incubation, HCC cell lines were washes and cytokine-activated NK cells were added, and assay was continued as functional assay described in sections **2.5.1** and **2.5.2**. ADCC was measured as enhanced CD107a, IFN-γ and TNF-α expression in the presence of cetuximab.

2.6 NK cell proliferation assay and maturation phenotype

After purification, unstimulated NK cells were stained with CellTrace CFSE (ThermoFisher Scientific, UK), according to manufacturer's instructions. Briefly, NK cells were resuspended in PBS at 1x10^6/ml and CellTrace CFSE dye was added at 1ul/ml. Cells were incubated for 20 mins at room temperature with gentle agitation. The dye was quenched by adding complete culture medium, containg 10% serum, and incubating the cells for 5 mins. Cells were then washed and resuspended in complete NK MACS medium and cultured as described in *NK cell stimulation* section. At day5, 7 and 9 of *in-vitro* culture, NK cells were stained for surface markers as described in the section ***2.3 Hepatocellular carcinoma cell lines***

Hepatocellular carcinoma (HCC) cell lines, HepG2, Huh7, PLC were cultured in complete DMEM, 10% fetal bovine albumin (F9665, Sigma-Aldrich, UK), and 5% PenStrep (10378016, Life technologies, UK); the liver cancer cell line SNU475 was maintained culture in complete RPMI, supplied with 10% fetal bovine albumin and 5% PenStrep. Cells were kept in a humidified incubator at 37°C with 5% CO2 and routinely tested for mycoplasma.

NK cell surface staining and assessed using a three laser FACS Aria flow cytometer (BD Bioscience, Oxford, UK). Data were analysed using FlowJo v.10.9.0.

2.7 Immunoblotting

After purification, NK cells were treated with cytokines from condition2 (IL-12+15+18 for16 hours, then IL-2 only till the end of culture) or condition3 (IL-12+15+18+21 for16 hours, then IL-2 only till the end of culture), for 1hour, 2hours, 5hours, 16hours or 24hours. At the end of each culture period a proportion of NK cells were lysed in RIPA Buffer (CellSignalling Technology), supplemented with PMSF (Sigma, 174 µg/ml), Protease inhibitor (Sigma, 1:100 final dilution, and Phosphatase inhibitor cocktail (CellSignalling Technology, 1:100 final dilution). A 10% polyacrylamide gel (ThermoFisher Scientific) was used to separate proteins, which were then transferred to nitrocellulose membranes and blcked with 5% BSA (Sigma). Proteins were probed with antibody against phosphorylated STAT3 (Tyr705, D3A7, Rabbit mAb, CellSignalling Technology) or β-actin (8H10D10), CellSignalling Technology). HRP-linked

secondary antibody and chemiluminescence reagents (ThermoFisher) were used to detect protein bands, which were visualised using the ChemiDoc-It Imaging system (UVP). All antibodies were used at the concentration recommended by the manufacturer.

2.8 Cytokine release assays

HCC cell lines and CAR-NK cells were co-cultured at a 1:1 E:T ratio and incubated in a humidified incubator at 37°C and 5% CO_2 for 24 hours. Supernatants of the co-culture were frozen at -80 ˚C and then used to measure the levels of the cytokine IFN-γ secreted. The levels of IFN-γ secreted by the CAR-NK were measured using the Biolegend ELISA Human IFN-γ kit (ELISA MAX Deluxe Set Human IFN-γ, Biolegend), following manufacturer instructions.

2.9 Lentiviral transduction of HCC cell lines to express GFP

HepG2 and PLC cell lines were added to a 6well plate at $2x10^6$/ml in serum-free DMEM medium. GFP positive lentivirus particles were diluted in 1ml serum-free DMEM and then added on top of each cell line. Cells were centrifuged for 2hrs at 400g and 32˚C and then kept in a humidified incubator at 37°C and 5% CO_2. After 24 hours incubation, complete DMEM media with 10% fetal bovine albumin was added on top and cell were placed in a humidified incubator at 37°C and 5%CO_2. After this, cells are cultured as described above in section 2.3. GFP expression was analysed on day7 of culture, using a MACSQuant Analyzer 10 Flow Cytometer. Cells were then sorted using the MACSQuant Tyto Cell Sorter, to obtain a cell population which is ≥95% GFP positive.

2.10 Chimeric Antigen Receptor (CAR)-NK development

2.10.1 Glypican-3(GPC3)-CAR construction

A second generation GPC3-CAR was constructed, as described earlier (218). Three constructs were made, incorporating three different scFv sequences: GC33 scFv, YP7 scFv and 9F2 scFv. These were combined in frame with CD8 hinge and transmembrane (TM) domain, 4-1BB/CD137 co-stimulatory domain, and CD3ζ activation domain, both intracellular (IC)

domains. CAR constructs were then inserted into a lentiviral plasmid backbone, under control of a human EF-1α promoter. All materials for this were provided by Miltenyi Biotec (Miltenyi Biotec B.V. &Co. KG, Bergisch Gladbach, Germany).

2.10.2 Lentiviral vector production

The plasmids encoding each CAR construct, packaged into baboon envelope (BaEV) pseudotyped lentiviral vectors (LV), were generated by transient transfection of HEK 293T cells, as shown before (219,220). All materials for this, was supplied by Miltenyi Biotec (Miltenyi Biotec B.V. &Co. KG, Bergisch Gladbach, Germany).

2.10.3 Lentiviral transduction of NK cells

Purified NK cells were seeded in a flat bottomed 48-well plate (0.5 x 10^6 cells in 500µl), either with condition2 (IL-12+15+18 for16 hours, then IL-2 only till the end of culture) or condition3 (IL-12+15+18+21 for16 hours, then IL-2 only till the end of culture). Two days after isolation, NK cells were transduced with lentiviral particles for the expression of CAR. Lentiviral particles and Vectofusin-1 (Miltenyi Biotec, 2.5µg/ml final concentration per well) were mixed at 1:1 volume and incubated at room temperature for 7min. While the LV-Vectofusin mixture is incubating, equal volume as the volume of the LV-Vectofusin mix was carefully removed from the wells containing the cells, to keep the final cell concentration the same (0.5 x 10^6 cells in 500µl). The lentivirus-Vectofusin-1 mixture was then added on top of the NK cells, in a serum-free NK-MACS medium, supplemented with 1% NK-MACS supplements. Cells were centrifuged for 2hrs at 400g and 32˚C and then kept in a humidified incubator at 37°C and 5%CO_2. After 24 hours, 50% of the medium was replaced with fresh NK-MACS medium, containing 5% human plasma and fresh cytokines. Workflow of the transduction procedure is added below, in **Figure 4.**

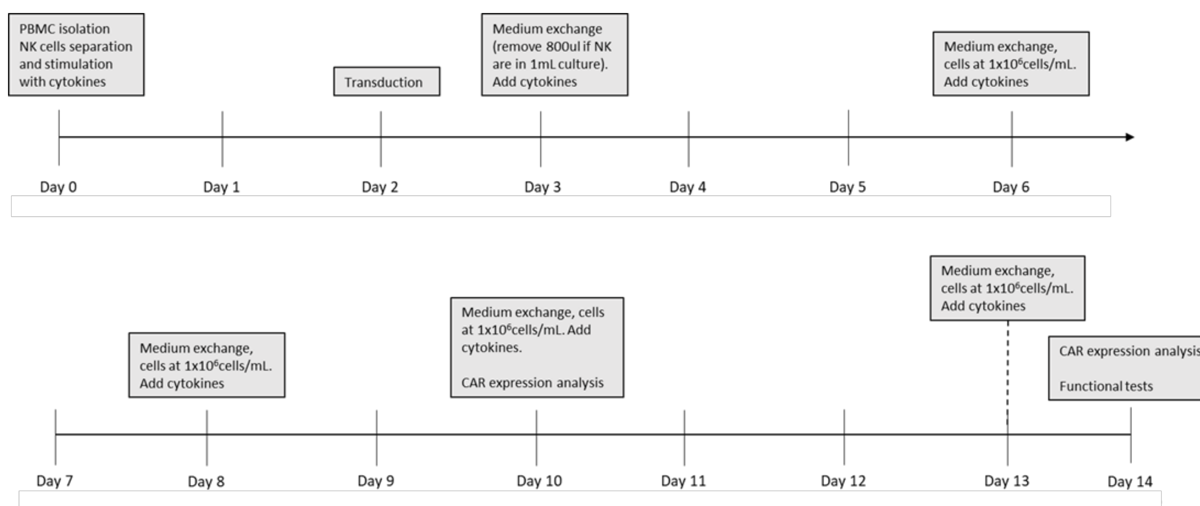


Figure 4. Workflow for CAR-NK Cell transduction

2.10.4 Glypican-3 expression analysis

Glypican-3 expression on HCC cell lines as well as CAR-NK was analysed by flow cytometry, on the MACSQuant Analyzer 10 Flow Cytometer. Anti-GPC3-APC (Acro Biosystems, GP3-HA2H9) was used to detect GPC3.

2.10.5 Functional analysis of CAR-NK

2.10.5.1 CAR-NK cells specific lysis assay

HCC cell lines HepG2 and PLC were stained with CellTrace Violet Cell Proliferation Kit (Invitrogen), according to manufacturer instructions, and co-cultured with CAR-NK cells at an E:T ratio of 2:1 or 1:1, for 4hrs at 37˚C. At the end of the co-culture, cells were stained with propidium iodide (Invitrogen), and NK cell specific lysis of Violet-stained HCC target cells was assessed by flow cytometry, using the MACSQuant Analyzer 10 Flow Cytometer. NK cell lysis was defined as follows:

$$NK\ specific\ lysis = \frac{(\%HCC\ lysis\ in\ coculture) - (\%spontaneous\ HCC\ lysis)}{100 - \%spontaneous\ HCC\ lysis} * 100$$

2.10.5.2 Over time analysis of CAR-NK cytotoxicity

Analysis of CAR-NK cells cytotoxicity over time, was done using real-time monitoring via the IncuCyte S3 system (Sartorius, Goettingen, Germany). The assay was set up similarly as explained in section 2.10.5.1. However, this time GFP+ transgenic HCC cell lines (2.9) were used and co-cultured with CAR-NK cells at E:T ratio of 1:1 or 2:1. Phase contrast and green fluorescence images were captured with 10x magnification every 2 hours for 48 hours.

2.10.6 NK cell metabolism analysis via NMR

This section is taken from the thesis of Genevieve Rogers (Ref: PhD thesis under preparation, Genevieve Rogers, University of Southampton, 2023) as this was a collaborative work.

The methodology used for this is taken from Rogers et al (221).

2.10.6.1 Natural killer cell culture and experimental set up

NK cells were purified from healthy donor PBMC, as described in section **2.1**. NK cells were expanded with cytokines IL-2, IL-12, IL-15, IL-18, IL-21 (R&D System, UK) for 16 days, as described in section **4.7**.

An initial volume of 1 mL of NK cell suspension was cultured per condition, with the appropriate cytokine(s), at a starting cell density of 1×10^6 cells/mL in well plates. **Figure 5** sets out the structure of the NK cell culture: the experiment follows a set of repeating cycles, *k*, of defined lengths. This includes an incubation period, culture volume and cell number measurements, and a cell passage. After the first incubation at 37°C and 5% CO_2 (in cycle k_1) of 16 hours, the following cycles include an incubation of 72 hours. At the beginning of each step, cells are at 1×10^6 cells/mL, in a known volume, V_{k-1}, containing a known quantity of metabolites, M. After the incubation, the volume of the solution is measured to quantify the evaporation of water from the sample during this time. NK cells are re-suspended and 10 ul of cell suspension is removed for cell counting on an automated cell counter (BioRad, UK). Well plates are centrifuged at 300 G for 3 minutes to allow for supernatant sampling, of volume β_k. These samples were frozen for later NMR analysis to determine the change in concentration, C_k, of a panel of 28 metabolites, from which the change in the amount of each metabolite, ΔM, can be calculated. A volume of new media, γk, is added to each well to

replace the supernatant sampled and to account for cell expansion (cells always kept at 1 × 10^6 cells/mL), and cell suspensions are moved to a larger well plate or flask when necessary. The next cycle then begins. This process is repeated over 16 days, with supernatant samples taken and media refreshed every 3 days. Using this process, the metabolic rates per cell per hour can be determined and compared between different cytokine conditions across different donors.

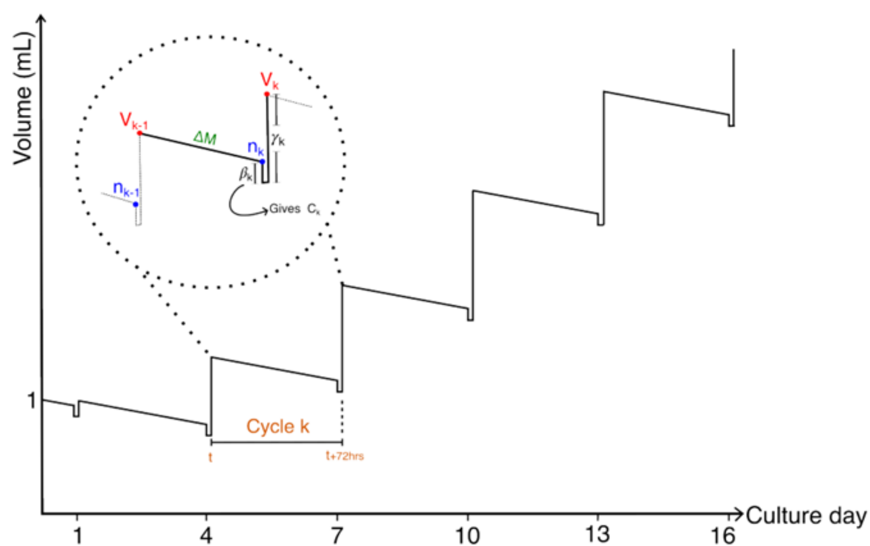


Figure 5. Experimental set-up for analysis of NK cells metabolism during *in-vitro* culture

2.10.6.2 NMR analysis of NK culture supernatants

Samples were diluted 2:1 with a combination of standards including DSS, DMSO and D2O in phosphate buffer (pH 7.4) (Sigma Aldrich, UK). This dilution resulted in a final concentration of 1mM DSS, 1mM DMSO, and 15% v/v D2O. Inclusion of DSS and DMSO functions as internal standards for chemical shift alignment and concentration quantification, respectively, while D2O functions as a lock signal.

A Bruker SampleJet sample changer was used to automatically load the samples into the NMR spectrometer. A Bruker 16.4T NMR spectrometer, corresponding to a proton resonance frequency of 700MHz, with an Avance Neo console and TCI Prodigy CryoProbe™was used to obtain ^{1}H spectra.

NMR spectra were acquired using the PROJECT pulse sequence (222) with 32 echoes and a total echo delay of 64 ms. The 90° pulse was calibrated to 8.5 µs at a power of 14.7 W, amounting to a nutation frequency of 11.9 kHz. Water signals were suppressed using continuous wave (CW) presaturation, applied throughout the 5 s recycle delay time at 3.76 × 10−5 W power. Before acquisition two dummy scans were taken. Subsequently, 65.5k data points were acquired over a spectral width of 20 ppm. Each spectrum was averaged over 256 scans.

NMR data were processed using home-built routines written in Julia 1.6 {bezanson_julia_2017} with NMR package written by Marcel Utz \{utz_nmr_2022}" (223) (Marcel Utz. NMR Package for Julia Programming Language, October 2022. URL https://github.com/marcel-utz/NMR.jl. original-date: 2017-12-04T22:14:48Z). Free induction decays were zero-filled to 32k points, and apodized with 1 Hz of Lorentzian line broadening.

2.11 Statistics

Data were analysed with GraphPad Prism 8, using One-Way ANOVA test, Two-way ANOVA test or paired t-test analysis where appropriate and specified in the figure legend. Significance values are indicated as *P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001.

2.12 Ethics

Ethical approval to collect sample from healthy individual was granted by West Midlands–Black Country Research Ethics Committee (Rec No 19/WM/0262).

Chapter 3 Effect of cytokines on proliferation, phenotype, and function of NK cell

3.1 Introduction

Natural killer cells (NK) are a promising source to use in adoptive cell immunotherapy, as they have an efficient endogenous capacity to recognize and kill tumour cells. However, they represent only a small fraction of human lymphocytes, which ranges from 5% to 15% of circulating lymphocytes, with a typical half-life of two weeks (5,6). NK cells are subdivided into different subpopulation with very distinct phenotypes and effector functions. To this end, increasing the cytotoxic potential and the number of NK cells *ex vivo* is a crucial step for the development of immunotherapy prior to clinical use.

Various approaches for *ex vivo* modulation of NK cells have been investigated, using cytokines (10,68,224,225). Specifically, the cytokines interleukin (IL)-2, IL-12, IL-15, IL-18, IL-21 have been evaluated for the impact on NK cell expansion and effector functions, either alone or in combination (32,35,84,226,227), providing promising results. However, further research is needed better understand the impact of cytokine modulation on NK cells and how these can be fully exploited for better immunotherapy developments. Selection of cytokine combination is an important factor as cytokines can upregulate or downregulate receptors responsible for NK cell maturation, activation, inhibition, and proliferation, and hence increasing their sensitivity against tumour cells that express the corresponding ligands. For example, IL-21 has been shown to trigger apoptosis, resulting in shorter lifespan on NK cells *in vitro*, therefore, alongside selection of cytokine combination, exposure time to specific cytokines is also critical for the enhancement of NK cells features (81,228–230).

Another way to expand and modulate NK cells *ex vivo* is the use of irradiated accessory cells such as antigen presenting cells, known as feeder cells. These methods lead to robust yields of NK cells, up to 10,000-fold expansion within five weeks of *in vitro* culture (84). Although

feeder cells have been used to support clinical trial studies, the use of these cells for future evaluations is limited to one institution. Furthermore, feeder cells need to be irradiated before use and modified for the expression of accessory such as membrane-bound IL-21 (77,84) or IL-15 (231), which increases the workload and timeline for the development of NK immunotherapy. Additionally, despite being irradiated and therefore inactivated, feeder cells can still cause high risks in a clinical setting (232).

The lack of adequate NK cell numbers for adoptive immunotherapy is still a great hindrance to the development of NK-cell based therapies. Additionally, as the protocols developed so far are not optimal, there is a need to improve current procedures and gain better understanding of NK cells manipulation *ex vivo*.

By using a feeder-free system, this chapter aims to identify the effect of different cytokine combinations on NK cell proliferation, phenotype, and functions. This is achieved using NK cells derived from the peripheral blood of healthy donors, purified and cultured *ex vivo,* using combinations of the cytokines IL-2, IL-12, IL-15, IL-18, and IL-21. Given its anti-senescence (77,84), yet controversial role in NK cell expansion (229,230), IL-21 is central to the current analysis to better understand the role of this cytokine on NK cell functions. I hypothesised that addition of IL-21 in *ex vivo* cultures, would result in enhanced NK cell proliferation and therefore I asked the following questions:

1. Does IL-21 increase NK cell expansion and is this affected by exposure time to the cytokine?

2. Does IL-21 increase NK cell effector functions, or is there a trade-off for proliferation?

3. Does IL-21 augments the functions of memory-like NK cells, generated with the combination of IL12+15+18 for 16 hours?

Therefore, I aimed to investigate a novel cytokine combination for enhanced *ex vivo* NK cell proliferation and activation.

3.2 Cytokine-activated NK cells show increased expansion

One of the most important features of developing an immunotherapy using primary immune cells is to generate a high yield of cells to use for adoptive transfer. Thus, optimising protocols for *in vitro* NK cell culture is key for this. To this end, NK cells were isolated from healthy volunteers and expanded *in vitro*, to address the effect of different cytokines (**Figure 6**). Several studies have shown how cytokines activate NK cell *ex vivo* and cause their expansion (233–236). A lot of focus has been put on the group of common γ-chain receptor cytokines, which comprise interleukin (IL)-2, IL-4, IL-9, IL-15, and IL-21. However, the effects of IL-21 on NK cell proliferation have not been fully elucidated. Therefore, IL-21 role is central in this analysis, and it is used in combination with other cytokines, to evaluate which condition gives optimal results.

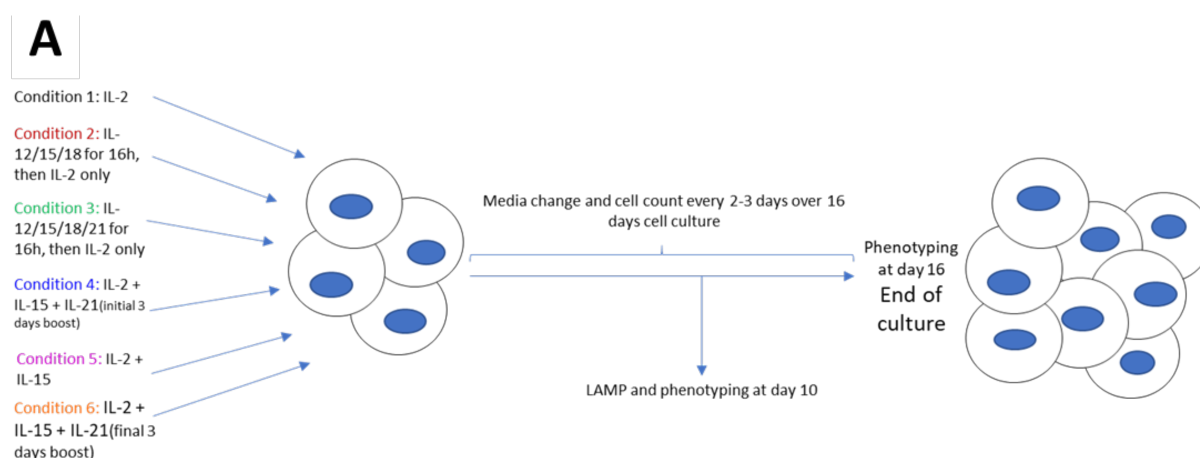


Figure 6. Schematic representation of NK culture conditions.

Representation of NK cell culture with the cytokine concentrations and experimental workflow. The concentration of cytokines used from here forward are the same as described here. After culture setup, media and cytokines were refreshed every 2/3 days, when cell count was also carried out. At day 10, NK cells were analysed for phenotype and function; phenotypic evaluation was then repeated at the end of the culture at day16.

The following cytokine combinations and concentrations were used in preliminary experiments:

- **Condition 1**: 500 U/ml IL-2. NK cells require a minimum supply of cytokines to survive. IL-2 and IL-15 have a similar effect on NK cells (237,238) and for this analysis IL-2 was chosen.
- **Condition 2**: IL-12 (10ng/ml) + IL-15 (20 ng/ml) + IL-18 (50 ng/ml) for 16h. Medium was then changed, and cells were cultured with only 500 U/ml IL-2 for the rest of the culture. This combination of cytokines is known to generate memory-like NK cells (35,65).
- **Condition 3**: IL-12 (10ng/ml) + IL-15 (20 ng/ml) + IL-18 (50 ng/ml) + IL-21 (25 ng/ml) for 16h. Medium was then changed, and cells were cultured with IL-2 (500 U/ml) only for the rest of the culture. In this combination, IL-21 is added together with the memory-like inducing cytokine combination, to evaluate IL-21 effects.
- **Condition 4**: IL-15 (20 ng/ml) + $IL\text{-}21_{\textit{initial 3 days}}$ (25 ng/ml). Media was then changed on day 3, and culture was continued with IL-15 only. This combination is used to understand the effect of IL-21 over a longer period, when used together with IL-15. This protocol replicates the work carried out by *Wagner et al* (63).
- **Condition 5**: IL-2 (500U/ml) + IL-15 (20 ng/ml). Discussions with our industrial partner Miltenyi, suggested the addition of this protocol, which is regularly used in their laboratories, allowing a comparison with their data.
- **Condition 6**: IL-2 (500 U/ml) + IL-15 (20 ng/ml) + $IL\text{-}21_{\textit{final 3 days}}$ (25 ng/ml). IL-21 was added only three days prior to degranulation assay; after that, media was changed, and culture was continued with IL-2+IL-15 only. This condition was added to evaluate the impact of adding IL-21 at a later time point of culture.

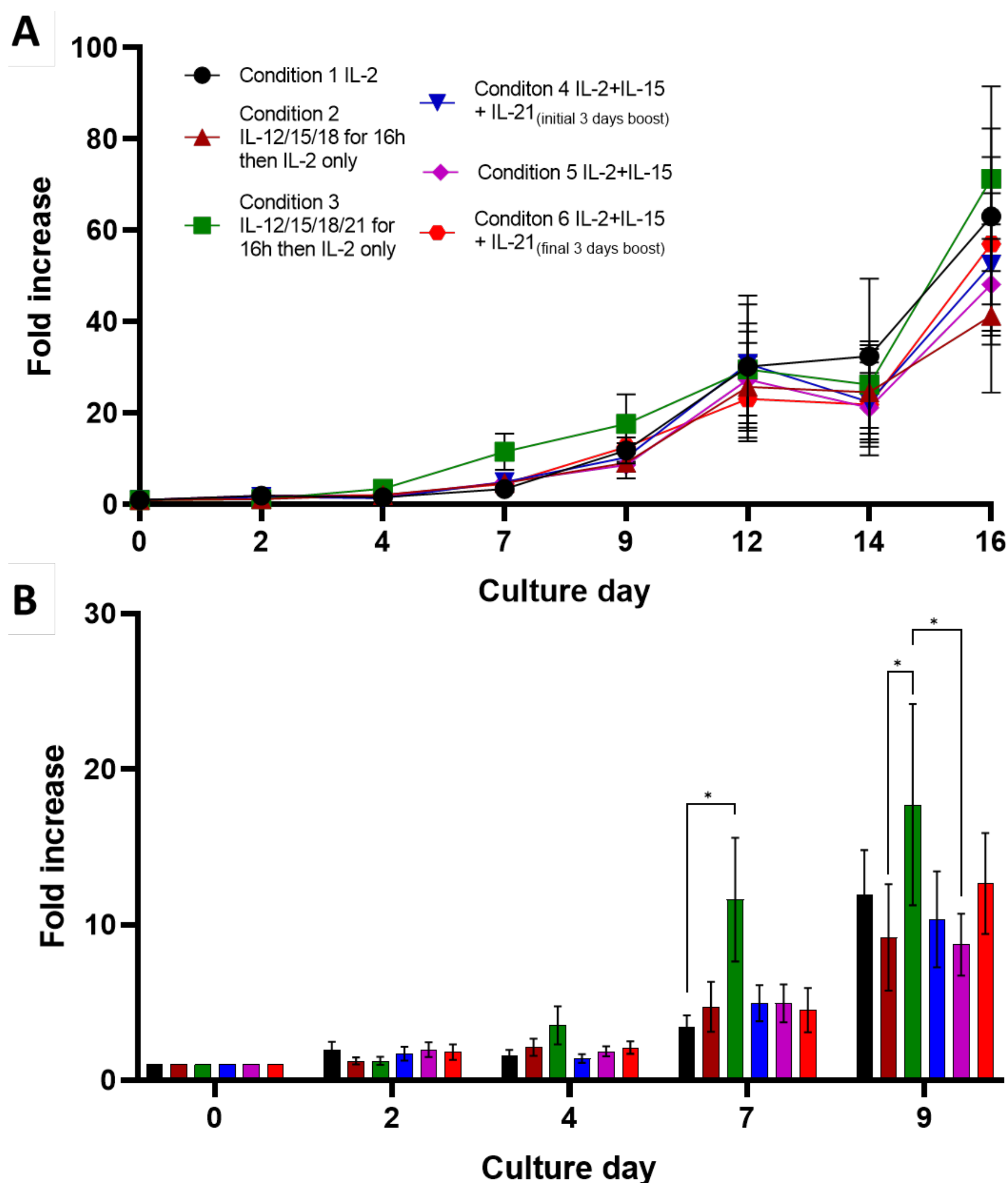


Figure 7. Expansion of NK cells treated with different cytokines combinations.

Peripheral blood derived NK cells are expended *in vitro* in the presence of different cytokines combinations. Each condition represents the average of 8 different donors (± SEM). **A**. Average of NK proliferation indicating the different culture conditions used. **B**. Average of NK proliferation per condition in the first 9 days of *in vitro* culture. Data shown as mean ± SEM (n=8), analysed by Two-Way ANOVA, and Tukey's correction multiple comparison test. (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001).

The expansion of NK cells was recorded over 16 days, with different cytokine combinations showing different effects on the cells. Condition 1 (IL-2 only) elicited expansion which was more prominent in the later phase of the culture, after 12 days of culture, reaching a 60-fold expansion at day 16 compared to day 0, and sustained an increased cell expansion up to the end of the culture on day 16 (**Figure 7A**). Condition 2 replicated the cytokines cocktail needed to generate memory-like NK cells (IL-12+IL-15+IL-18) (35), causing a similar expansion to condition 1 up to day 12 with a 20-fold expansion. After this time point, the memory-like cytokine combination causes a slow proliferation, reaching a 40-fold expansion by day 16 at the of the culture. Addition of IL-21 to the memory-like cytokine cocktail (condition 3, **Figure 7**) showed a surprising increase in NK cell expansion, resulting in the highest NK cell expansion overall (**Figure 7A**). Increased cell expansion was more prominent during the initial stages of cell culture; at day 7 cell expansion was higher than IL-2 only (condition 1), reaching a ~12-fold expansion, and at day 9 cell expansion was significantly higher than IL-2 only and the memory-like cytokine combination (condition 2), reaching a ~19-fold expansion (**Figure 7B**). IL-21 (condition 3) elicited a 70-fold expansion at day 16 compared to day 0, which was higher than all the other culture condition analysed (**Figure 7A**).

Adding IL-21 at different time points of the cell culture, either at the start (condition 4) or just before carrying out functional assays (condition 6), did not make a difference in cell expansion. Condition 4, 5 and 6, induced a similar NK cell growth, which was similar to condition 1 but lower than condition 3 (**Figure 7A**).

A paired analysis between condition 2 and 3 (**Figure 8**) shows that the addition of IL-21 to the cytokine mix increases cell expansion. However, adding IL-21 at different time points of the cell culture, either during the initial 3 days (condition 4) or just before carrying out functional assays so day 7 to day 10 (condition 6), did not make a difference in cell expansion (**Figure 7**). Thus, cytokines modulate NK cell growth, and the effect of the memory-like cytokine cocktail on NK cell proliferation, can be augmented by the addition of IL-21 (condition 3).

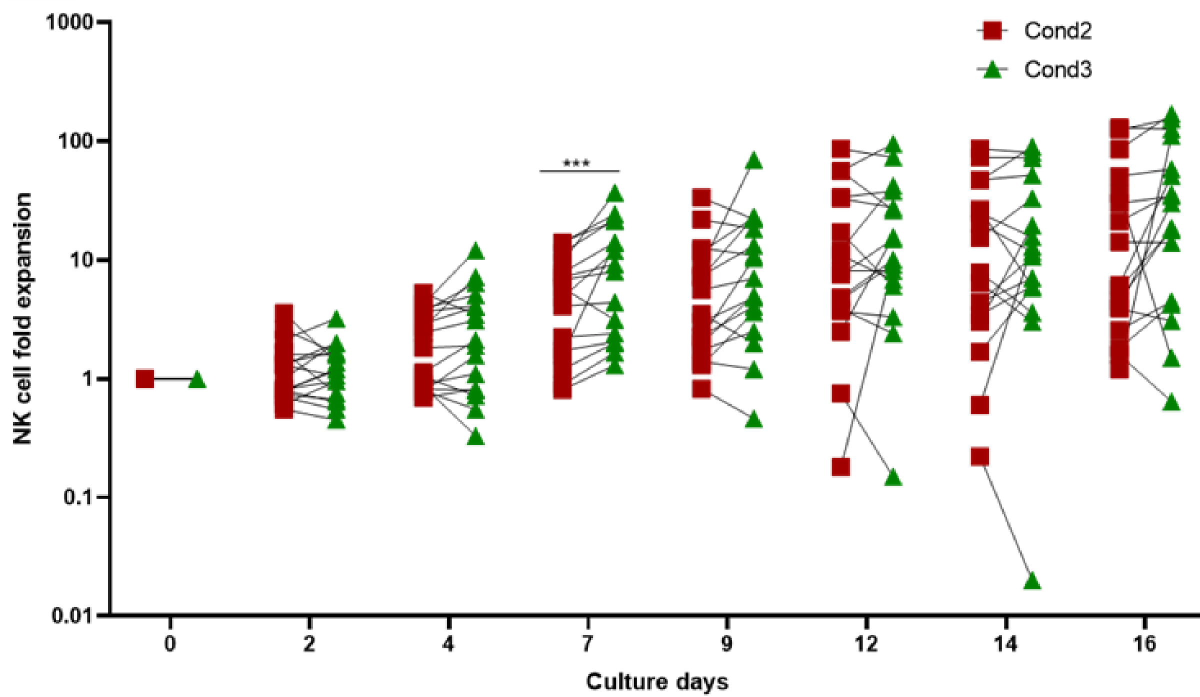


Figure 8. Addition of IL-21 to the memory-like-inducing cytokine cocktail shows increased expansion

Comparison between condition 2 (IL12+15+18 for 16hrs, then IL-2 only) and condition 3 (IL12+15+18+21 for 16hrs, then IL-2 only) on the effect on NK cell proliferation. Data presented as matched independent donors, analysed by multiple paired t-test, false discovery rate approach and with Two-stage step-up (Benjamini, Krieger, and Yekutieli) correction for multiple comparison test. (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001).

3.2.1 IL-2 sustains an increased NK cell expansion

To be able to compare our results to previously published data on memory-like NK cells (35), freshly isolated NK cells were cultured with either IL-2 or IL-15 (1 ng/ml) for maintenance after priming for 16 hours. There was a trend towards IL-2 helping to maintain a much higher NK cell expansion over 20 days. Additionally, when used alone, IL-2 caused a greater NK cell expansion (**Figure 7**) than IL-15 alone (**Figure 9**). IL-15 alone induced a lower than 10-fold expansion which was maintained for 20 days of *in vitro* culture (**Figure 9**). IL-2 only induced a much higher cell expansion, which was already higher than 10-fold expansion at day 7, reaching a 60-fold expansion at day 16 (**Figure 7**).

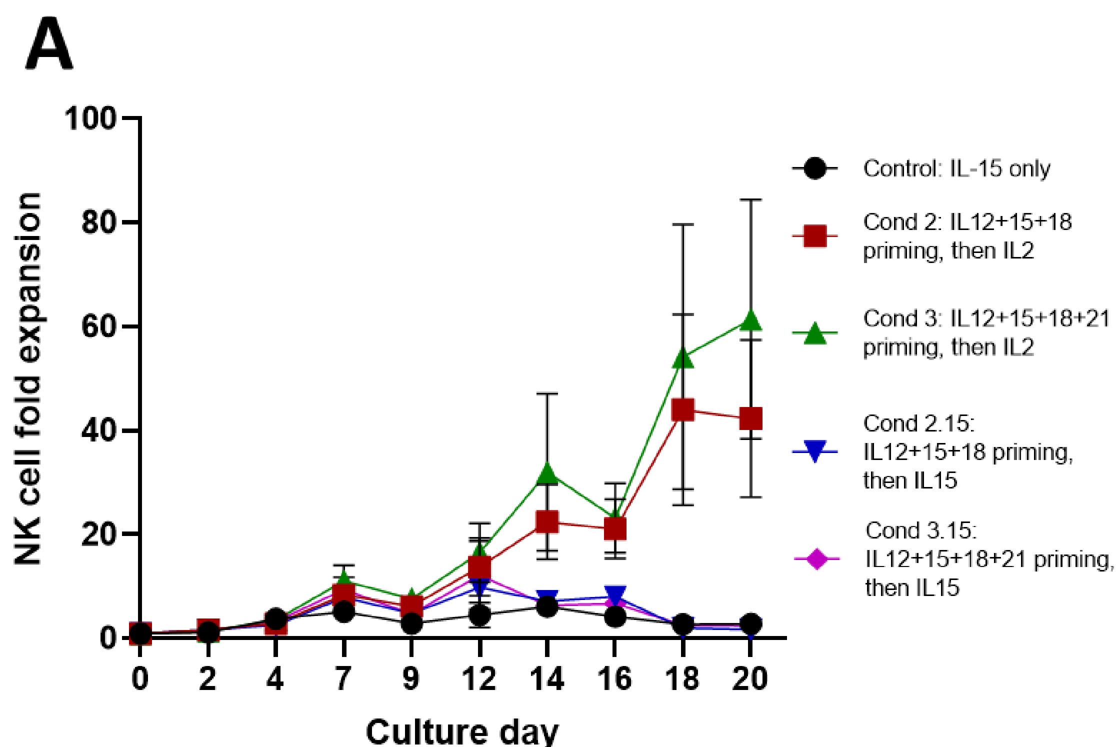


Figure 9. Comparison of IL-2 and IL-15 for maintenance after NK cell activation.

Expansion of NK cells using either IL-2 or IL-15 for cell maintenance after 16h priming. Each condition represents the average of 5 different donors (± SEM). Data analysed by Two-Way ANOVA, and Tukey's correction multiple comparison test. (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001).

3.3 Phenotype of cytokines activated NK cells

As cytokines can modulate cell receptor expression, the phenotype of the cytokine-activated NK cells was analysed by flow cytometry (**Figure 11**). Representative flow cytometry plots are shown in **Figure 10**. The expression of activating receptors NKG2D, NKG2C, CD16, NKp30, NKp40 and NK46, and of the inhibitory receptors NKG2A and CD96 was analysed.

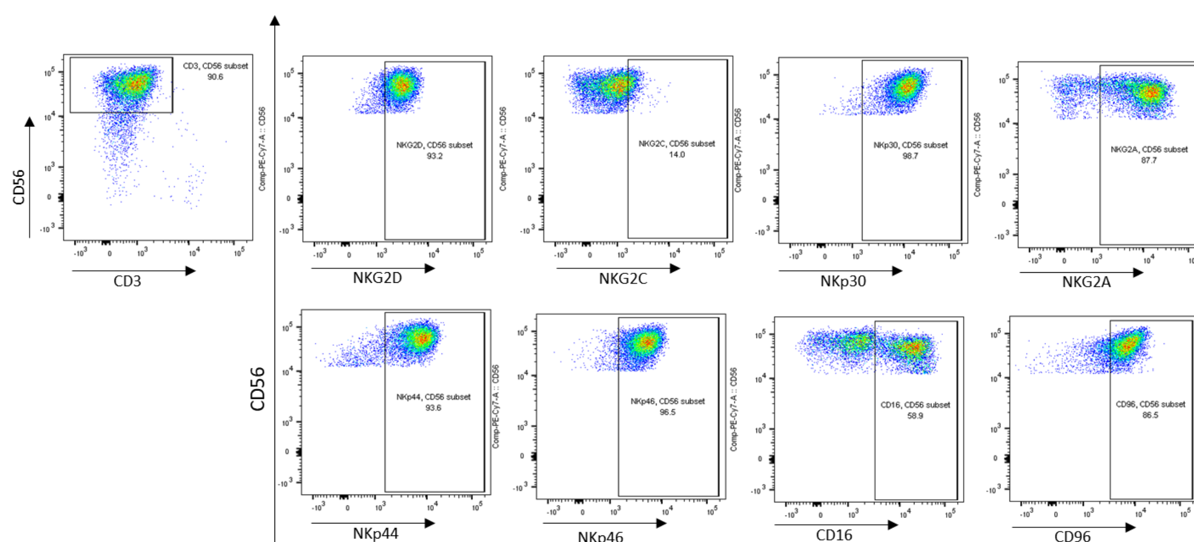


Figure 10. Gating strategy for the expression of NK surface markers

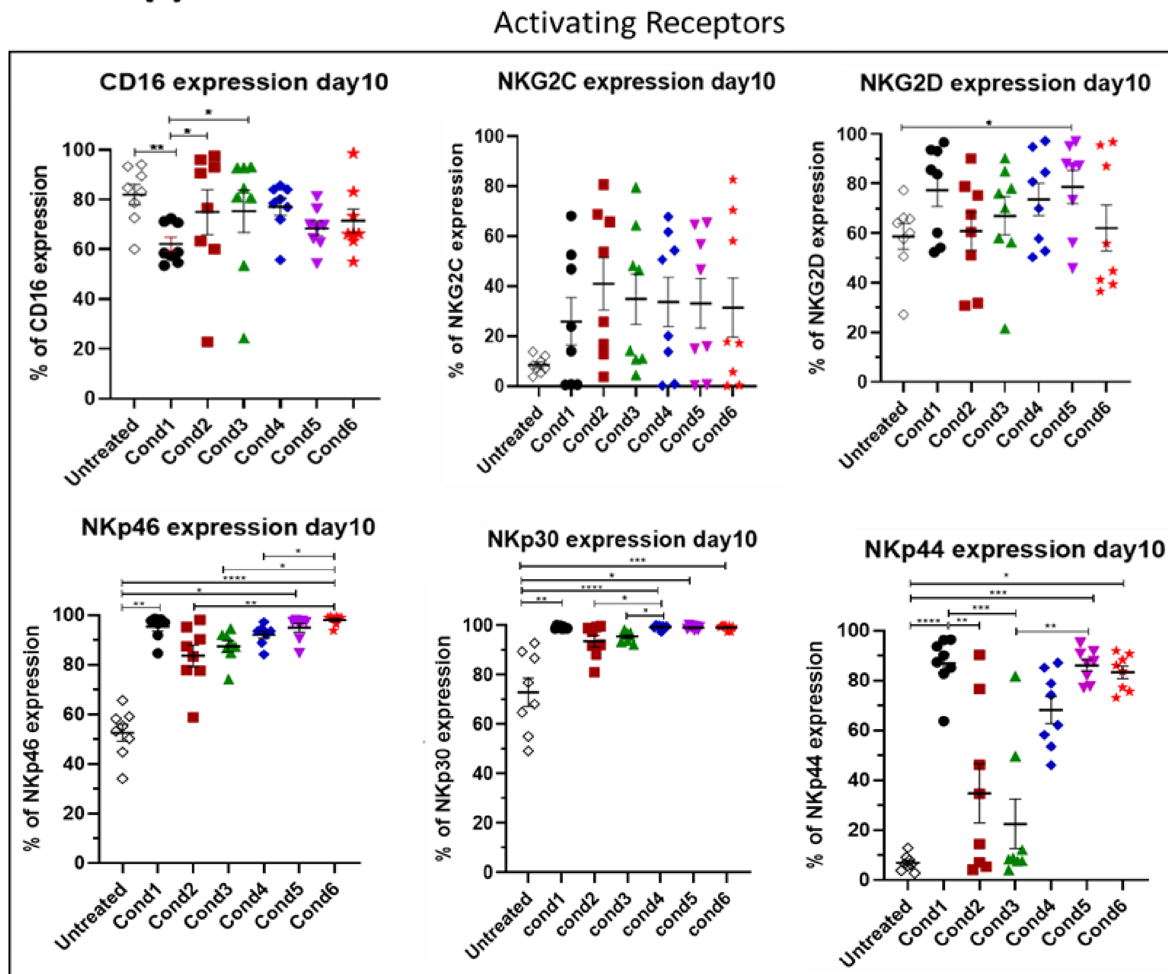
Representative flow cytometry plots from a healthy donor, showing receptor expression from peripheral blood NK cells after 10 days of cytokine activation.

This multifactorial analysis shows a very heterogeneous population of NK cells after cytokine activation. A contributing factor to this outcome is donor response to cytokines which introduce variability and heterogeneity. This causes a variable receptor expression, with a large spread compared to untreated NK cells measured at baseline levels, before cytokines were introduced in the culture (**Figure 11A (i) and (ii)**). CD16 showed an increased trend with condition 3 (**Figure 11A (i)**). NKp30, NKp46 and CD96 showed a great increase in expression compared to untreated NK cells (**Figure 11A)**, seen until day16 (**Figure 11B**). NKG2A was highly expressed, and it remained so to the end of the *in vitro* culture (**Figure 11B**).

Cytomegalovirus (CMV) infection is known to induce NKG2C expression, which marks adaptive memory-like NK cells (66,130,239). Although the CMV status of the donors was not known, NKG2C expression was increased with all the cytokines combinations compared to untreated NK cells, albeit with a very widespread range of expression among the donors,

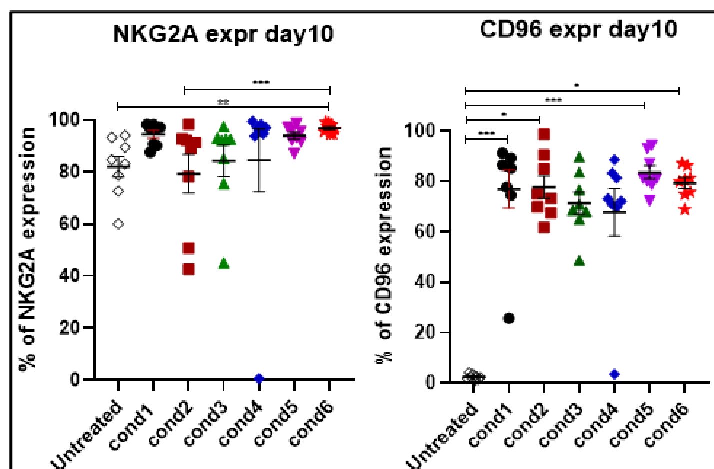
maintained until day 16 (**Figure 11A** and **B**). This suggests that NKG2C expression can be stimulated *in vitro,* via cytokine activation. Interestingly, NKp44 expression, was reduced with both condition 2 and 3, whereas the other conditions caused an increased expression compared to untreated NK cells, and this increase was maintained up to day 16 (**Figure 11**).

A (i)



(ii)

Inhibitory Receptors



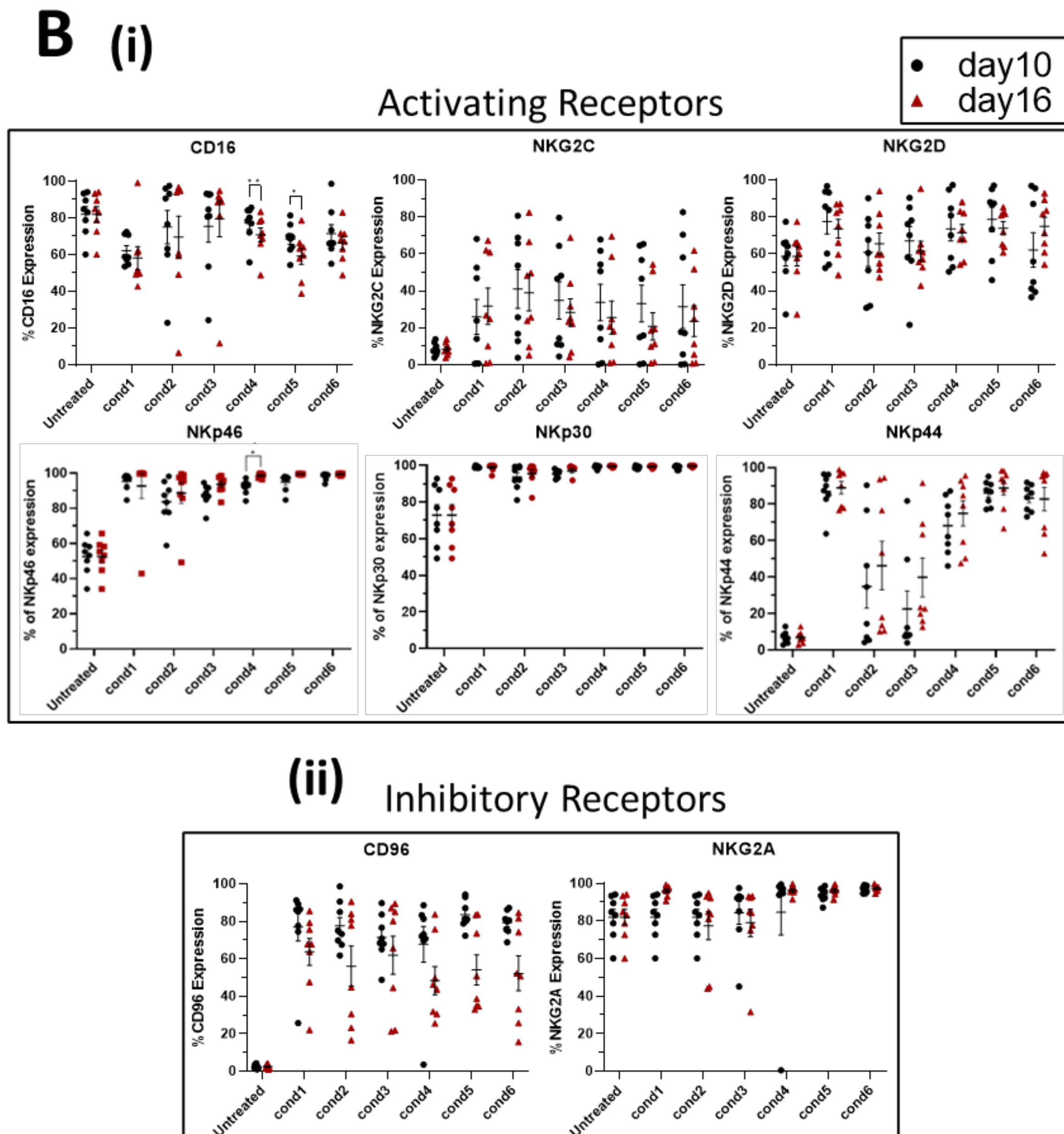


Figure 11. Evaluation of peripheral NK cell phenotype activated by cytokines.

A. Activating (**Ai)** and inhibitory (**Aii**) receptor expressed by NK cells, stimulated by cytokines on day 10 of *in vitro* culture. Data shown as mean of 8 different donors ± SEM. Data was analysed with Friedman's test, using Dunn's multiple comparison adjustment. **B.** Comparison between day 10 and 16 of *in vitro* culture of NK cells' (**Bi**) activating and (**Bii**) inhibitory receptor expressed by cytokine stimulated NK cells. Data shown as mean of 8 different donors ± SEM and analysed with Multiple paired t tests, corrected for multiple comparisons using the Bonferroni-Dunn method. (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001).

To understand the relationship and the influence of receptor expression on NK cell proliferation, correlation analysis was carried out (**Figure 12**). Expression of the strong inhibitory receptor NKG2A, does not negatively correlate with proliferation, independently of the cytokines used in the culture. This is in line with very recent investigations, showing that NKG2A expression helps to maintain NK cell expansion, despite what was originally hypothesised (240). When cultured with IL-2 only (condition 1, **Figure 12A**) NKG2D and NKG2C expression had a positive effect on NK cell expansion. When NK cells were cultured with the memory-like inducing cytokine combination (IL-12+15+18, condition 2, **Figure 12B**), CD16 and NKG2D expression positively correlated with NK cell expansion, whereas NKp44 expression had a negative impact on expansion. The same results were seen with the addition of IL-21 (IL-12+15+18+21 for 16 hours, condition 3, **Figure 12C**), CD16 expression positively correlated with NK cell expansion, whereas NKp44 expression had a negative impact. Longer exposure to IL-21 (condition 4, **Figure 12D**) indicated a positive correlation with NKG2D and expansion. Expression of NKG2C and NKp30 positively correlated with NK cell expansion when NK cells were cultured with cultured IL-2+15 (condition 5, **Figure 12E**). Although not statistically significant, a trend of positive correlation between NKG2C and NKG2D and NK expansion was observed with condition 6. Thus, receptor expression influences expansion of NK cells, and both are dependent on cytokine stimulation.

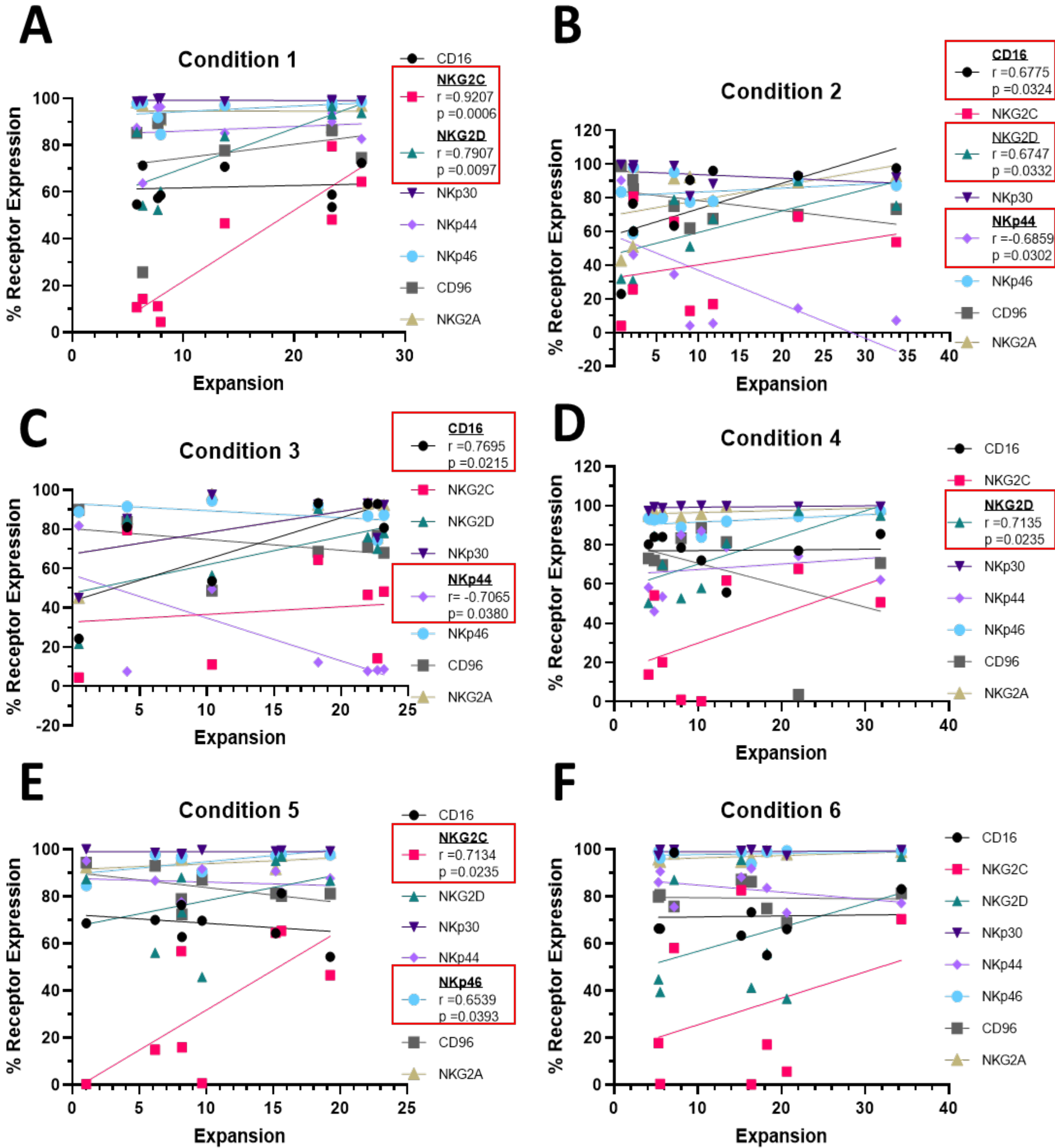


Figure 12. Correlation analysis between receptor expression and NK cell fold expansion

Receptors expression was correlated with fold expansion of NK cells, at day 10 of in vitro culture. Data analysed with one-tail Pearson correlation.

3.4 NK cells activated by cytokines are not terminally differentiated.

Cytokines also affect the maturation stages of NK cells. This is an important factor to consider when developing a novel immunotherapy, to avoid using exhausted and anergic cells. NK cells primed for 16h with IL-12+15+18 and/or IL-21, (Condition 2 and Condition 3, respectively, **Figure 6**), were analysed for the expression of markers, such as CD16, CD57, CD117, CD94 and CD122 (**Figure 1**, (2)), present at different stages of NK cell maturation (**Figure 13**). Cytokine-activated NK cells were initially analysed for the expression of CD16 and divided into CD16+ and CD16-. Within each subpopulation, the expression of CD57 and CD117 was analysed, as illustrated in the quadrant plot in **Figure 13A**. CD16, CD57 and CD117 are expressed in different stages of the NK cell maturation process (**Figure 1**) (2,5,32). To further understand NK cell phenotype, CD94 and CD112 expression was analysed in the largest NK subpopulation according to CD16, CD57 and CD117 expression.

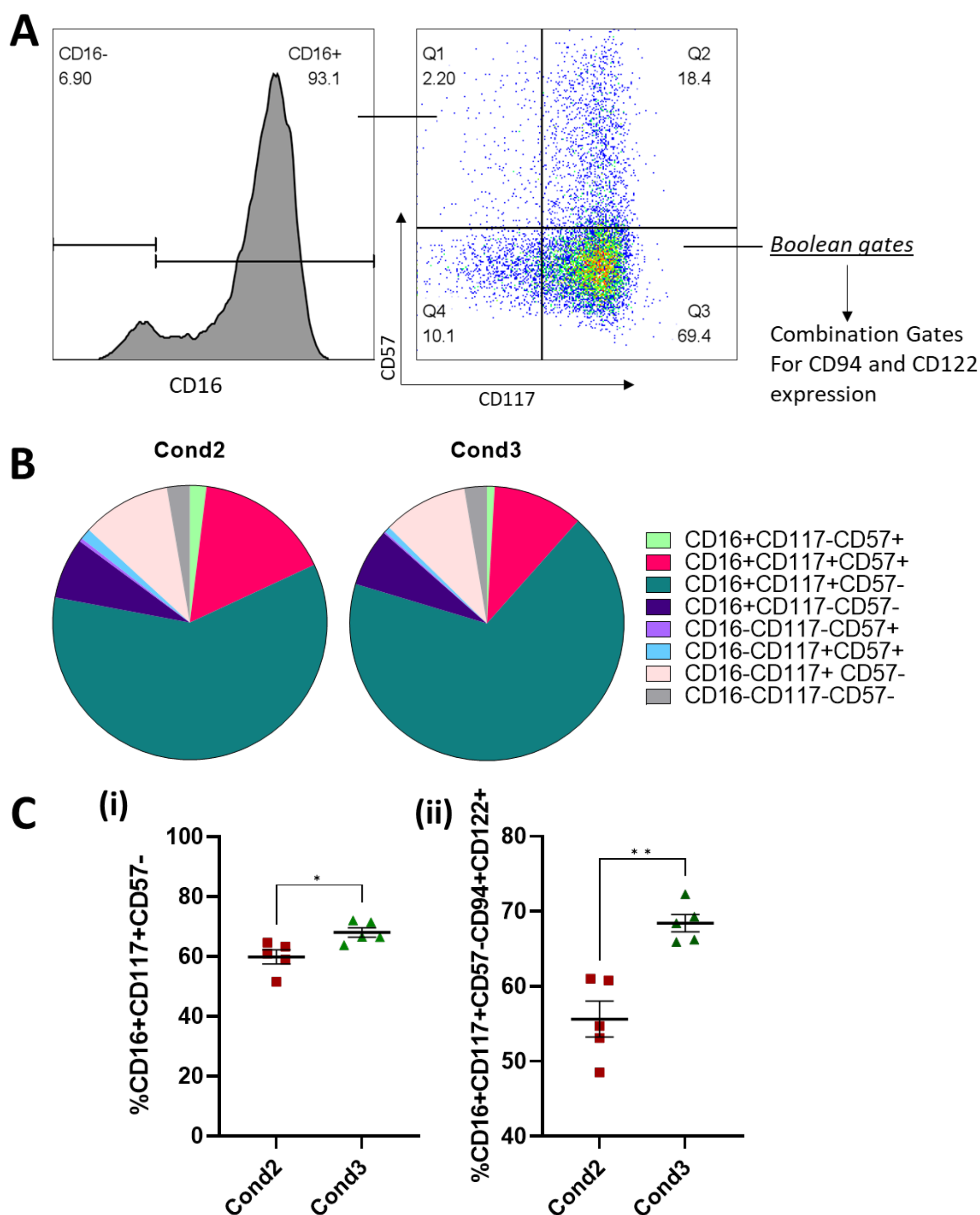


Figure 13. Maturation stages of cytokine-activated NK cells

A Flow cytometry plots illustrating gating strategy used to determine NK cell maturation on day 10 of cytokine stimulation, with condition 2 and condition 3. CD56+CD3- NK cells are gated according to CD16 expression and divided into CD16+ and CD16-. Within both subpopulations, the expression of CD57 and CD117 was analysed, as illustrated in the quadrant plot. This was then used to plot graph in (**B**) and (**C i**). Lastly, to understand the expression of CD122 and CD94, the *Boolean gates* and then the *Combination Gates* tool from FlowJo was applied to the quadrant (Q3, from **A**) showing the biggest subpopulation. This was then used to plot graph shown in (**Cii**). **B**. Expression of NK subpopulations according to the expression of CD16, CD117, CD57. **C**. Comparison between cond2 and cond3 of the most prominent NK cells subpopulation, according to the expression of CD16±CD117±CD57± (**C i**) and CD94±CD122± (**C ii**).

Figure 13B, shows that more than 50% NK cell population was composed of $CD16^{+}CD117^{+}CD57^{-}$ cells, indicating a young, yet transitioning population, where both CD16 and CD117 were expressed (2). Cells, however, were not terminally mature and differentiated (28) as there was no CD57 expression, which was only seen in smaller subpopulations (**Figure 13B**). Both condition 1 and 2, significantly contributed to the development of the $CD16^{+}CD117^{+}CD57^{-}$ subpopulation, however this was higher with condition 3 (**Figure 13Ci**). Next, this subpopulation was analysed for the expression of CD94± and CD122± (**Figure 13A** and **B**). Positive expression for both markers (CD94+ and CD122+) was the highest subgroup present, with condition 3 showing a greater expression than condition 2 (**Figure 13Cii**). CD122 is the β-chain receptor used by common-gamma chain cytokines (IL-2 and IL-15), and marks NK cell lineage commitment. Its expression (**Figure 13Cii**) was consistent with previous findings of terminally differentiated NK cells (CD16+CD57+), expressing low levels of CD122 (32). As the cytokine cocktails analysed here showed a less differentiated NK cell phenotype, (CD16+CD57-), CD122 expression was high. These results indicate that *in vitro* culture with IL-21 confers NK cells a very heterogeneous phenotype with intermediate maturity, which favours cell expansion and proliferation.

3.5 Cytokine-activated NK cells show anti-tumour activity against HCC

In addition to increasing NK cell numbers, *ex vivo* expansion aims to obtain cells that are functional and have high anti-tumour activity. The function of the cytokine-activated NK cells was tested by carrying out a degranulation assay against different HCC cell lines (**Table 6**). Cytokine-activated NK cells were tested for their ability to degranulate, which represents killing activity. This was established by measuring the expression of CD107a (241).

Figure 14 shows that condition 2 and condition 3 caused the lowest NK activation, while conditions 4, 5 and 6 caused a higher activation, and thus degranulation. Despite the results not being statistically different, the NK degranulation trend with condition 2, 3, 4, 5 and 6 was seen with HepG2, SNU475 and Huh7 cell lines. This is an interesting observation, given that the cell lines represent different differentiation stages of HCC (**Table 6**), which might suggest that there would be different degranulation patterns.

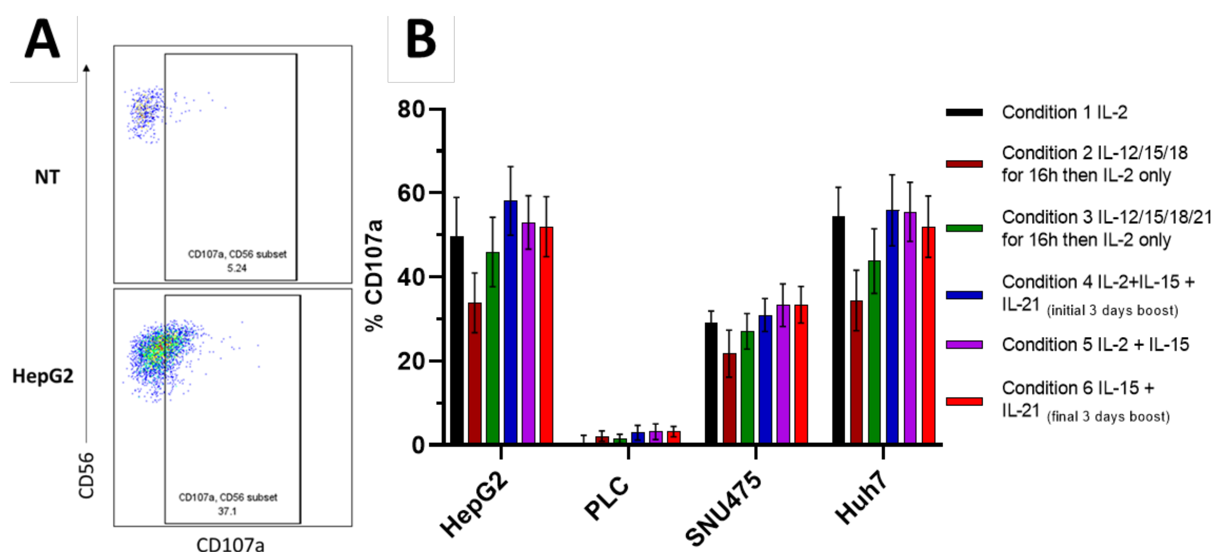


Figure 14. NK cell degranulation after 10 days of cytokine stimulation.

A. CD56+CD3- NK cells activation after 4hrs incubation with HCC cell lines. Representative example of gating for NK cells activation, measured as percentage of NK cells positive for CD107a expression. NT= no target, used as control and background activation, which was subtracted from the co-culture graph. Average plot in (**B**). **B.** Cytotoxicity of NK cells after 10 days of cytokines stimulation, incubated with the HCC cell lines HepG2, PLC, SNU475, Huh7. E:T ratio of 1:1, n=10.

Cell line	Characteristics	NKG2D ligand expression	HLA-C (KIR ligand) and HLA-E (NKG2A ligand)
HepG2	Well differentiated hepatoma cell line	None	Low
PLC	Well differentiated hepatoma cell line	MICA, ULBP2 +/-	Medium
SNU475	Poorly differentiated primary HCC cell line	MICA, ULBP2	Medium /High
Huh7	Well differentiated hepatoma cell line	None	Low

Table 6. NKG2D, KIRs and NKG2A ligand expression by HCC cell lines (65).

The HCC cell lines used in this analysis express different levels of ligands for both activating and inhibitory receptors (**Table 6**). Expression of ligands for the activating receptor NKG2D did not result in higher degranulation, in fact SNU475 resulted in low degranulation for all the conditions used, and PLC resulted in the lowest CD107a expression (**Figure 14**). The opposite was true for Huh7 and HepG2, which expressed no ligands for NKG2D (**Table 6**). In addition to NKG2D ligands, HCC cell lines showed a variable expression of HLA-C (**Table 6** and **Figure 15**), a highly polymorphic ligand both in receptor biding and function recognised by activating and inhibitory KIRs (57,185,242,243), and HLA-E which is a ligand for the inhibitory receptor NKG2A (244). This also contributes to the outcome of NK degranulation.

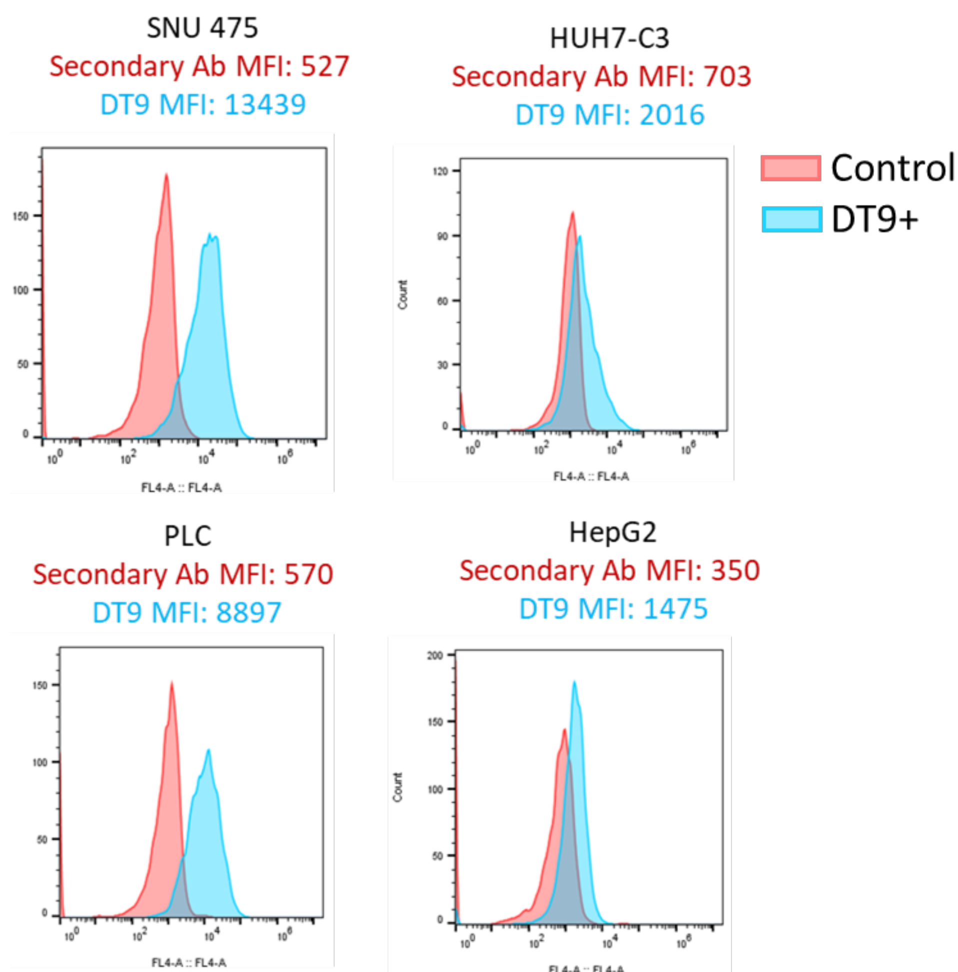
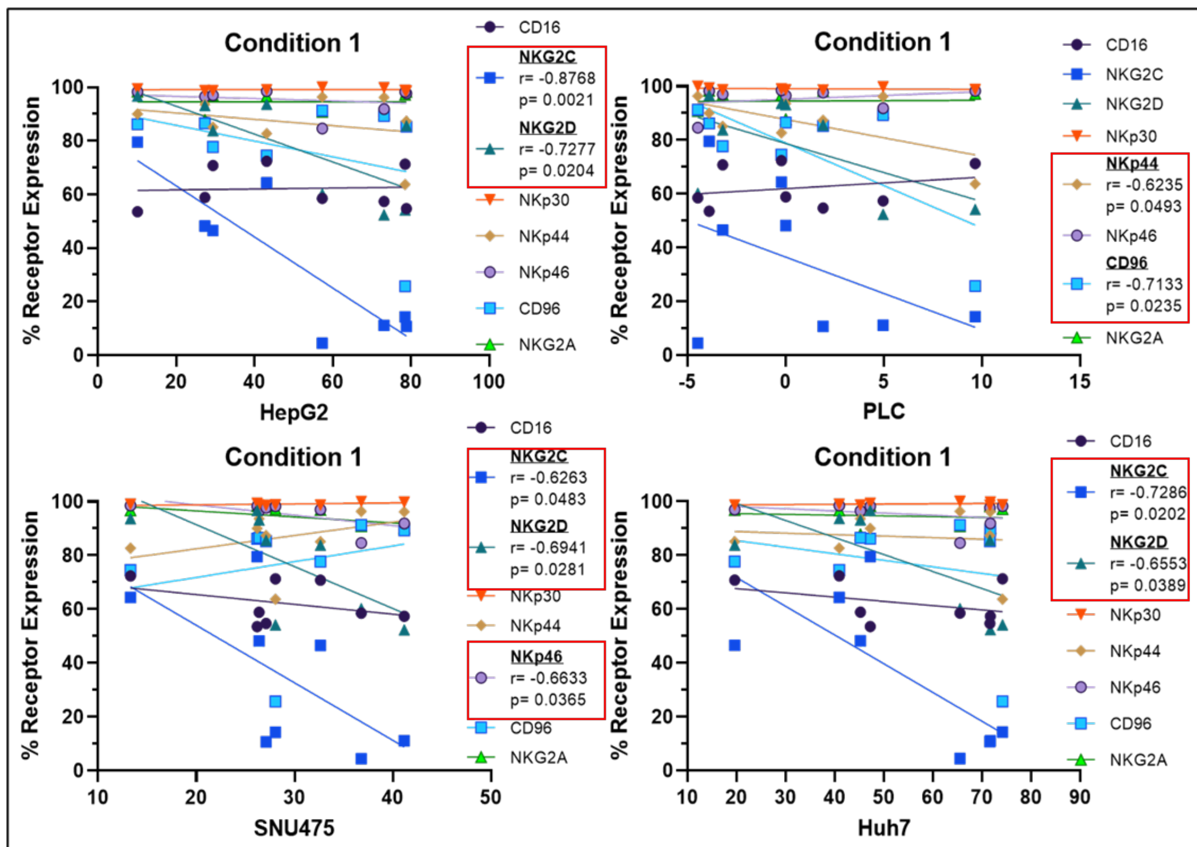


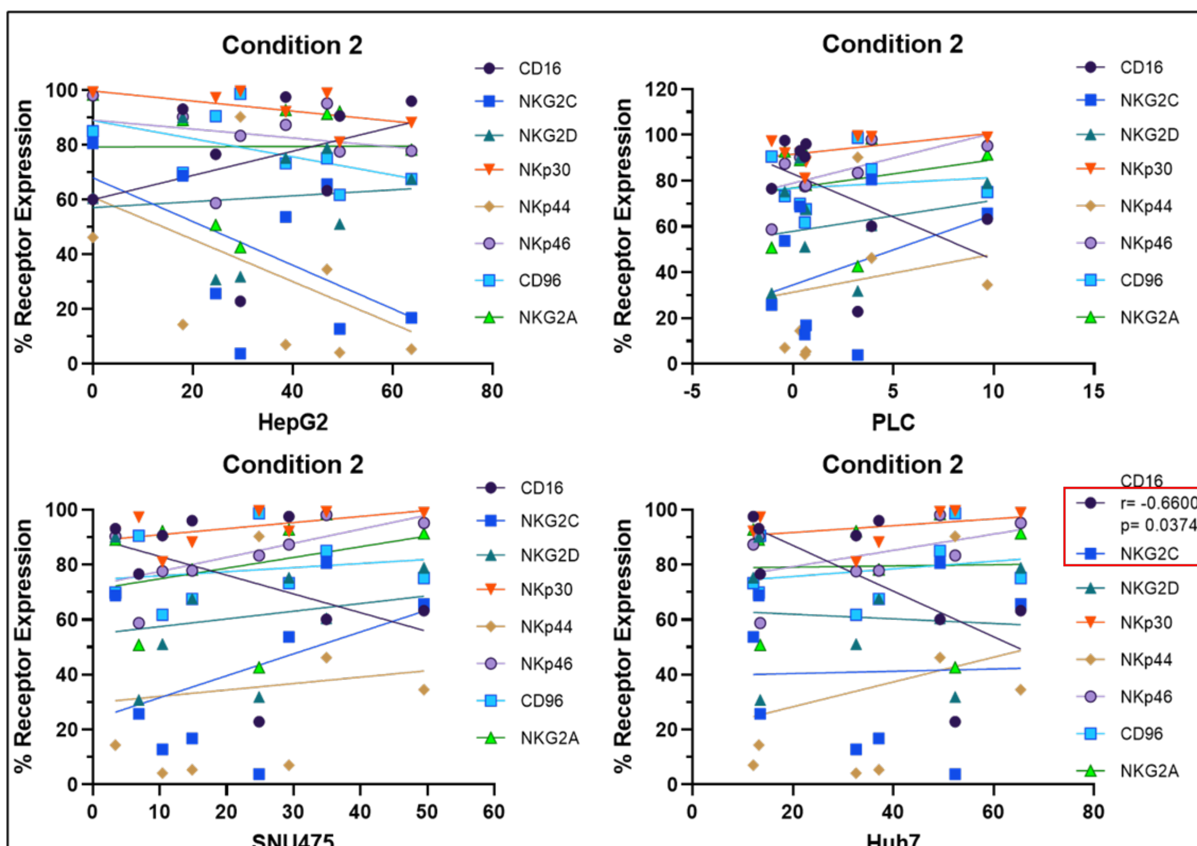
Figure 15. HLA staining of HCC cell lines

DT9 antibody recognises HLA-C and HLA-E expression. Huh7 and HepG2 show little to no expression of HLA-C or HLA-E, SNU475 and PLC show a higher expression.

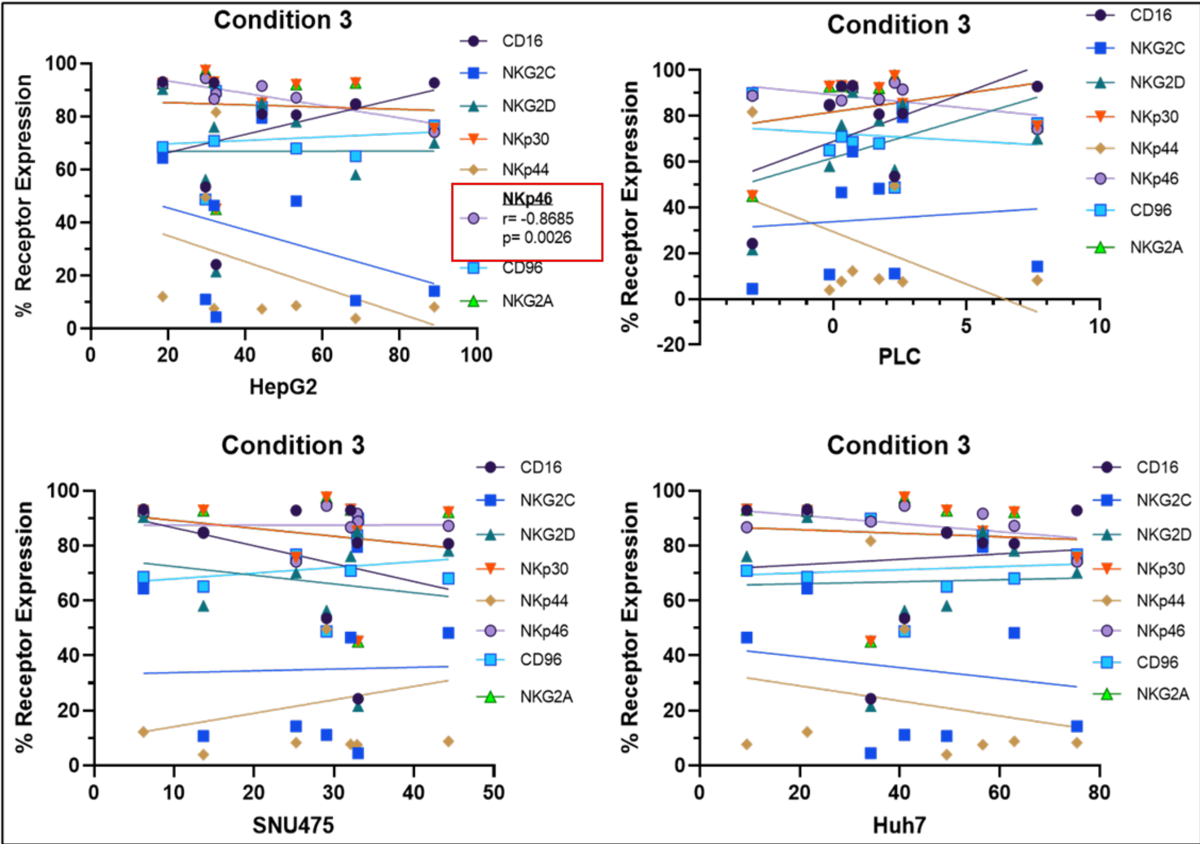
It has been shown that degranulation of NK cells is the product of several activating receptors acting in synergy (245,246). To this end, correlation between receptor expression and function against HCC cell line was analysed (**Figure 16**). In the presence of IL-2 only (condition 1, **Figure 16A**) NKG2D and NKG2C expression had a negative effect on NK cells degranulation against the HCC cell lines HepG2, SNU475, and Huh7. Although not statistically significant, a similar trend was observed with PLC. Additionally, NKp44 and CD96 contributed to a negative outcome of degranulation with PLC, and NKp46 expression showed a negative correlation with SNU475 cell line. When NK cells were cultured with the memory-like inducing cytokine combination (IL-12+15+18, condition 2, **Figure 16B**), the range of receptors explored did not influence NK cells degranulation, except CD16 when NK cells are incubated with Huh7 cell

line. Addition of IL-21 to condition 2 (IL-12+15+18+21 for 16 hours, condition 2, **Figure 16C**), did not impact NK cell degranulation with any of the HCC cell lines. However, a longer exposure to IL-21 (condition 4, **Figure 16D**) gave a negative correlation with NKG2D and degranulation with HepG2 and Huh7. Expression of NKp30 and NKG2A negatively correlated with degranulation against PLC cell line. During *ex vivo* culture of NK cells with IL-2+15 (condition 5, **Figure 16E**) NKG2D and NKG2C expression had a negative effect on NK cells degranulation against the HCC cell lines HepG2, and SNU475; but NKG2C only negatively correlated for degranulation against Huh7. Although not statistically significant, a similar trend was observed with PLC, for both NKG2C and NKG2D. When IL-21 was added to NK cell culture between day 7 to 10 (condition 6), CD16, NKG2C, and NKG2D expression had a negative impact on degranulation against HepG2 and Huh7 cell lines. Taken together these results show that NK cell activation is dependent on the specific threshold to overcome to elicit NK cell activation and antitumour functions. This is determined by the activating and inhibitory ligands expressed by the target cells as well as the cytokine stimuli received during *in vitro* culture. It is interesting to see that the strong inhibitory immune checkpoint NKG2A, does not have a negative impact on NK cells activation, expect when NK cells are stimulated with condition 4 and cocultured with PLC cell line.


A
Condition 1
% Receptor Expression
HepG2
CD16
NKG2C
r= -0.8768
p= 0.0021
NKG2D
r= -0.7277
p= 0.0204
NKp30
NKp44
NKp46
CD96
NKG2A
PLC
NKp44
r= -0.6235
p= 0.0493
CD96
r= -0.7133
p= 0.0235
SNU475
NKG2C
r= -0.6263
p= 0.0483
NKG2D
r= -0.6941
p= 0.0281
NKp46
r= -0.6633
p= 0.0365
Huh7
NKG2C
r= -0.7286
p= 0.0202
NKG2D
r= -0.6553
p= 0.0389


B
Condition 2
% Receptor Expression
HepG2
PLC
SNU475
Huh7
CD16
r= -0.6600
p= 0.0374
NKG2C
NKG2D
NKp30
NKp44
NKp46
CD96
NKG2A

C



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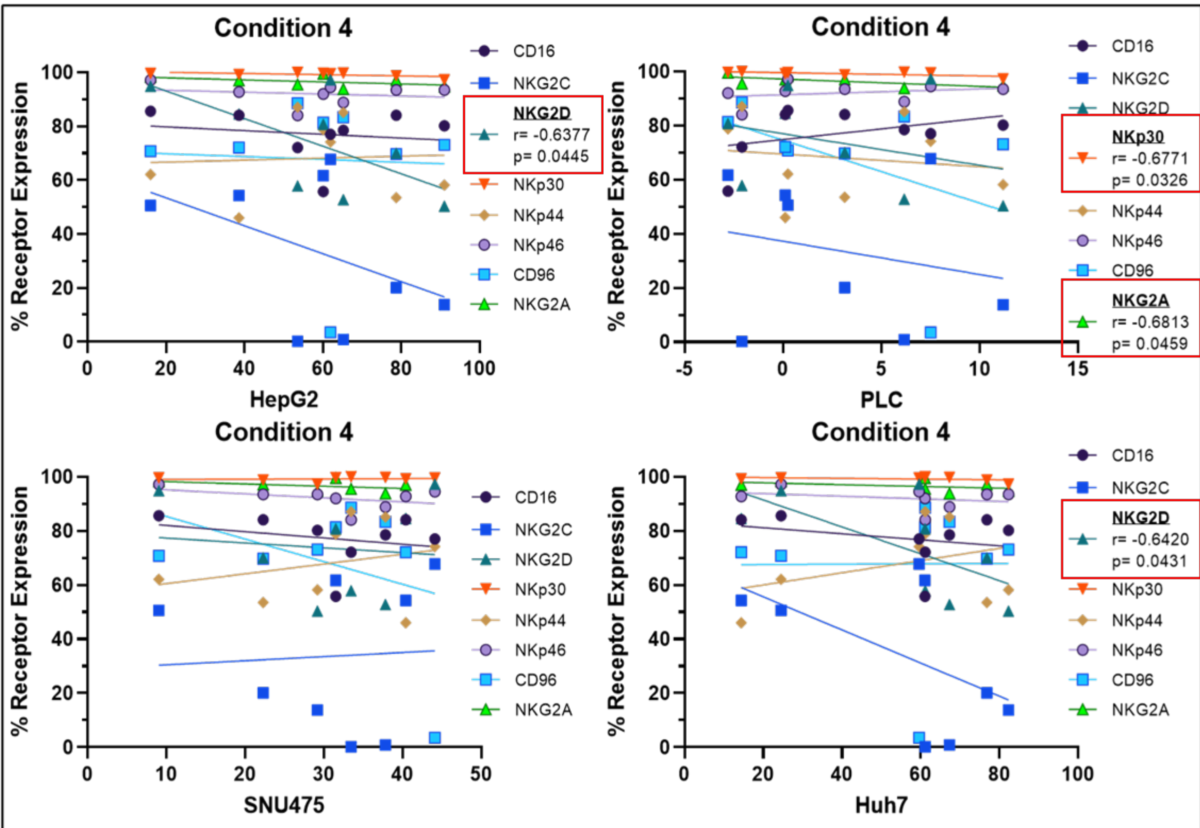

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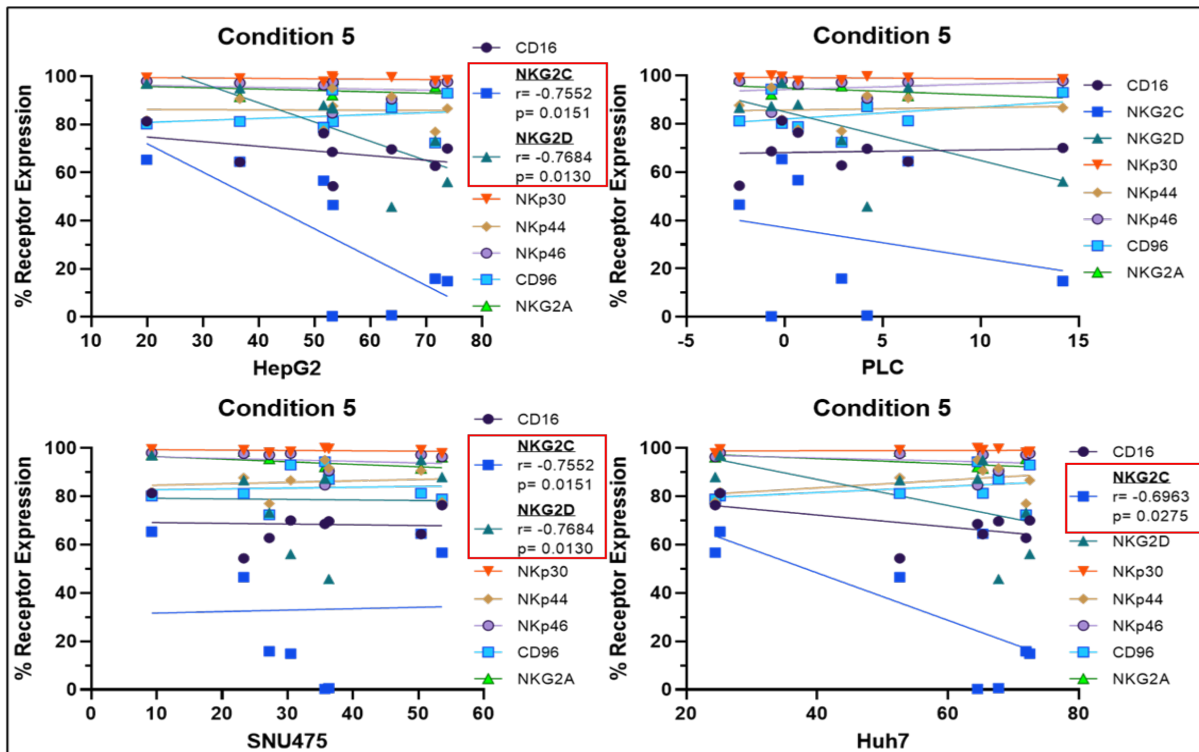

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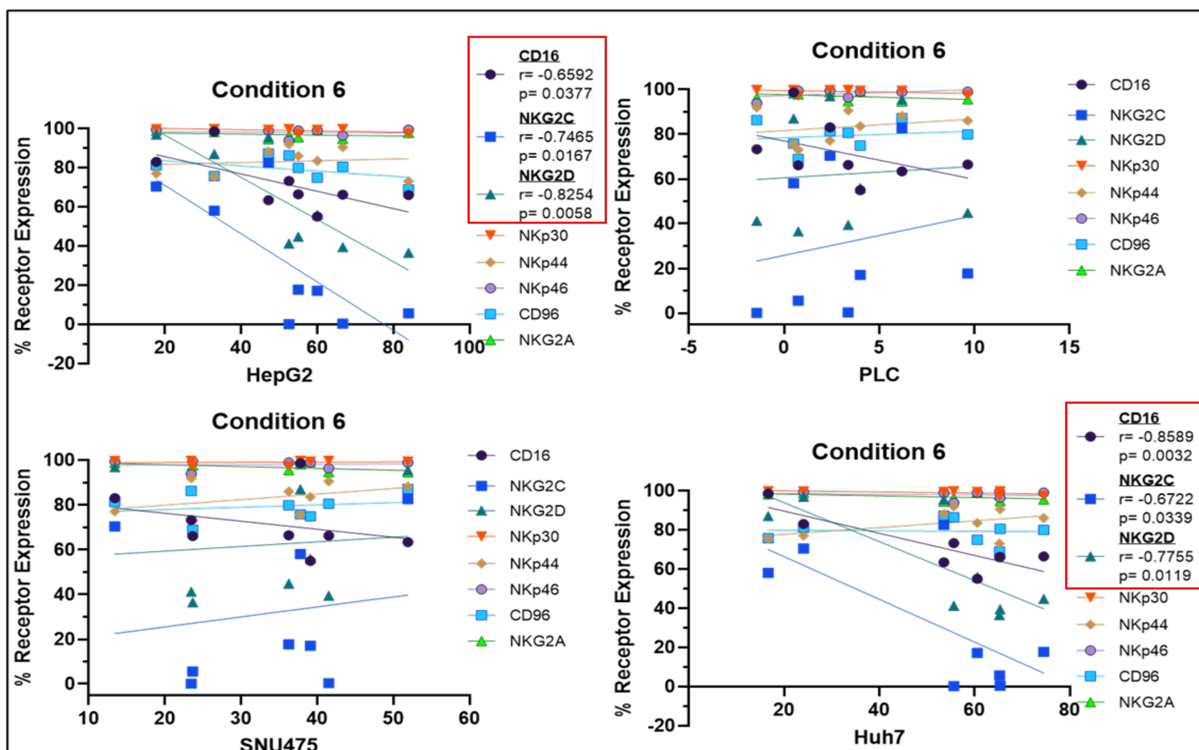


Figure 16. Correlation analysis between receptor expression and degranulation of NK cells

Receptors expression was correlated with degranulation of NK cells, both analysed at day 10 of *in vitro* culture. Data analysed with one-tail Pearson correlation.

3.6 Discussion

This chapter investigates how NK cells can be manipulated *in vitro*, to increase their cytotoxicity and proliferation which can later be utilized for the development of immunotherapy. The main finding was that short exposure of memory-like NK cells to IL-21 (condition 3) leads to increased cell proliferation, especially in the first 9 days of *in vitro* culture (**Figure 7, Figure 8, Figure 9**). Interestingly, cells grown using this condition were healthier and proliferated more than those grown using the memory-like cocktail IL-12+15+18 for 16h (condition 2) or the other conditions analysed (condition 4, 5 and 6). Moreover, condition 3 aids the proliferation of NK cells with early to intermediate maturity (CD16+CD117+CD57-CD94+CD122+, (**Figure 13**), which show antitumour activity (**Figure 14**).

3.6.1 IL-21 increases NK cell expansion

NK cells are traditionally associated with innate properties, lacking the enhanced and specific response typical of T and B cells. However, recent reports have shown the ability of NK cells to gain memory-like features, with enhanced response upon reactivation (130). Over the past decade, copious amount of energy has been invested in understanding and developing memory-like NK (ML-NK) cells for cancer treatment. This is because ML-NK cells respond more potently to cancer than conventional NK (35,66,95). First observed in mice, ML-NK cells show increased cytotoxicity and proliferation compared to conventional NK (cNK) cells (95). *Romee et al* (35) showed that pre-activation with the cytokines IL-12+15+18 for 16h, followed by *in vitro* rest with small amount of IL-15 and re-stimulation with cytokine cocktail (IL-12 +IL-15 or +IL-18), induces “memory-like” NK cells. Interestingly, despite their novel discovery, they found that the memory-like NK cells did not have enhanced degranulation ability. This is in line with other studies (66,141) and it is also seen in our data (**Figure 14**). Though, it is encouraging to see that the memory-like NK cells, still have high levels of degranulation (**Figure 14**, condition 2 and 3), suggesting high NK cell activity. These results differ from other studies, where the degranulation of memory-like NK cell is increased (65,247). This might be a consequence of different concentrations of cytokine used as well as the exposure time of the NK cells to the cytokines. For example, *Zhuang et al* (65), showed increased degranulation by memory-like NK cells, however they used a control group that was treated with only 100 U/ml of IL-2. In the present study the memory-like NK cells were compared with a control

group that was cultured with 500 U/ml IL-2. Although the degranulation results obtained do not show statistical differences, the conditions with IL-21 (mainly condition 4 and 5, **Figure 14**) show a slightly elevated percentage of CD107a$^+$ NK cells. Interestingly, this conflicts with the observation of *Wagner et al* (63), who were able to show that IL-15+IL-21$_{boost}$ (labelled in this thesis as condition 4) NK cells had a greater degranulation than IL-15 only NK cells. The discrepancy in these results can be attributed to the variation of experimental designs, such as exposure time to IL-21, the combination and concentration of cytokines used, controls, maturation stage and origin of NK cells.

IL-21 triggers apoptosis, resulting in shorter lifespan *in vitro*, thus it is important to regulate exposure time to the cytokine, as well as its concentration (10). Although the current study and *Wagner, et al* (63) added IL-21 to the culture for three days, it was added at different time points. Here, we added IL-21 for the first 16h of *in vitro* culture (condition 3, **Figure 6**), or in the first three days of culture (days 1 to 3, condition 4 **Figure 6**), or three days before the degranulation assay (days 7 to 10, condition 5 **Figure 6**). *Wagner et al*, added IL-21 three days before the degranulation assay, during days 3 to 6 of culture. Because NK cells respond to stimuli received from the surrounding environment, it is possible that this minute difference in the protocols can have a significant impact on the cells' functionality. In both studies, NK cells are at different points of their growth when IL-21 is added, therefore we hypothesize that IL-21 impact on NK cell function is dependent on cell cycle. For instance, it could be possible that IL-21 stimulates expression of genes involved in the replication of NK cells but have a lesser impact on genes involved in NK cell function. This hypothesis could help explain the much higher cell expansion achieved in the current study with the IL-21 containing protocols, compared to *Wagner at al* study.

The goal of the current work was to establish a feeder-cell free protocol, for efficient expansion of primary NK cells, to then be used for the development of chimeric antigen receptor (CAR)-NK immunotherapy. Here we show that short exposure to IL-21, in combination with the memory-like cytokine cocktail (named condition 3), significantly increases proliferation, up to ~70 folds at day16 (**Figure 6**, **Figure 7**, **Figure 8**). None of the

investigations done on memory-like NK cells analyse the use of IL-21, hence the results shown here provide the basis for a novel NK cell culturing regimen. The increased proliferation seen with the use of IL-21 was also reported by other studies. These, however, use membrane-bound IL-21 to feeder cells (77,82,84,228,248–250), which increases NK cell proliferation to up to 10,000-fold after 5 weeks of culture(84) . Using feeder cells induces additional signal in the NK cells that lead to a greater expansion (84). While this large-scale NK cell expansion offers advantages, the presence of feeder cells introduces potential safety concerns for the final product used in patients. This could have detrimental clinical consequences. Additionally, we were able to demonstrate that the benefit of IL-21 increased proliferation is still maintained in a feeder cell-free culture system, which also comes with no risks of contaminations for immunotherapy.

3.6.2 IL-21 combines phenotype of canonical CD56^{bright} and CD56^{dim} NK maturation stages

IL-21 pleiotropic effect is also seen on the expression of surface receptors on NK cells (63,81,83,251–253). In line with other investigations (63,77,84), this study shows that IL-21 upregulates CD16 expression compared to untreated NK cells (**Figure 11**), which is essential for antibody-mediated cellular cytotoxicity (ADCC). This means that cytokine-activated NK cells can bind to endogenous antibodies already attacking the tumour cells, further activating the NK cells, and increasing their killing potential. Additionally, this indicates that cytokine-activated NK cells could be used as a combination therapy with monoclonal antibodies, to augment their anti-tumour activity.

NK cells can be divided into immature CD56^{bright} and mature CD56^{dim} cells, with clear phenotypic and functional differences. CD56^{bright} are CD16^{-}, NKG2A^{+}, CD117^{+}, CD57^{-}. Conversely, CD56^{dim} are CD16$^{lo/-}$, NKG2A^{lo}, CD117^{-} and CD57^{+}; this last marker expressed in the last stage of NK cell maturation process (**Figure 1**) (2,78). Here we show that cytokine-activated NK cells have a CD16^{+}CD57^{-} phenotype, significantly higher with condition 3, indicating that IL-21 does not cause cells to be terminally mature in the short term, as ~60% of the NK population does not express CD57 (**Figure 13**), and leading to the combination of features canonical for CD56^{bright} and CD56^{dim}. NK cells expressing CD57 are in their later stages

of maturation and are defined by having limited proliferation (28), therefore the downregulation of CD57 can explain the increased proliferation observed here with IL-21. It has also been shown that expanding NK cells with IL-21 induces telomeres lengthening, which reduces cell senescence and contributes to increased NK cell expansion (77). This is dependent on STAT3 signalling that directly upregulates the expression of human telomerase reverse transcriptase (hTERT), mediating cellular immortalization (77,254), suggesting why IL-21 might contribute to increased expansion upon short exposure (condition 3: IL-12+15+18+21 for 16h, then IL-2 only).

The results shown here of NKG2A, NKp30 and NKp46 upregulation (**Figure 11**) are consistent with other studies (63,77,252), and can be attributed to the IL-21 effect. However, NKG2A can be also upregulated by other cytokines, such as IL-2, IL-15, or IL-12 (255). Its upregulation was shown to be a hallmark of memory-like NK cells, together with CD94, NKp30 and NKp46 increase (130,142). CD94 and NKG2A form a heterodimer and the CD94/NKG2A receptor has been shown to significantly correlate with memory-like NK cells inhibition and treatment failure in acute myeloid leukemia (AML) patients, indicating that CD94/NKG2A limit the enhanced antitumour activity of ML-NK cells (130). Additionally, these data suggest that NKG2A$^+$ NK cells have a high proliferative capacity which might be receptor-dependent and maintained throughout the culture, in line with their development stage, as NKG2A expression indicates that the cells are not terminally mature and are characterized by high expansion. Thus, despite its inhibitory role, NKG2A seems to be involved in other processes of the life of an NK cell, such as education and self-tolerance (22,32,256) and, perhaps, proliferation as shown here. This is also supported by the increased expression of CD94 (**Figure 13**), however, further analysis is needed. The results seen in this chapter, are in line with a very recent study on the role of NKG2A in NK cell expansion, indicating that NKG2A is essential in maintaining NK cell expansion capacity. NKG2A mediates this by reducing apoptosis because of excessive activation, and by reducing CD57 expression (240). This suggests that NKG2A inhibitory signalling is required to avoid activation-induced apoptosis and sustain expansion when NK cells are strongly stimulated. To maintain self-tolerance, expansion of NK cells lacking inhibitory receptors such as NKG2A is dampened to protect against strong autoimmunity, which could lead to inflammation and tissue damage.

Interestingly, in concert with low CD57- and high NKG2A+, CD117 expression might also support NK cell proliferation with condition 3 (**Figure 13**). CD117 is present on immature Hematopoietic Progenitor Cells (HPC), expressed very early on in the maturation process of NK cells (**Figure 1**), however, its presence aids proliferation and it does not impair NK cells degranulation (**Figure 14**). This is in line with previous studies, which show the increased expression of CD117 (226). So, IL-2 enhances and sustains CD117 expression, and we hypothesise this is enhanced by the IL-21 priming in condition 3. This, however, would need to be experimentally validated.

IL-21 has no effect on NKG2D as cytokine-activated NK cells show a similar expression as untreated NK cells (**Figure 11**). This is in contrast with previous reports, showing downregulation of DAP10 which mediates NKG2D signalling (63,77,252,253). It has been shown that IL-21 also downregulates DAP12 expression, that regulate NKp44 (63,77,252,253). The main role of NKp44 is to eliminate viral infected cells or tumour cells (49,50), but this is dependent on the isoform of the receptor expressed which in turn is determined by the cytokine milieu (257,258). NKp44 expression is not detected in resting peripheral NK cells and on freshly *ex vivo* isolated NK cells. However, it is upregulated by cytokines such as IL-2, IL-15 and IL-1β (45) It has been indicated that NKp44 expression can also be modulated by the NF-kB pathway, and this increases NK cell activation (116). Conversely, **Figure 11** shows that IL-2 and/or IL-15 counteracts IL-21 negative impact on NKp44 expression. Therefore, the decreased expression seen with condition 2 and condition 3, could be the result of IL-12 inhibition, in line with other studies (227).

Cytokines strongly modulate NK cells cytotoxicity, proliferation, and phenotype. As shown here, IL-21 plays an important role, generating a phenotypic and maturation profile which increases NK cell proliferation and maintains cells' antitumour activity. These findings encourage the use of the protocol elucidated here for the development of NK cell-based immunotherapy.

Chapter 4 Understanding intracellular signalling of NK cells in response to cytokine stimulation and analysis of their metabolism

4.1 Introduction

Cytokines mediate their effect on NK cell by signalling through transcription factors, such as STAT proteins or NF-kB (69,259,260). These transcription factors traditionally pair with specific cytokine families. For example, γ-chain receptors cytokines IL-2, IL-15 and IL-21 signal through STAT1, STAT3 and STAT5, with IL-2 and IL-15 activating primarily and strongly STAT5, and IL-21 signalling predominantly via STAT3. IL-12 mainly leads to the activation of STAT4, however, it has been suggested to also activate STAT1, STAT3 and STAT5 (69,261), whereas IL-18, member of the IL-1 family of cytokines, is the main driver of NF-kB (100). However, these are not the only interactions possible, as STATs and NF-kB can be activated by multiple cytokine signals and certain cytokine families can activate different transcription factors. Therefore, the pleiotropic effect of both cytokines and transcription factors hinders the possibility to precisely define signalling pathways, and to assign the molecular changes seen in NK cells to specific transcription factor-cytokine pairs.

In addition, cytokines are known to cooperate, synergise or antagonise other cytokines to enhance or downregulate NK cell effector functions, phenotype, metabolism, maturation, and proliferation. For example, IL-12 and IL-18 synergise to increase IFN-γ production (262–265). IL-2 and IL-15 both support and increase proliferation (266). IL-18 has also been shown to synergize with IL-15 to increase proliferation (103). IL-15 also synergises with IL-21 to increase NK proliferation, cytokine secretion, cytotoxicity and expression of receptors such as KIR (110,252,267). However, this is in sharp contrast with other findings, showing antagonism between IL-21 and IL-15 for the proliferation of murine NK cells (229,268). Moreover, IL-12, IL-15 and IL-18 have been shown to cooperate to generate cytokine-induced memory-like NK cells (88). It is clear that this interplay increases the complexity of cytokine-transcription factor interactions, further hindering the deconvolution of the cytokine signalling network in

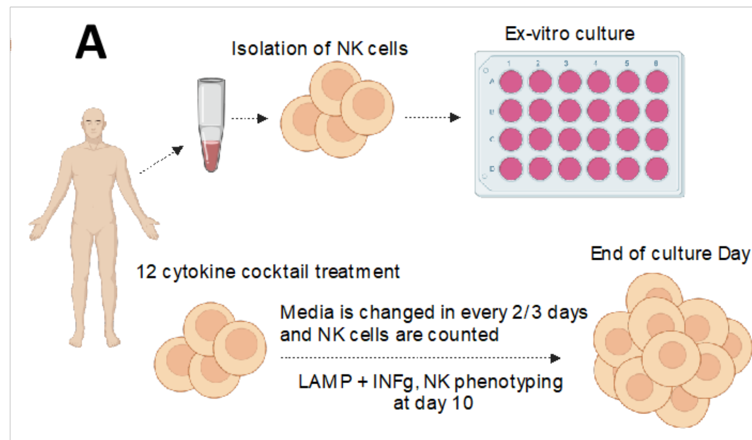
NK cells. Understanding the mechanisms by which cytokines shape and modulate NK cells is key to using these protocols for new NK cell-based therapies. As such, we aimed to evaluate NK cells profile in the presence of cytokine combinations, involving IL-2, IL-15, IL-12, IL-18, IL-21. NK cells were screened for their proliferation, maturation, and effector functions. Additionally, their metabolism was also studied using nuclear magnetic resonance (NMR) spectroscopy. Lastly, to better understand the intricate signalling pathways initiated by cytokines, mathematical modelling was used to elucidate the complex web of interaction in NK cells, which can be applied to improve current culturing protocols.

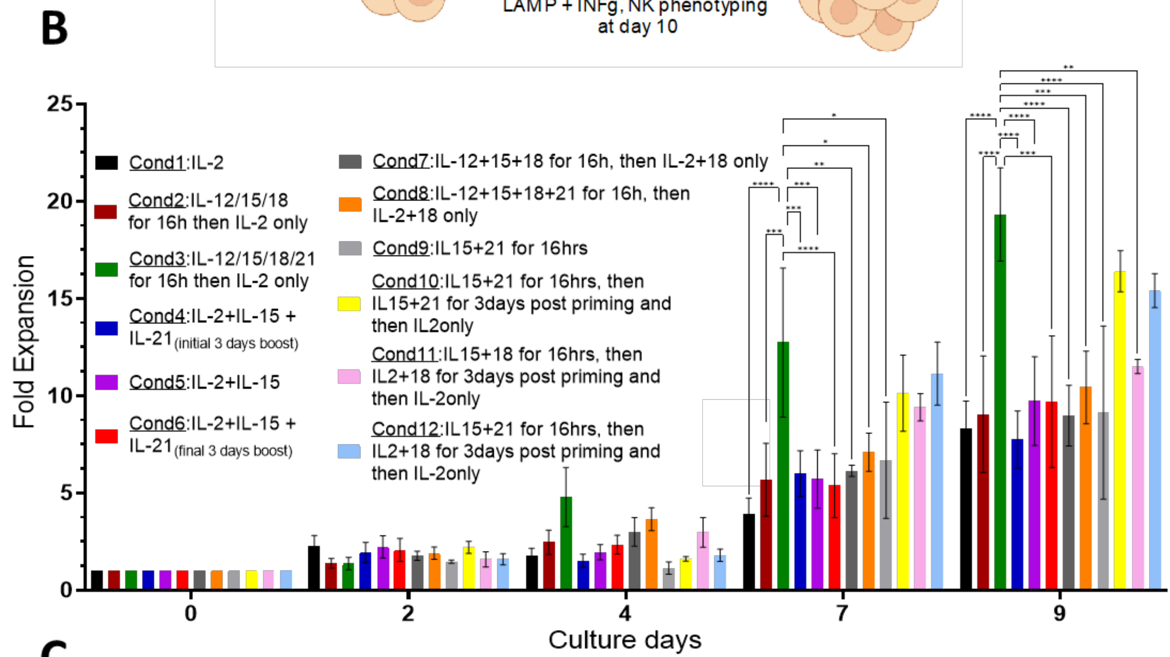
4.2 Testing different cytokine conditions for NK cell proliferation

To further understand the effect of cytokine on NK cells, other conditions alongside those investigated in **Figure 7** were analysed (**Figure 17**). To this end, NK cells were purified from peripheral blood of healthy donors and cultured *in vitro* with different combinations of the cytokines IL-12, IL-15, IL-18, and IL-21 (**Figure 17A**). The experimental design involved a priming step of 16 hours, followed by a post-priming consisting of a different cytokine combination for up to three days, and finally a maintenance regimen of IL-2 alone or IL-2 in combination with IL-15, as shown in **Figure 17**. Concentration of cytokines were the same as those used in **Figure 6**. The following extra conditions were tested:

- **Condition 7**: IL-12 (10ng/ml) + IL-15 (20 ng/ml) + IL-18 (50 ng/ml) for 16h priming. Medium was then changed, and cells were cultured with IL-2 (500 U/ml) + IL-18 (50 ng/ml) for 3 days post-priming. After this, IL-2 (500 U/ml) only was used for the rest of the culture.
- **Condition 8**: IL-12 (10ng/ml) + IL-15 (20 ng/ml) + IL-18 (50 ng/ml) + IL-21 (25 ng/ml) for 16h. Medium was then changed, and cells were cultured with IL-2 (500 U/ml) + IL-18 (50 ng/ml) for 3 days post-priming. After this, IL-2 (500 U/ml) only was used for the rest of the culture.
- **Condition 9**: IL-15 (20 ng/ml) + IL-21 (25 ng/ml) for 16h. Medium was then changed, and cells were cultured with IL-2 (500 U/ml) only for the rest of the culture.
- **Condition 10:** IL-15 (20 ng/ml) + IL-21 (25 ng/ml) for 16h. IL-15 +IL-21 combination was kept for 3 days post-priming. After this, IL-2 (500 U/ml) only was used for the rest of the culture.
- **Condition 11:** IL-15 (20 ng/ml) + IL-18 (50 ng/ml) for 16h. Medium was then changed, and cells were cultured with IL-12 (10ng/ml) + IL-18 (50 ng/ml) for 3 days post-priming. After this, IL-2 (500 U/ml) only was used for the rest of the culture.
- **Condition 12:** IL-15 (20 ng/ml) + IL-21 (25 ng/ml) for 16h. Medium was then changed, and cells were cultured with IL-12 (10ng/ml) + IL-18 (50 ng/ml) for 3 days post-priming. After this, IL-2 (500 U/ml) only was used for the rest of the culture.

Amongst the regimens analysed, condition 3 (IL-12+15+18+21 priming), showed the highest and best proliferation, and its effect of increased NK cell proliferation as early as day 4, with a 5-fold cell expansion (**Figure 17B and C**). By day 9 of culture, under all conditions, cells had a proliferation between 10 to 20 times, with the IL-21 primed regimens condition 3, 10 and 12 giving the best proliferation overall (**Figure 17B**). High cell proliferation with condition 3 was maintained till day 16, when it reached a 100-fold expansion (**Figure 17B and C**).


A
Isolation of NK cells
Ex-vitro culture
12 cytokine cocktail treatment
End of culture Day
Media is changed in every 2/3 days and NK cells are counted
LAMP + INFg, NK phenotyping at day 10


B
Fold Expansion
Culture days
Cond1:IL-2
Cond2:IL-12/15/18 for 16h then IL-2 only
Cond3:IL-12/15/18/21 for 16h then IL-2 only
Cond4:IL-2+IL-15 + IL-21 (initial 3 days boost)
Cond5:IL-2+IL-15
Cond6:IL-2+IL-15 + IL-21 (final 3 days boost)
Cond7:IL-12+15+18 for 16h, then IL-2+18 only
Cond8:IL-12+15+18+21 for 16h, then IL-2+18 only
Cond9:IL15+21 for 16hrs
Cond10:IL15+21 for 16hrs, then IL15+21 for 3days post priming and then IL2only
Cond11:IL15+18 for 16hrs, then IL2+18 for 3days post priming and then IL-2only
Cond12:IL15+21 for 16hrs, then IL2+18 for 3days post priming and then IL-2only

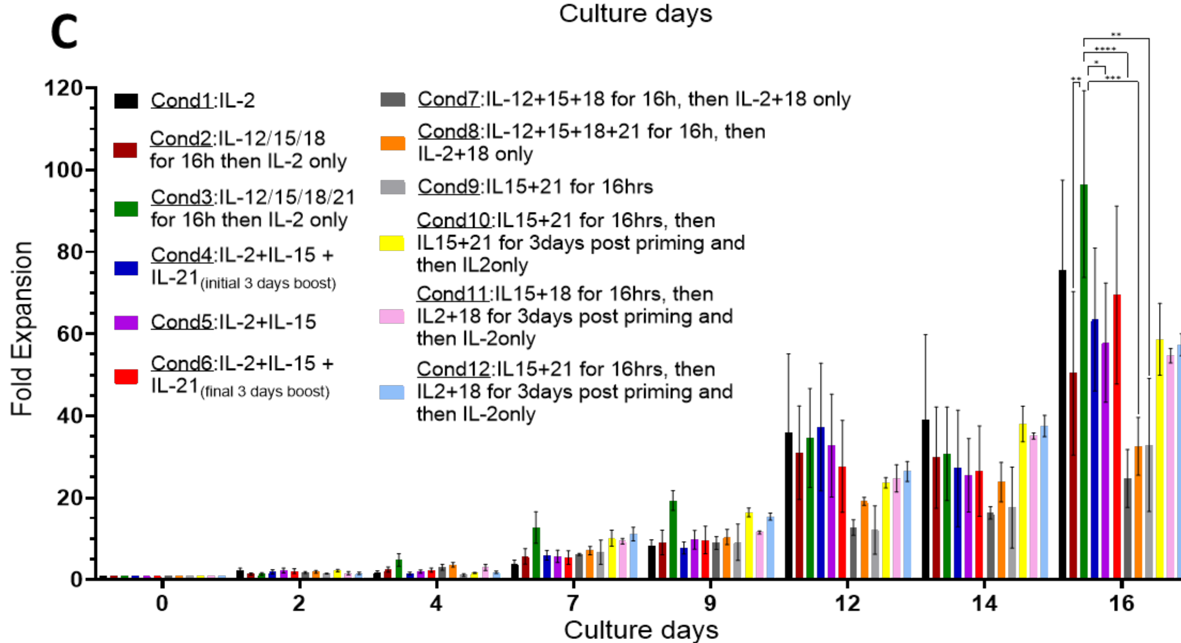

C
Fold Expansion
Culture days
Cond1:IL-2
Cond2:IL-12/15/18 for 16h then IL-2 only
Cond3:IL-12/15/18/21 for 16h then IL-2 only
Cond4:IL-2+IL-15 + IL-21 (initial 3 days boost)
Cond5:IL-2+IL-15
Cond6:IL-2+IL-15 + IL-21 (final 3 days boost)
Cond7:IL-12+15+18 for 16h, then IL-2+18 only
Cond8:IL-12+15+18+21 for 16h, then IL-2+18 only
Cond9:IL15+21 for 16hrs
Cond10:IL15+21 for 16hrs, then IL15+21 for 3days post priming and then IL2only
Cond11:IL15+18 for 16hrs, then IL2+18 for 3days post priming and then IL-2only
Cond12:IL15+21 for 16hrs, then IL2+18 for 3days post priming and then IL-2only

Figure 17. Testing different cytokine conditions for NK cell proliferation.

A Schematic representation of experimental approach for NK cell *in vitro* culture. Purified NK cells were cultured *in vitro* with different cytokine treatments. Cell proliferation was monitored every two/three days till the end of the culture. At day10, NK cells were analysed for their receptor expression and activation. **B**. and **C**. Proliferation of NK cell at day 2, day 4, day 7, day 9, day 12, day 14, and day 16, treated with the regiments highlighted in the text. Data shown as mean ± SEM, n=8 for conditions 1 to 6, n=4 for condition 7 and 8, n=3 conditions 9 to 12. All data was analysed by two-way ANOVA, and Tukey's multiple comparison test (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001).

4.3 STATs and NF-kB interaction after cytokine stimulation determines NK cell proliferation *in vitro*

Binding of IL-21 to its receptor leads to the activation of Janus kinase 1 (JAK1) and JAK3, which phosphorylate and hence activate STAT proteins, mostly STAT3 (80). To understand the importance of IL-21 in NK cell proliferation, STAT3 inhibition was investigated by using the inhibitor S3I-201. Additionally, IL-18, which leads to the activation of NF-kB pathway, has also been shown to affect NK cell proliferation (103,269,270), and its role in NK proliferation was investigated by using the NF-kB inhibitor PS1145.

NK cells were cultured using condition 2 (IL-21 absent) or condition 3 (IL-21 present) in the presence of either inhibitor during the priming stage (first 16 hours after NK purification). These results show that proliferation was dependent on both pathways for both conditions (**Figure 18A** and **B**). However, in condition 2, the effect of STAT3 inhibition was greater than that for NF-kB, whereas the reverse was true for condition3, where addition of IL-21 rescues proliferation (**Figure 18A** and **B**). Direct comparison of condition 2 and 3 on individual donors confirmed that inhibition of the NF-kB pathway had a more profound effect in condition 3 than in condition 2; the reverse was true for STAT3 (**Figure 18B**). Phosphorylation of STAT3 was tested in the absence of any inhibitors and was found to be more evident in condition 3 than condition 2 (**Figure 18C**). Overall, this suggests that IL-21 exerts its pro-proliferative effect via STAT3.

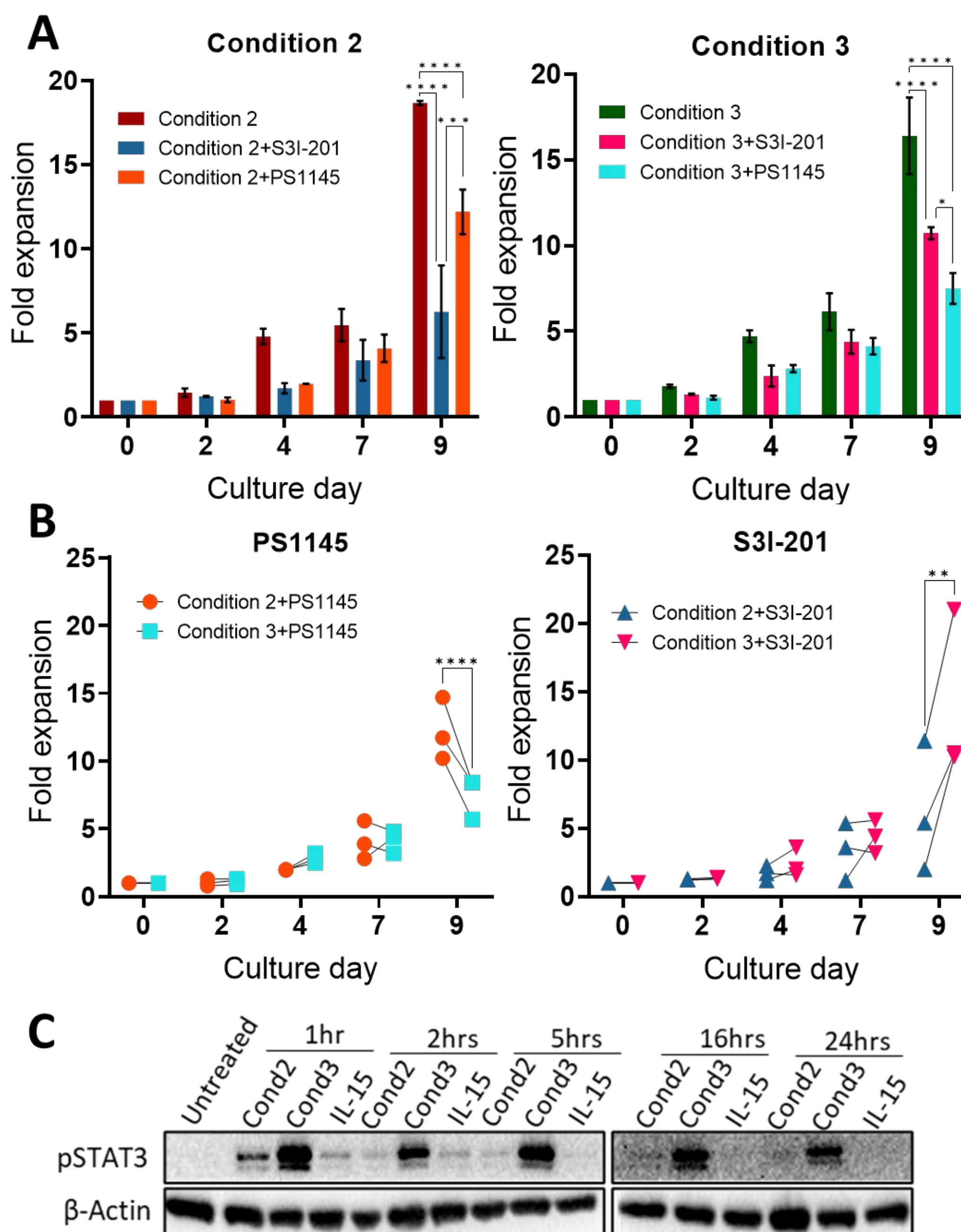


Figure 18. Proliferation of NK cells with either STAT3 or NF-kB inhibitor

Isolated NK cells were cultured with condition 2: IL-12+15+18 priming for 16hrs, then IL-2 only, and condition 3: IL-12+15+18+21 priming for 16hrs, then IL-2 only. During the priming stage, NK cells were treated either with 75 μM S3I-201 (STAT3 inhibitor) or 10 μM PS1145 (NF-kB inhibitor), cells were then washed and cultured only with IL-2 only. Data shown as mean ± SEM, n=3 (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001). **A**. Fold expansion of condition 2 and condition 3 with and without inhibitors. Data analysed by two-way ANOVA, and Tukey's multiple comparison test **B.** Comparison of fold expansion between condition 2 and condition 3 in the presence of inhibitors. Data analysed by two-way ANOVA, and Bonferroni's multiple comparison test **C.** Isolated NK cells were cultured with condition 2, condition 3 or IL-15 only (1ng/ml), for 1hr, 2 hrs, 5hrs, 16hrs or 24hrs; no inhibitors were present. pSTAT3 and β-actin protein levels were then detected by immunoblotting.

However, the intricate intracellular signalling system in NK cells is complex due to crosstalk between STAT and NF-kB signalling pathways (88,111). There is a lack of detailed knowledge about the mechanism of crosstalk and about the quantification of activated STAT/NF-κB that primarily affect proliferation when cytokine combinations are used. Therefore, to investigate cell proliferation further, mathematical modelling developed by Indrani Nayak was performed, incorporating the transcription factors STATs and NF-kB complex signalling downstream of IL-2, IL-12, IL-15, IL-18, IL-21. Based on my experimental work, Indrani Nayak, from *Steve and Cindy Rasmussen Institute for Genomic Medicine, Abigail Wexner Research Institute, Nationwide Children's Hospital, Columbus, Ohio* performed the mathematical modelling analysis in this collaborative project.

The *in silico* analysis generated various possible imputation maps that explored potential hypotheses for primary and secondary STAT/NF-κB signalling pathways induced by specific cytokines **(Appendix 1A)**. This was based on the experimental observation that STAT3 and NF-κB inhibition downregulate NK proliferation when cultured with both condition 2 and 3 (no IL-21 and with IL-21 present, respectively) (**Figure 18**). Next, regression models were carried out to determine the effect of the interplay (synergistic or antagonistic) between the STATs and NF-κB during priming and post-priming in NK cell proliferation. Four possible scenarios of STAT/NF-κB interactions that regulate NK cell expansion were considered: **(i)** STATs and NF-κB induced in the *priming phase* but not in the post-priming (PP-I) stage regulate NK cell proliferation. **(ii)** STATs and NF-κB induced in the *PP-I phase* but not in the priming stage regulate NK cell proliferation. **(iii)** STATs and NF-κB induced in the *priming- and post-priming (PP-I)* regulate NK cell proliferation *additively*. **(iv)** STATs and NF-κB induced in the *priming- and post-priming (PP-I) synergize or antagonize* and regulate NK cell proliferation (**Appendix 1A**). To predict fold change for each observed condition, the regression model was trained with the other 11 observed conditions using Leave-one-out cross-validation (LOOCV) (271,272). The strength of each model is assessed by calculating the R-square value for the overall predictions across the 12 observed conditions (**Appendix 1A**). The model predicted that the best cytokine combination to describe expansion in NK cell populations is imputation map 28 (IL-2 signals through STAT5, STAT4, STAT1, IL-12 signals through STAT4, IL-15 through STAT5, IL-18 through NF-κB, STAT3, IL-21 signals through STAT3, STAT1), selected among

those feasible imputation maps with a pairwise synergy between the STATs and NF-κB induced in the priming and post-priming stimulation phases (lowest cross-validation error and highest R-square) (**Appendix 1B**). The optimum imputation map and the synergy between STATs and NF-κB between priming and post-priming predicts that the cytokine combination in condition 3 generates maximum fold expansion compared to other conditions (**Appendix 1C**), which is validated by the experimental data (**Figure 17C**). The model also identified condition 7 as yielding the lowest fold expansion (**Appendix 1C**), in agreement with the experimental data (**Figure 17C**).

Furthermore, employing the optimal map 28, the model was used to predict the weighted average of NK cell fold expansion (**Appendix 1E**) at day 9 for conditions 2 and 3 with and without the presence of STAT3 and NF-κB inhibitors. Both STAT3-inhibitor (S3I) and NF-κB-inhibitor (NF-κBI) reduced fold expansion in condition 2, however, STAT3 inhibition caused a greater reduction in fold expansion than NF-kB inhibition, according to the experimental data (**Figure 18B**). The model was able to predict a reduction in fold expansion for condition 3 when STAT3 was inhibited, as observed in the experimental data (**Figure 18B**). However, the model was not able to replicate the reduction in fold expansion seen with condition 3 in the presence of NF-kB inhibitor (**Appendix 1E**), which in the experimental data showed a stronger impact on NK expansion than STAT3 inhibition (**Figure 18B**), indicating a limitation in model.

While the model has limitations, it suggests that condition 3 is an optimal culture condition for NK cells due to the presence of IL-21 during the priming stage. Conversely, condition 7 which does not have IL-21 either in the priming or post-priming phase, resulted in the lowest cell expansion.

4.4 Effects of STAT3 and NF-kB inhibition on NK cells phenotype and effector function

The effect of STAT3 and NF-kB inhibition (S3I-201 and PS1145, respectively) on phenotype, cytotoxicity and cytolytic capacity of NK cells was also analysed (**Figure 19** and **Figure 20**).

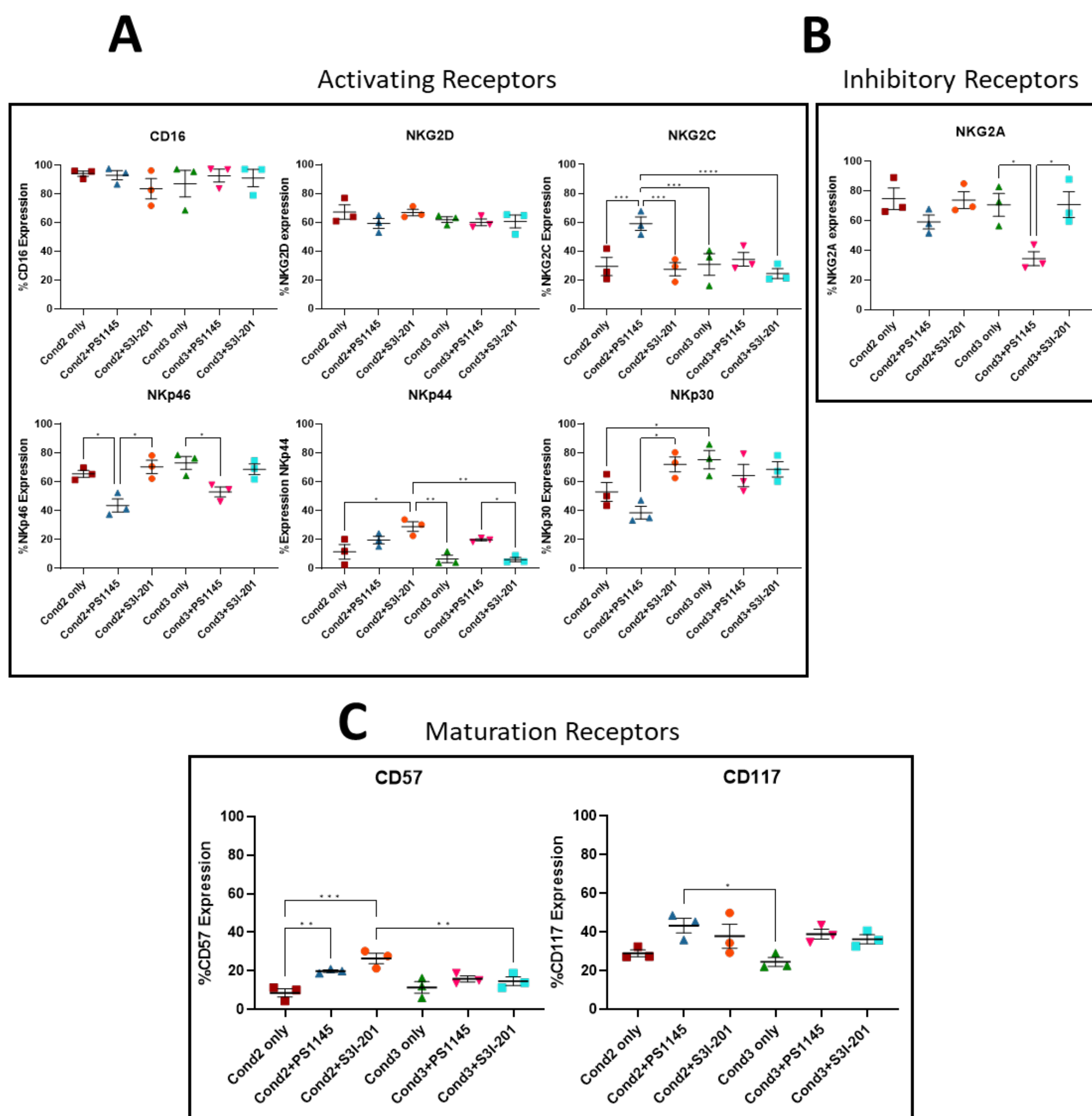


Figure 19. Effect of STAT3 and NF-kB inhibition on the phenotype of NK cells

A. Activating, **B**. inhibitory, **C**. maturation receptors expressed at day 10 of *in vitro* culture by cytokine-activated NK cells, cultured in the presence of STAT3 and NF-kB inhibitor during the 16hr priming stage. Data shown as mean of 3 different donors ± SEM. Data analysed by RM one-way ANOVA, and Tukey multiple comparison correction. (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001).

Inhibition of either transcription factor did not change CD16 and NKG2D expression, both with condition 2 and condition 3 (**Figure 19A**). Interestingly, NKG2C expression increased when cells were cultured with condition 2 in the presence of NF-kB inhibitor PS1145; no change was seen with condition 3. STAT3 inhibitor, S3I-201, did not change expression of NKG2C when cells were cultured with condition 2 or condition 3. NKp46 expression decreased in the presence of PS1145, with both conditions, whereas it was unchanged in the presence of S3I-201. NKp44 expression follows an opposite pattern; its expression was unchanged in the presence of PS1145. Addition of S3I-201, induces an increased expression of NKp44 with condition 2, however no change was seen with condition 3. Condition 3, though, causes a lower expression of NKp44 in the presence of S3I-201 than with PS1145. NKp30 expression with condition 3 is unaffected in the presence of both inhibitors. Conversely, with condition 2, NF-kB inhibition causes a lower expression than STAT3 inhibition (**Figure 19A)**. The inhibitory receptor NKG2A expression did not change with condition 2 in the presence of either PS1145 or S3I-201. However, addition of PS1145 with condition 3 caused a decrease in expression (**Figure 19B**).

Analysis of maturation markers CD57 and CD117 is consistent with our previous findings when NK cells are cultured with condition 2 or condition 3 alone (**Figure 19C**). CD57 expression increased when cells were cultured with both inhibitors, with condition 2. Its expression was unchanged with condition 3 and either inhibitor. Inhibition of either STAT3 or NK-kB did not change CD117 expression, both with condition 2 and condition 3 (**Figure 19C**).

To better understand how IL-21 and IL-18 affect NK cell functions and the interplay between cytokines, the expression of CD107a (a proxy used to determine degranulation and hence cytotoxic potential of NK cell (241)) and IFN-γ was evaluated. As described before, purified NK cells were cultured *ex vivo* with either condition 2 or 3 in the presence of either S3I-201 or PS1145 during the 16 hours priming. On day 10 of culture, NK cells were incubated with HCC cell lines HEPG2, PLC and SNU475. When co-cultured with HepG2, CD107a expression decreased in the presence of PS1145 and condition 2; however, inhibition of either STAT3 or

NF-kB does not affect CD107a expression when cells are treated with condition 3 (**Figure 20A**). The opposite is seen for IFN-γ production with both conditions (**Figure 20B**).

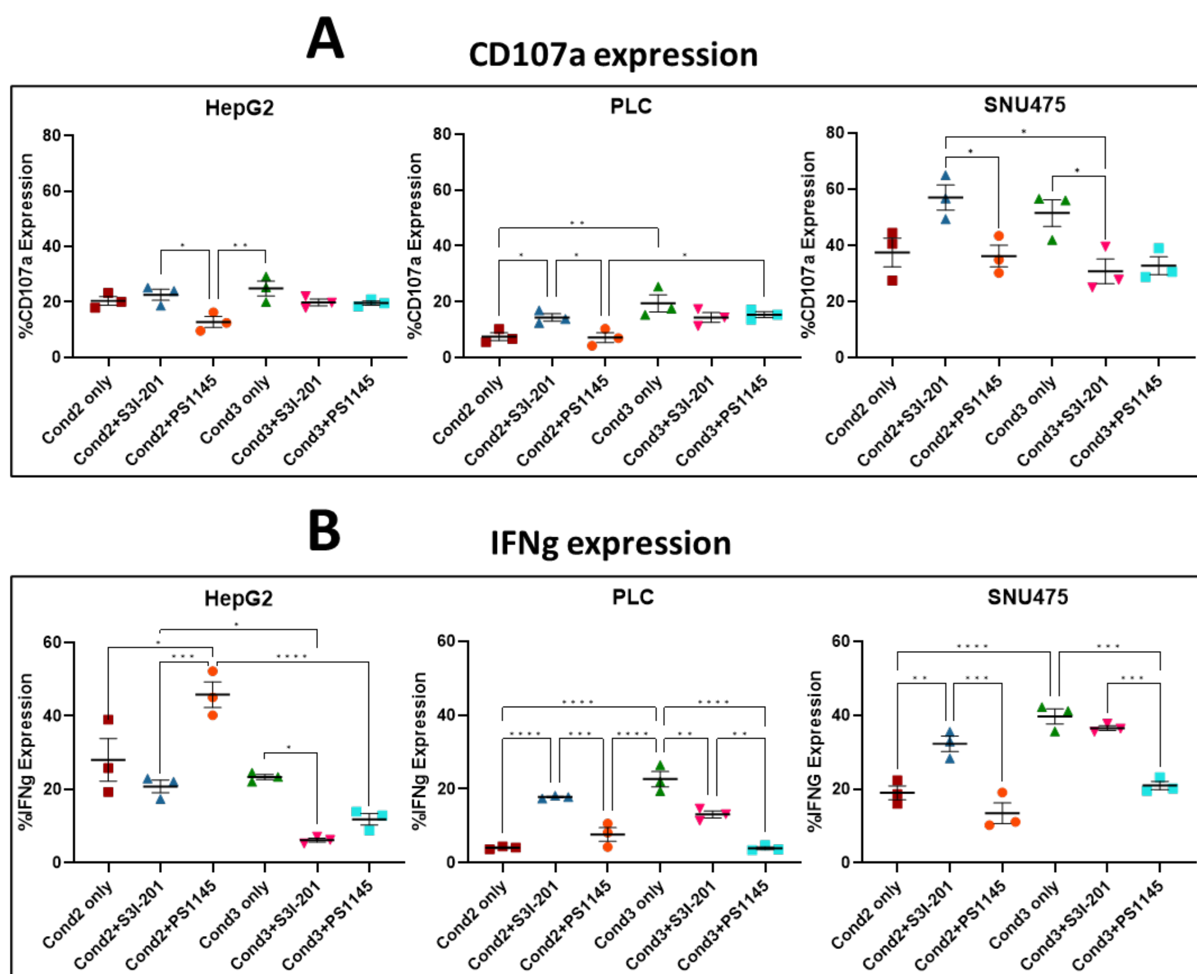


Figure 20. Effect of STAT3 and NF-kB inhibitors on NK cells activation against HCC cell lines

Purified NK cells were cultured with condition 2 (no IL-21 during the priming stage) or condition 3 (with IL-21 during the priming stage), treated either with 75µM S3I-201 (STAT3 inhibitor) or 10µM PS1145 (NF-kB inhibitor), cells were then washed and cultured only with IL-2. Data shown as mean ± SEM, n=3, analysed by one-way ANOVA, and Tukey multiple comparison correction (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001). **A.** Degranulation (CD107a expression) and **B.** Interferon-gamma (IFN-γ) expression by cytokine activated NK cells, on day10 of *in vitro* culture. NK cells were co-cultured with the HCC cell lines HepG2, PLC and SNU475 for 4hrs (E:T 1:1).

Inhibition of STAT3, caused an increase in CD107a expression by NK cells co-cultured with PLC and treated with condition 2; the opposite is seen with NF-kB inhibitor. No change is seen with either inhibitor for CD107a expression, when cells are treated with condition 3 in the presence of either inhibitor, compared to condition 3 only. However, condition 3 only causes a significantly higher degranulation of NK cells compared to condition 2 only (**Figure 20A)**. This is also true for INF-γ production. Its release is, however, affected with both inhibitors, which cause a lower INF-γ production compared to condition 3 only (**Figure 20B**).

Stimulation with the cell line SNU475, causes the highest degranulation overall with both conditions, compared to the other cell lines (**Figure 20A**). With condition 2, PS1145 treatment causes a lower degranulation than S3I-201 treatment, whereas with condition 3, S3I-201 treatment caused a lower CD107a expression than condition 3 only. Interestingly, STAT3 inhibition, caused a significantly lower CD107a expression with condition 3 than with condition 2, indicating that IL-21 mediates its action via STAT3 signalling. IL-21 caused a significantly higher IFN-γ production (condition 3 only vs condition 2 only, **Figure 20B**). Inhibition of NF-kB caused a lower IFN-γ production despite IL-21 presence, whereas STAT3 inhibition only caused an increase in IFN- γ when IL-21 was not present (**Figure 20B**).

Interpretation of these *ex vivo* data is complicated by the complex signalling pathways involved, which are not only affected by the cytokines and/or inhibitors added to culture system, but also by the endogenous expression of receptors on NK cells and ligands on tumour cells (**Table 6**).

4.5 Effects of different regiments on NK cell receptor expression and cytotoxicity against HCC cell lines

To determine how the different cytokine combinations (**Figure 17**) affected NK cell anti-tumour function, we studied their degranulation capacity (CD107a) and interferon gamma (IFN-γ) production using NK cells cultured under the different protocols. Of the twelve protocols investigated, the focus was directed to only five regimens: condition 2, condition 3, condition 9, condition 10 and condition 12. The rationale is that these conditions generated the greatest increase in proliferation, with condition 3 being the highest (**Figure 17B**); condition 2 replicates the cytokine combination used to induce memory-like NK cells.

After 10 days of cytokine stimulation, NK cells were tested in co-culture assays with three different hepatocellular carcinoma cell lines (HepG2, PLC and SNU475). As observed before (**Figure 14**), the introduction of IL-21 in the priming phase did not have a negative effect on CD107a or IFN-γ secretion. NK effector function was most notable for condition 12, which gave the strongest results for both CD107a and IFN-γ. This regimen contains IL-21+IL-15 in the priming phase and IL-18+IL-2 in the three days post priming, and strong cytotoxicity (CD107a) was seen especially against HepG2 and SNU475, but strong IFN-γ against all the cell lines tested.

To correlate the effector functions with cytokine conditions we studied receptor expression. Overall, there were no simple correlations of receptor expression with cytokine condition. In particular, expression of the inhibitory receptor NKG2A (**Figure 21C(ii)**) was high for all regimens tested and of the activating receptors CD16, NKG2D, NKG2C and NKp46 were similar amongst the different regimens (**Figure 21C(i)**). In line with what we observed earlier (**Figure 11**), there was a trend towards lower NKp44 and NKp30 in the conditions associated with IL-12+15+18 priming, independent of IL-21 inclusion (condition 2 and condition 3, **Figure 21C(i)**). Low expression of NKp44 expression in unstimulated NK cells aligns with other studies, suggesting that its expression is not constitutive, but it is upregulated upon cytokine stimulation (273,274).

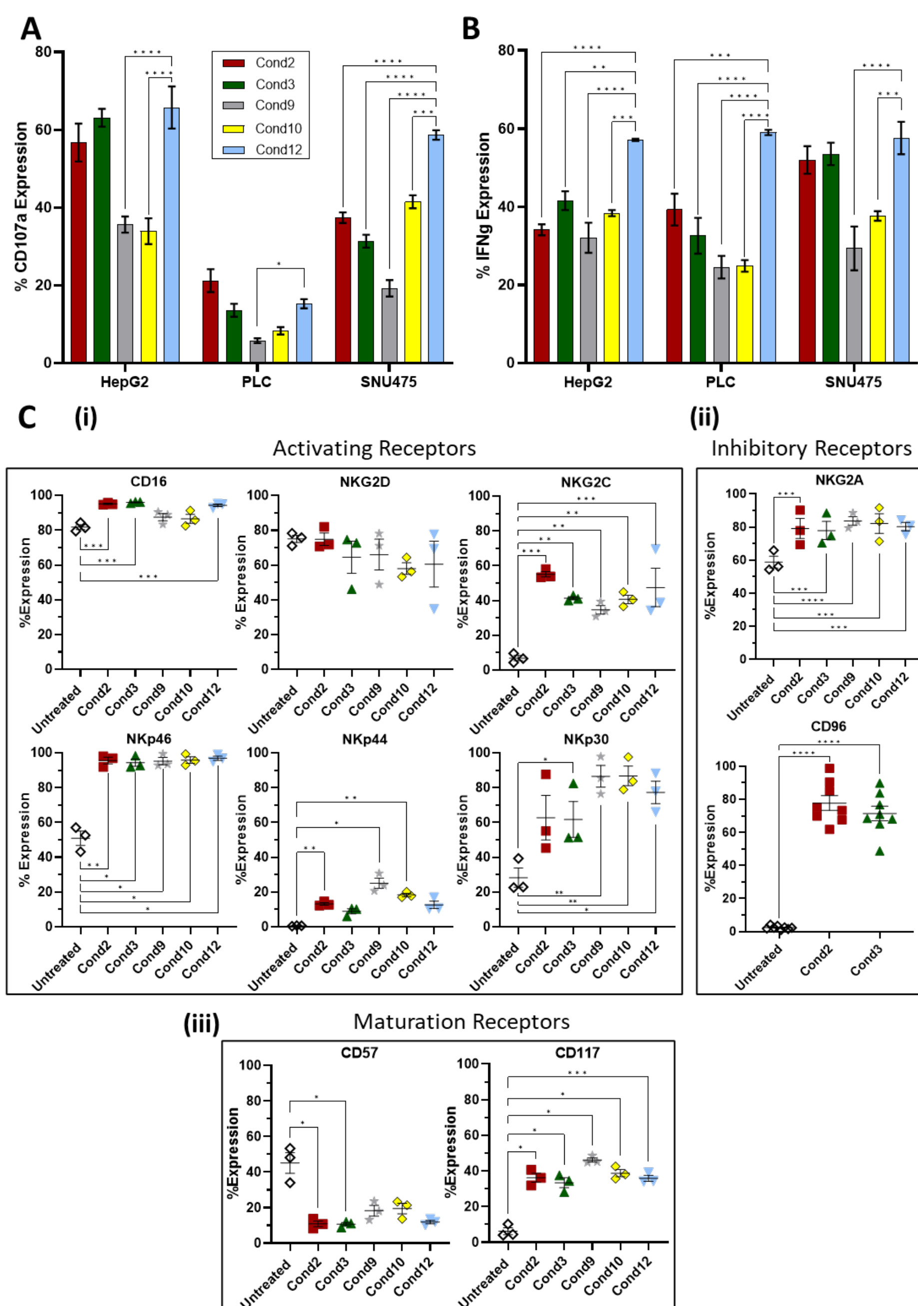


Figure 21. Effects of different regiments on NK cell receptor expression and cytotoxicity against liver cancer cell lines as a model for immunotherapy.

Isolated NK cells were assessed by flowcytometry at baseline level (untreated) or after treatment as at day 10, using flow cytometry. Data shown as mean± SEM (n=3). **A.** Degranulation (CD107a) of NK cells incubated for 4hrs with hepatocellular carcinoma cell lines (E:T 1:1). Data analysed by two-way ANOVA, and Dunnett's multiple comparison test (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001). **B.** Interferon-gamma (IFN-γ) released from NK cells incubated for 4hrs with hepatocellular carcinoma cell lines. Data analysed by two-way ANOVA, and Dunnett's multiple comparison test (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001). **C.** Expression of several phenotypical markers by NK cells stimulated by cytokines for 10 days**. C i.** Shows activating receptors; **C ii**. shows inhibitory receptors; **C iii**. Shows markers of maturation. Data shown as mean± SEM (n=3), analysed by RM one-way ANOVA, and Dunnett's multiple comparison test (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001).

The high expression of CD16 seen in the cytokine-expanded NK cells (**Figure 11** and **Figure 21**), prompted the analysis of their ADCC capacity against the HCC cell lined HepG2 and PLC. NK cells can elicit anti-tumour functions through CD16 during anti-EGFR (cetuximab) treatment (275). The selected cell lines show different EGFR expression, with PLC cells expressing EGFR at high levels while EGFR is hardly detectable in HepG2 cells (276). Surprisingly, with none of the condition tested (condition 1, condition 2, condition 3 and condition 12) there was no increased cell cytotoxicity or cytolytic activity as pre-treatment of the HCC cell lines did not cause an increase in the levels of CD107a, IFN-γ and TNF-α. This was observed with both cell lines tested, HepG2 and PLC (**Figure 22**).

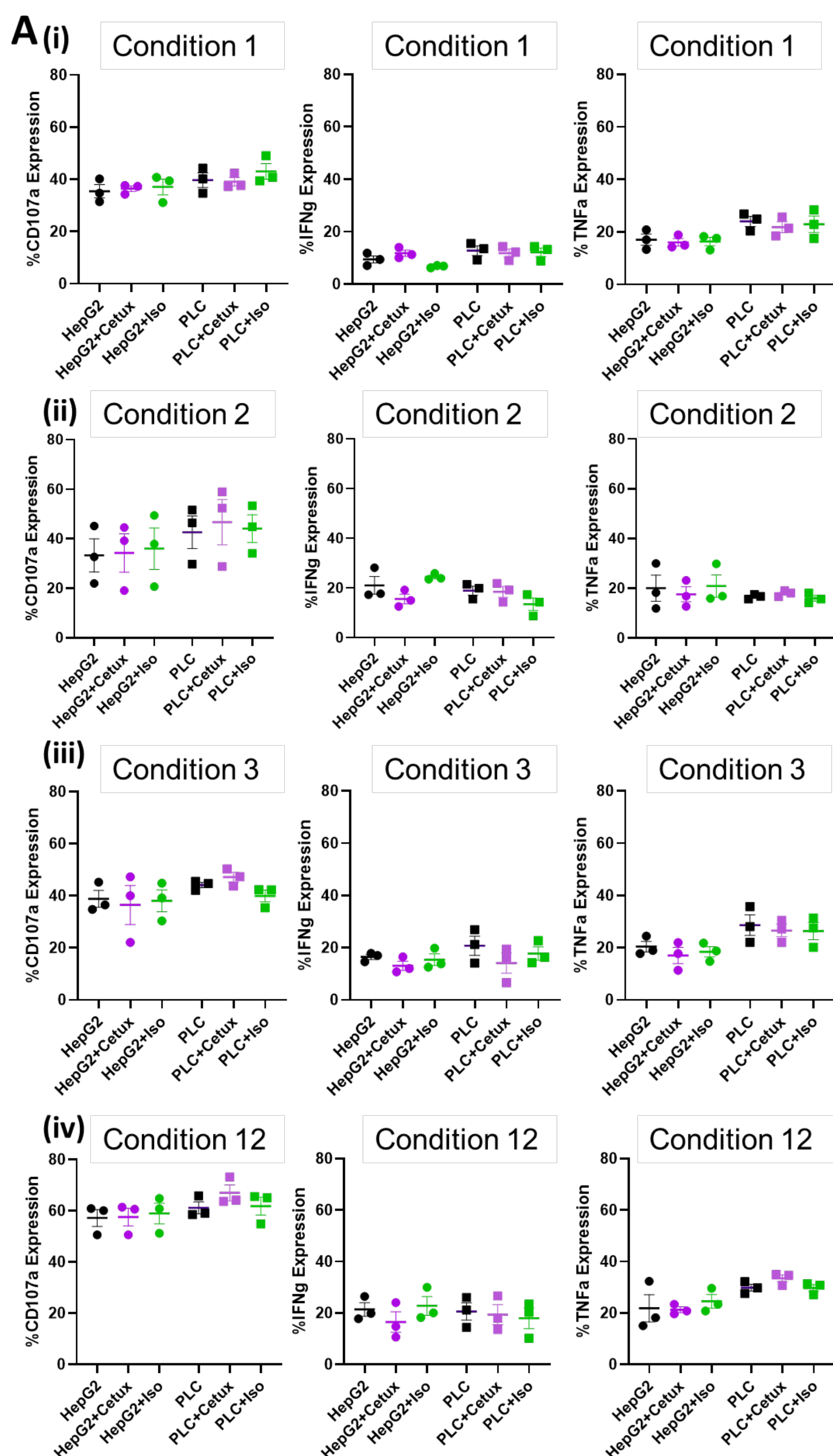


Figure 22. Cytokine-activated expanded NK cells do not show ADCC against HCC cell lines

Cytokine-activated NK cells were expanded for 10 days and co-cultured with cell lined HepG2 or PLC for 4 hrs (E:T 1:1). 20 minutes prior to co-culture with NK cells, tumour cells were incubated with the antibody cetuximab or isotype control (10µg/ml). ADCC was measured as expression of CD107a, IFN-γ, TNF-α. NK cell were cultured with condition 1: IL-2 only **(i),** condition 2: IL-12+15+18 for 16hrs, then IL-2 only **(ii),** condition 3: IL-12+15+18+21 for 16hrs, then IL-2 only **(iii),** condition 12: IL-21+15 for 16hrs, then IL-18+2 for 3 days and finally IL-2 only **(iv).**

4.6 Proliferation and maturation of different NK cells subpopulations

Given that various cytokine combinations modulate NK cell expansion differently, we analysed both the proliferative activity of NK cell, recorded as number divisions, and the maturation stage of the NK population (**Figure 23**). Focus was put only on condition 1, condition 2, condition 3 and condition 12. This is because condition 3 gave the highest proliferation (**Figure 17**); condition 2 was included to evaluate how the memory-like inducing cytokine combination compares to the other regimens; condition 12 was added because it induced the highest NK cell activation (**Figure 21**), and lastly, condition 1 was included to study NK cells with baseline cytokine conditions.

Proliferation peak was seen at day 7 for IL-21 containing regimes (condition 3 and 12). For these conditions, the proportions of NK cells that divided 7 times (population 7), or more, was greater than that seen at day 5 and 9, as shown by (**Figure 23B**). Conversely, condition 1 and 2 (which did not have IL-21) cells followed a slower and progressive proliferation, where the proportion of NK cells that underwent 7 or more cell divisions increased from day 7 to day 9. Condition 12, which had IL-21 and IL-15 in the priming step, as opposed to IL-12/15/18 plus IL-21, caused the greatest loss of the proportion of NK cells that had divided 7 or more times, however, there was an increase in the other subpopulations, specifically cells with at least 5 divisions, suggesting that IL-21 might induce a rapid proliferation.

After assessing their proliferation activity, cytokine-activated NK cells were further analysed for the expression of the maturation markers CD117 (immature), CD16 (mature) and CD57 (terminally differentiated) (**Figure 23D**). At early time points of culture (day 5, **Figure 23D**) NK cell maturation is heterogeneous and appears to be undergoing a transition. The most prevalent subpopulations are CD16+CD117+CD57- and CD16-CD117+CD57-, which are differentially affected by the different culture conditions, highlighting NK cell maturation as they acquire CD16. By day 7, the predominant NK cell subpopulation is CD16+CD117+CD57- (**Figure 23D**), suggesting that NK cell maturation is not only influenced by the cytokine combination but also by the exposure time to that specific combination. At day 9, the

maturation of NK cells changed once again. CD16+CD117+CD57- and CD16-CD117+CD57- are the most abundant across all the cytokine combinations, however, condition 3 and 12 also induced proliferation of CD16+CD117-CD57- subpopulation, partially seen also on day 5 and 7 (**Figure 23D**). This suggests that IL-21 might induce a more mature NK phenotype.

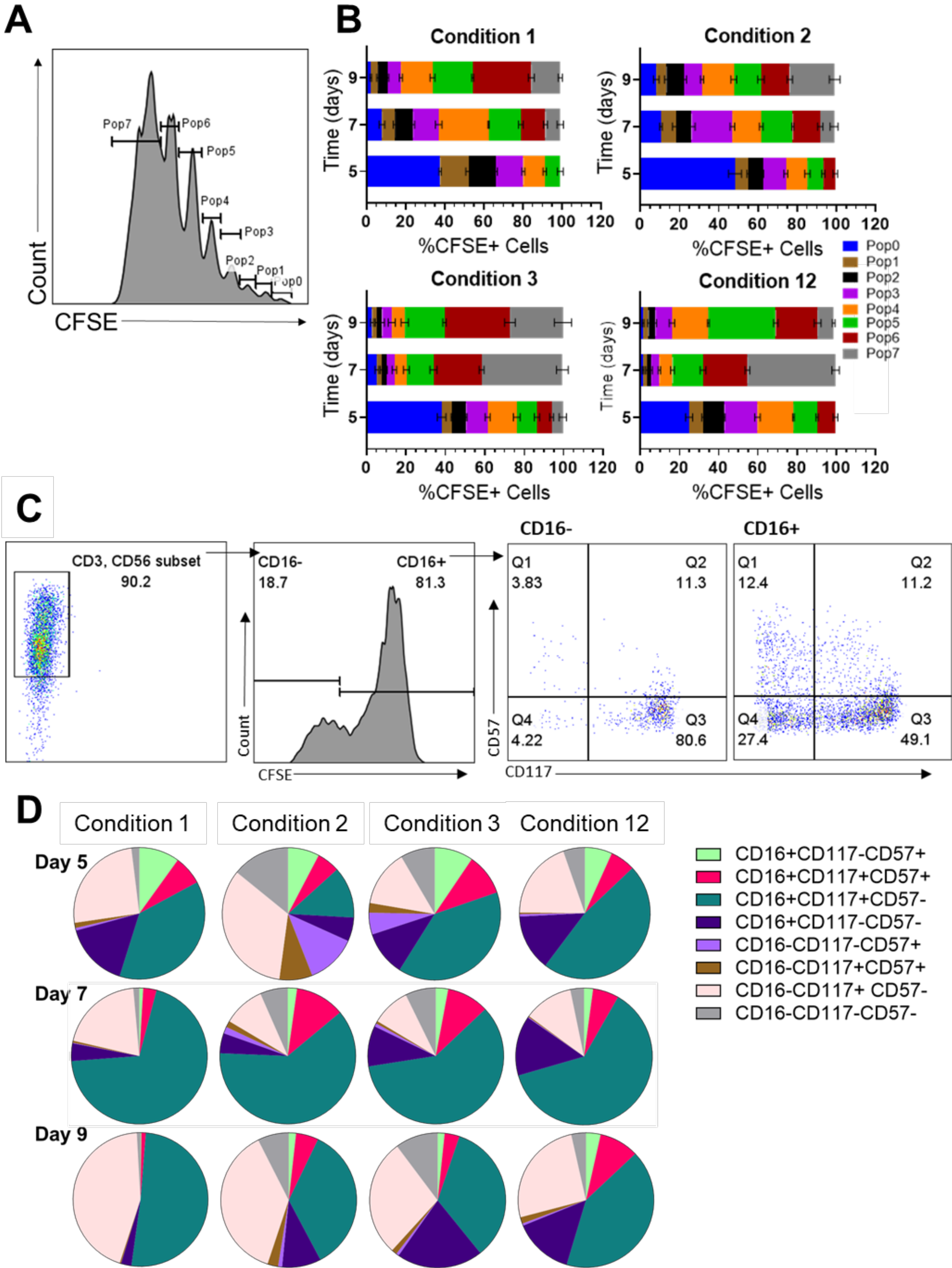


Figure 23. Proliferation and maturation of NK cell subpopulations.

Isolated NK cells were stained with CellTrace CFSE, cultured with condition 1, condition 2, condition 3, condition 12 and assessed by flowcytometry at day 5, 7 and 9 of *in vitro* culture. **A.** Representative example of gating strategy. NK cells were gated on each cell division, according to their CFSE percentage. **B.** Proliferation of cells, based on their CFSE percentage. Each division is marked by a difference in CFSE retention by the cells, marking the different populations (pop0 to pop7) within the whole heterogenous cell pool. Data shown as mean± SEM (n=4). **C.** Representative example of gating strategy to determine NK cell maturation. Whin the whole NK cell population, CD16+ and CD16- expression was determined, and in each subgroup the maturation of NK cells was established by plotting the expression of CD57 vs CD117. **D.** Maturation of NK cells, according to the expression of CD16±, CD57±, and CD117± at day 5, 7 and 9. Data shown as mean ± SEM (n=4). **C. D.** Values are expressed as a percentage of either CD16- or CD16+.

4.7 Metabolism analysis of cytokine-activated NK cells

These experiments were carried out together with Genevieve Rogers (Ref: PhD thesis under preparation, Genevieve Rogers, University of Southampton, 2023) as this was a collaborative work. In particular, after setting up the cell culture systems together, Genevieve Rogers carried NMR analysis of cytokine-activated NK cells' supernatant and I carried out the phenotype, functional assays, and data analysis.

4.7.1 Characterization of expansion, phenotype, and effector function of cytokine-activated NK cells

To confirm the results observed previously regarding expansion and effector functions, NK cells were isolated from different healthy donors and cultured with the following conditions:

- **Condition 1**: IL-2 (500 U/mL).
- **Condition 2**: IL-12 (10 ng/mL) + IL-15 (20 ng/mL) + IL-18 (50 ng/mL) for 16 hours, then IL-2 (500 U/ml) only for the remaining culture period.
- **Condition 3**: IL-12 (10 ng/mL) + IL-15 (20 ng/mL) + IL-18 (50 ng/mL) + IL-21 (25 ng/mL) for 16 hours, then IL-2 (500 U/ml) only for the remaining culture period.
- **Condition 12**: IL-15 (20 ng/mL) + IL-21 (25 ng/mL) for 16 hours, then IL-2 (500 U/mL) + IL-18 (50 ng/mL) for 3 days, and finally IL-2 (500 U/mL) only for the remaining culture period.
- **Condition 13**: IL-15 (10 ng/mL).

As seen before (**Figure 7**), condition 1 elicited a high cell expansion which became more prominent in later stages of the *in vitro* culture, at day 13 and 16, reaching an average of 50-fold and 250-fold expansion, respectively (**Figure 24**). Condition 2, which mimicked the cytokine cocktail needed for memory-like NK cells generation (35), resulted in the lowest expansion of NK cells, with a mean of just over 50-fold expansion at the end of the 16-day culture period (**Figure 24**). Hence, according with previous data (**Figure 24**, **Figure 17**), the memory-like inducing cytokine combination, causes a slow proliferation of NK cells. Interestingly, however, the addition of IL-21 to the memory-like inducing cytokine cocktail, caused an increase in NK cell expansion (condition 3, **Figure 24**), seen from day 7 of culture.

At the end of the culture period, at day 16, condition 3 yielded an average of 150-fold expansion, which was significantly higher than condition 2 (**Figure 24**). Condition 12, the other IL-21-based regimen, also induced a very rapid and high NK cell expansion, beginning as early as day 4 and continuing throughout the 16-day culture period, yielding an average of 250-fold expansion (**Figure 24**). As IL-15 is known to support NK cell expansion (10,77,84,237,277), condition 13 was added to the investigation. High dose IL-15 alone, causes a substantial fold expansion particularly noticeable from 10 day of *in vitro* culture, with a mean of 20-fold expansion. The expansion is sustained till day 16, reaching an average of 200-fold expansion (**Figure 24**).

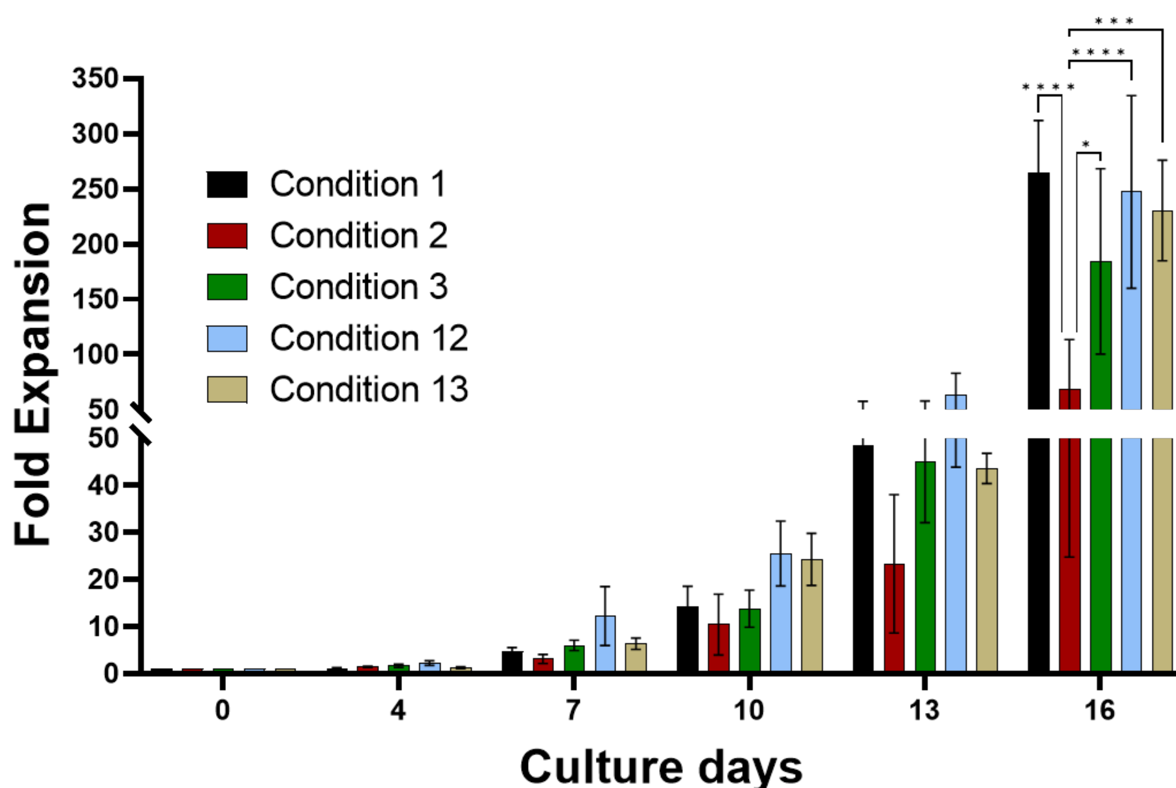


Figure 24. Proliferation of cytokine-activated NK cells

Peripheral blood derived NK cells are expended *in vitro* in the presence of different cytokines combinations. Each culture condition represents the average of 5 different donors (± SEM). Data shown as mean ± SEM (n=5), analysed by Two-Way ANOVA, and Tukey's correction multiple comparison test. (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001).

In accordance with earlier findings, IL-21 drives NK cell expansion (**Figure 7**, **Figure 17**, (77,84)), solidifying the potential of this cytokine for clinical applications aimed at achieving high yields of NK cells. However, IL-2 or IL-15 alone (condition 1 and 13 respectively, **Figure 24**) might appear to induce higher or equal fold expansion to IL-21-based regimens. A close examination, though, reveals a marked difference in NK cell maturation (**Figure 25**), showing that IL-21 not only promotes cell expansion but also plays a crucial role in the maturation and development of NK cells.

Analysis of the combined expression of the maturation markers CD16, CD57, CD117, and NKG2A, revealed that the most prominent subsets were CD16+CD57-CD117+NKG2A+, CD16+CD57-CD117-NKG2A+, and CD16-CD57-CD117+NKG2A+ with all culture conditions analysed (**Figure 25A**). A closer comparison, however, indicated that the proportions of these NK cells subpopulations were influenced by the culture condition adopted. Although not statistically significant, CD16+CD57-CD117+NKG2A+ expression was lowest with condition 3, with a mean of 22% expression, followed by condition 12 with average of 30% expression. Condition 1, condition 2, and condition 13 showed a comparable average of 35% expression (**Figure 25B**). Condition 3 induced the highest proportion of CD16+CD57-CD117-NKG2A+ NK cell, reaching an average of 35% of the total population. Conversely, condition 3 yielded the lowest proportion of CD16-CD57-CD117+NKG2A+ NK cells, which accounted for only 2% of the total population (**Figure 25B**). Condition 12, the other IL-21-containing regimen, showed an average expression of CD16-CD57-CD117+NKG2A+ of 13% of the total NK cell population. This was still lower than condition 1, condition 2 and condition 13 which yielded an average of 23%, 18% and 25% respectively (**Figure 25B**). Noteworthy is the expression of NKG2A, which was present in all the major NK cells subsets, regardless of the expression of the other maturation markers or culture condition used. This observations indicates a high expression of NKG2A in cytokine-stimulated NK cells, in line with other findings (240).

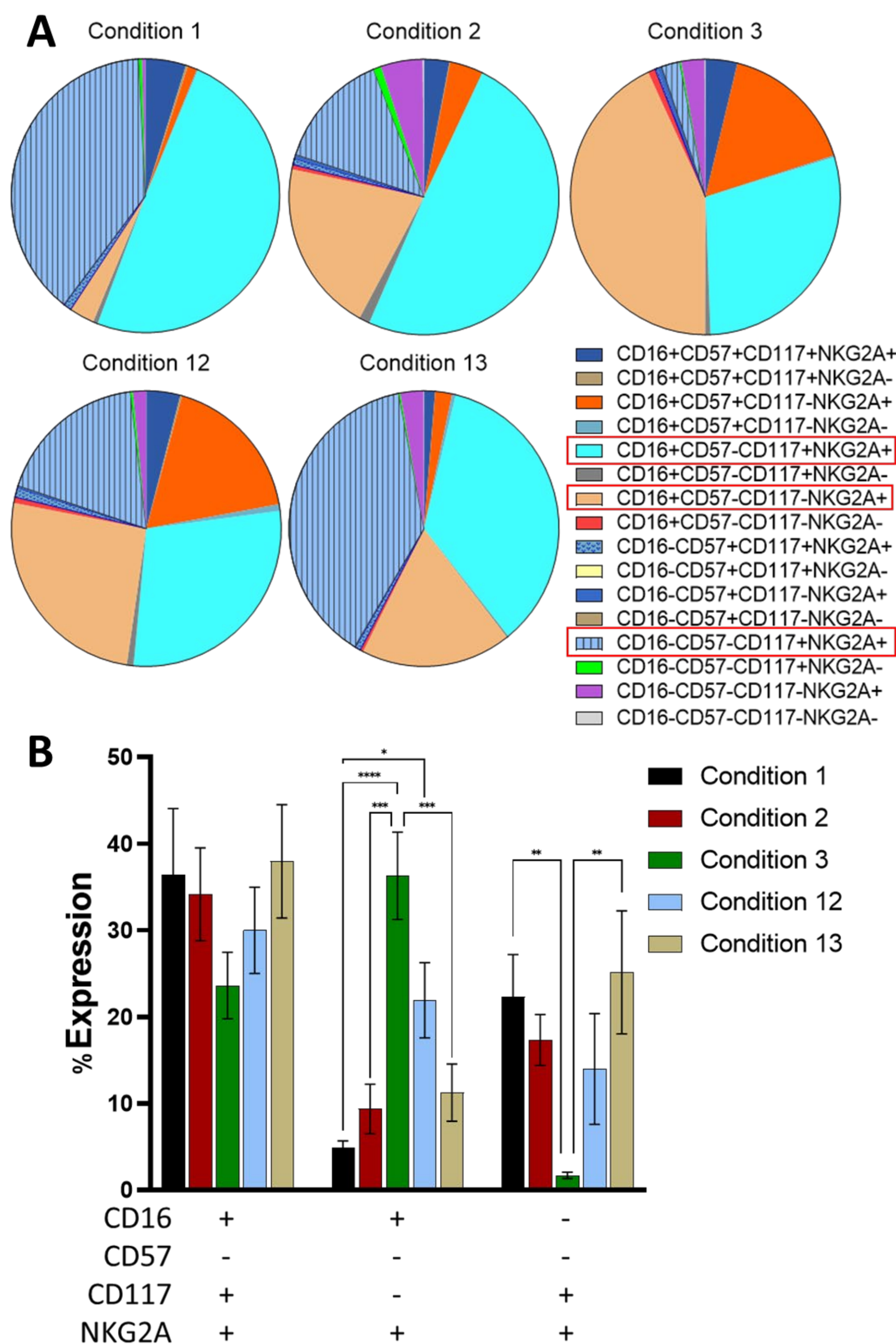


Figure 25. IL-21 favours the maturation of healthy NK cells.

Analysis of cytokine-activated NK cells at day 10 of *in vitro* culture. **A.** Maturation of NK cells with the different culture conditions. Cells were gated as CD56+CD3- and then the "*Boolean gates* and then the *Combination Gates"* tool from FlowJo was applied to explore the whole NK population, based on the expression of CD16, CD57, CD117, and NKG2A. **B**. Analysis of the most prominent NK cells subpopulations obtained from **A**. Data shown as mean ± SEM (n=5), analysed by Two-Way ANOVA, and Tukey's correction multiple comparison test. (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001).

Further characterization of cytokine-activated NK cells was done by studying the expression of activating and inhibitory receptors. All culture conditions induced over 90% expression of the activating receptor NKG2D (**Figure 26A**). NKp30 expression was slightly reduced when NK cells were cultured with condition 2 and 3 at day 10 and, although not statistically significant, this trend was maintained till day 16. This was also observed by NKp46 expression (**Figure 26A**). NKp44 expression (**Figure 26A**) was significantly reduced, both at day 10 and day 16, by exposing NK cells to condition 2, 3. In accordance with other studies (63), IL-21 from condition 3 and condition 12 induced a lower expression of NKp44. Unstimulated NK cells showed low levels of NKp44 which aligns with other research. This suggests that NKp44 is not constitutively expressed, but its expression is upregulated upon cytokine stimulation (273,274). All the NCR receptors (NKp30, NKp46, NKp44) are greatly upregulated upon cytokine stimulation (**Figure 26A**). IL-21 in condition 3 induced an increase in NKG2C expression, compared to the other condition, reaching an average of 45% by day 10. At day 16, all culture condition induced a similar average expression of 50% (**Figure 26A**).

The goal of this research project was to understand the behaviour of cytokine-activated NK cells in the context of the liver cancer, hepatocellular carcinoma. To this end, we probed cells for the expression of the “liver-homing” chemokine CXCR6, which binds to the ligand CXCL16 only expressed by hepatic sinusoidal endothelial cells (278,279). While only a small fraction of peripheral blood NK cells expresses CXCR6 (280–282), exposure to the cytokines IL-2 and IL-15 increased its expression to an average of 30% and 15-20%, respectively, of the total NK cell population compared to untreated NK cells at day 0 (**Figure 26C**). Condition 2 induced a modest increase in CXCR6 expression, with an average of 5% of the total NK population compared to untreated cells. Conversely, condition 3 induced a more substantial expression of CXCR6, resulting in an average of 20% at day 10 of in vitro culture. However, this expression decreased to slightly above 1% of the total NK cell population by day 16. Lastly, condition 12, induced a stable expression of CXCR6, of 10%, which was maintained till the 16-day culture period (**Figure 26C**).

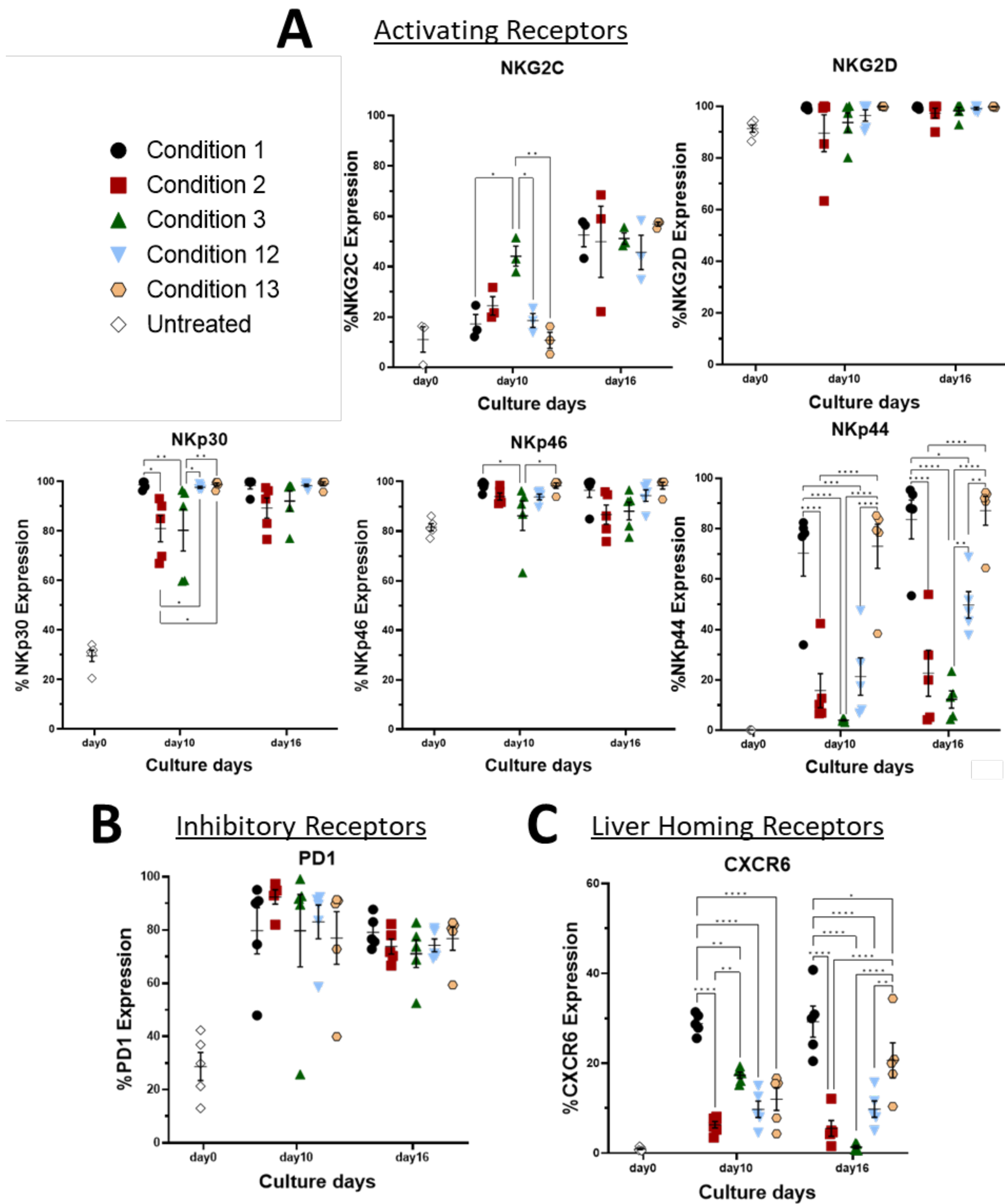


Figure 26. Phenotype of cytokine activated NK cells over the 16-day culture period

A. Activating **B.** Inhibitory and **C.** "Liver homing" receptors expressed by cytokine-stimulated NK cells, at day 0 (untreated), day 10 and day 16 of *in vitro* culture. Data shown as mean of 5 different donors ± SEM. NKG2C data shown as a mean of 3 donors ± SEM. Data was analysed by Two-Way ANOVA, and Tukey's correction multiple comparison test (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001).

An important inhibitory immune checkpoint receptor is the programmed cell death protein 1 (PD-1). Although healthy peripheral blood NK cells only express minimal levels of PD-1, its expression is induced by glucocorticoids in combination with the cytokines IL-12, IL-15, and IL-18 (283). However, the mechanisms underlying the de novo expression of PD-1 on NK cells and what induces PD-1 upregulation are not fully understood. Therefore, PD-1 was analysed when cells were cultured with condition 1, 2, 3, 12, and 13 (**Figure 26B**). Cytokine stimulation induced an increase in PD-1 expression in all conditions analysed, reaching an average of 90% after 10 days of *in vitro* culture. By the end of the 16 days culture period PD-1 expression decreased to an average of 80%, consistent across all conditions (**Figure 26B**). This suggest that in vitro culture of peripheral blood NK cells stimulated with cytokines upregulates PD-1 expression, however, this is not determined by specific cytokines.

Cytokine stimulation not only modulates NK cell expansion and receptor expression but governs NK cells effector functions. To assess their activation, cytokine activated NK cells were co-cultured with four different hepatocellular carcinoma cell lines, HepG2, PLC, Huh7, and Huh7-CO102 (**Figure 27**). In accordance with what observed before (**Figure 21A** and **B**), condition 12 induced significantly higher CD107a expression than the other conditions against both HepG2 (average 55%) and PLC (average 65%) cell lines. Similarly, condition 12 induced the significantly highest CD107a expression when NK cells were co-cultured with cell lines Huh7 and Huh7-CO102 (**Figure 27A**). While still capable of activating NK cells against hepatocellular carcinoma cell lines, all other conditions exhibited similar levels of activation, averaging at 40% for HepG2 and PLC and 15% for Huh7 and Huh7-CO102 cell lines (**Figure 27A**). Condition 1 induced the lowest expression of IFN-γ during co-culture of NK cells with HepG2, whereas the other culture conditions showed a comparable level of 20% average expression. Similar results were observed with the cell line PLC, although findings did not reach statistical significance. Condition 13 promoted the highest expression of IFN-γ (18% mean) upon co-culture of NK cells with Huh7 cells, in contrast the other culture conditions showed a comparable level of 5% average expression. Co-culture of NK cells with Huh7-CO102 induced an average of 5% IFN-γ expression, regardless of culture conditions (**Figure 27B**). TNF-α expression was consistent across all culture conditions, with an average 20% expression, when NK cells were co-cultured with HepG2 cell line. Condition 3 and 12 exhibited a trend to

induce higher TNF-α expression (average 30%) in NK cells co-cultured with PLC, while condition 2 induced the lowest expression of 18%. Co-culture of NK cell with the cell lines Huh7 and Huh7-CO102 induced an average of below 10% TNF-α expression, regardless of culture conditions (**Figure 27C**).

Taken together these data show that although large donor variation, IL-21 consistently induced an early and rapid NK cell expansion, generating a population of mature NK cells. However, NK cells activation varies when using different donors' batches especially when tested against PLC cell line.

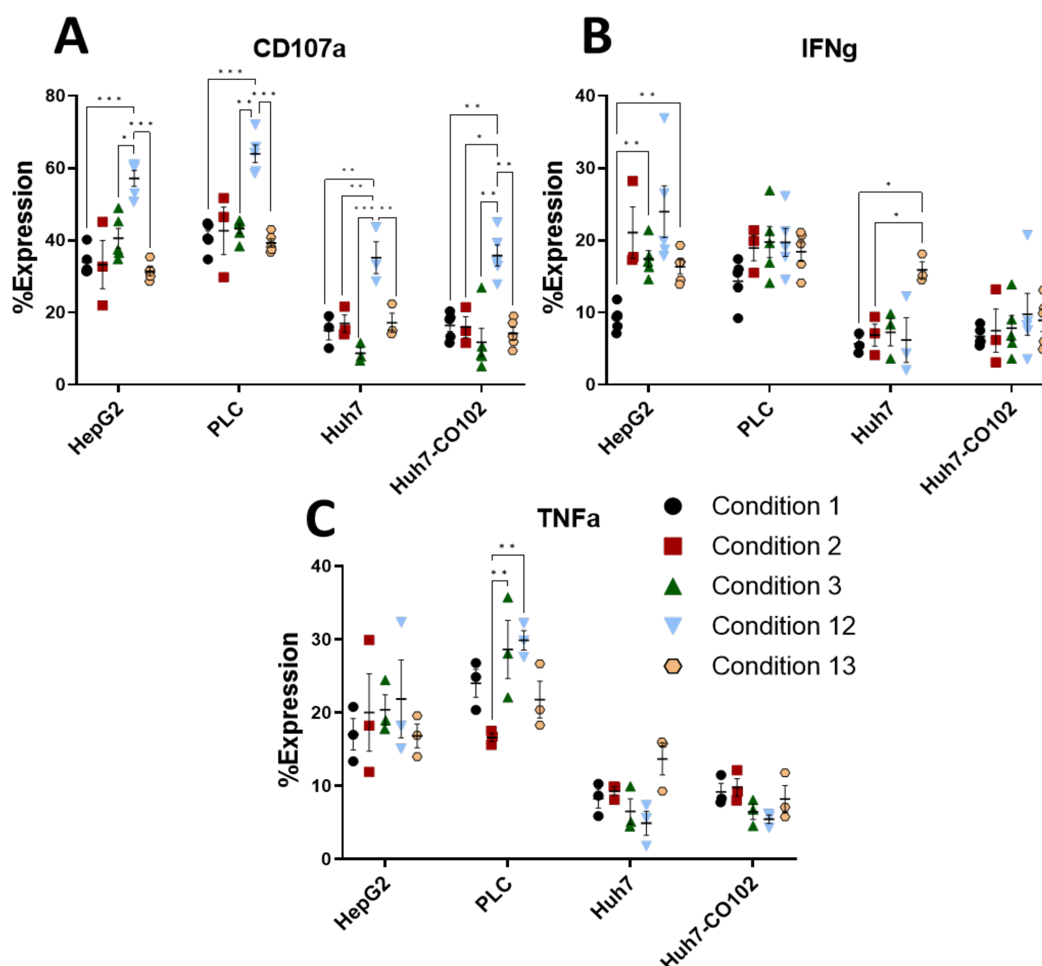


Figure 27. Effector functions against hepatocellular carcinoma cell lines of cytokine-activated NK cells at day 10 of *in vitro* culture

A. CD107a **B.** IFN-γ **C.** TNF-α. of NK cells after 10 days of cytokines stimulation, incubated with the HCC cell lines HepG2, PLC, Huh7, and Huh7-CO102. E:T ratio of 1:1, n=5. Huh7 for all effector function analysis, n=3. TNF-α expression was analysed with n=3. Data was analysed by Two-Way ANOVA, and Tukey's correction multiple comparison test (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001).

4.7.2 Quantitative analysis of cytokine-activated NK cells metabolism

To further evaluate cytokine-activated NK cell profile, their metabolism was analysed via nuclear magnetic resonance (NMR) spectroscopy. Out of the metabolites analysed, changes in lactic acid and glucose best represented the metabolism of cytokine activated NK cells (**Figure 28**). Glucose is used as the principal metabolic fuel, taken up from the media and utilised through glycolysis, the citrate-malate shuttle (CMS), the tricarboxylic acid (TCA) cycle and OxPhos to provide energy required for cells functions. Lactic acid is formed as a by-product of glycolysis, and therefore analysis of glucose and lactic acid changes might provide a view of NK cells metabolism (118,284). Condition 1 (**Figure 28B**) caused an increase in lactic acid production, with a peak at day 13, and decreasing by day 16. This is mirrored by glucose take up from the media, peaking at day 13 (condition 1, **Figure 28C**). The inconsistency seen at day 16 between lactic acid and glucose might be the result of lactic acid being converted to pyruvate and used in the TCA cycle. Condition 2 (**Figure 28B**) caused the greatest production of lactic acid, compared to the other cytokine conditions and this was more prominent at day 4 and 7 of culture. It caused a very rapid increase in lactic acid production, peaking at day 4 and being maintained till day 7. After day 7 there was a gradual decrease in lactic acid production till day 16. Glucose uptake increased throughout the culture time, peaking at day 13 (**Figure 28C**). Condition 3 causes the greatest variation both in lactic acid production and glucose uptake. It causes a very rapid increase in lactic acid production, seen as early as day 4, which slowly decreased till day 10 and rapidly increased at day 13 sharply decreasing by day 16 (condition 3, **Figure 28B**). Glucose uptake decreased after day 1, peaking at day 16 of culture (Condition 3, **Figure 28C**). Lactic acid production when NK cells were cultured with condition 12 increased after day 1 and was stable till day 13, after which it quickly dropped (Condition 12, **Figure 28B**). Glucose uptake decreased compared to day 1 and it was stable till day 13, when it showed a peak uptake (Condition 12, **Figure 28C**). Condition 13 (**Figure 28B**) caused lactic acid production to increase after day 1, with a peak at day 10. Glucose uptake, however, was the opposite, showing a decrease compared to day 1 and with a peak seen at day 13 (Condition 13, **Figure 28C**).

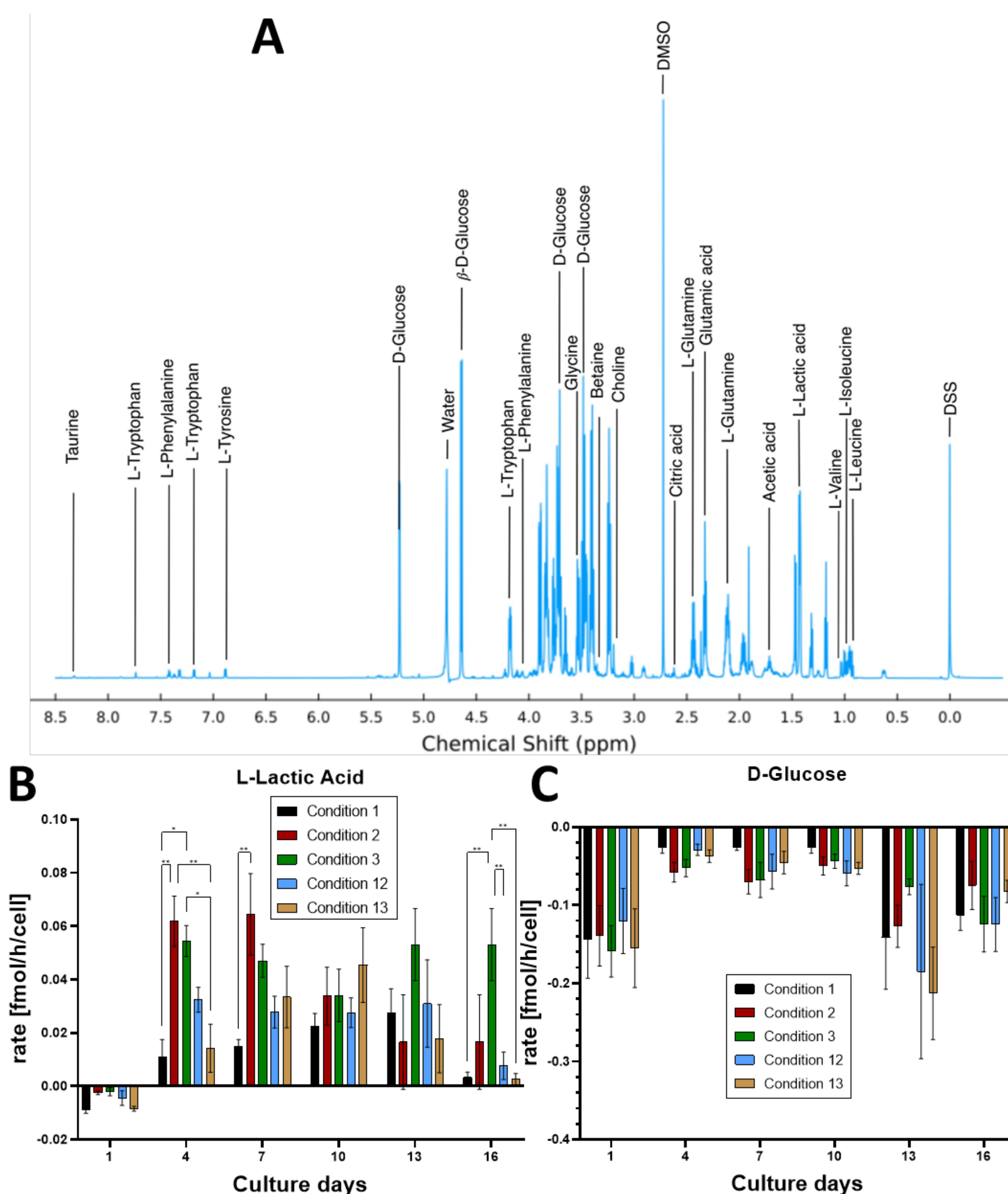
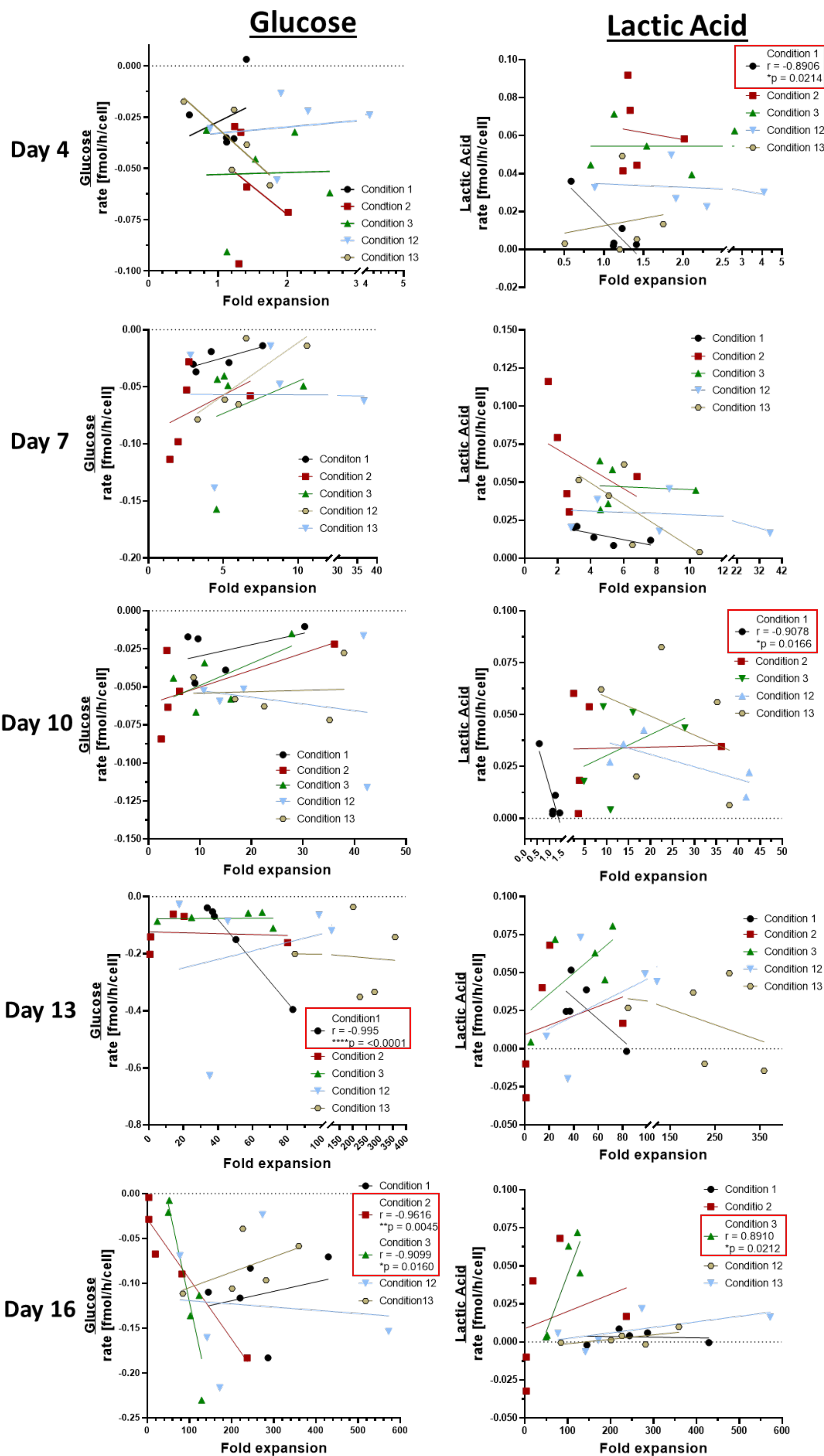


Figure 28. Metabolic changes in cytokine-stimulated NK cells

A. Typical proton NMR spectrum utilized for metabolic analysis obtained from NK culture media, indicating each metabolite present in the culture by reference to the Human Metabolome Database (285). Spectra were generated for all the NK culture samples and metabolites quantification was done by applying spectral decomposition to detect a change in signal, as shown in (221). **B.** L-Lactic acid produced by NK cells. **C.** D-Glucose taken up by NK cells. Metabolites in **B**. and **C**. were measured every three days for a total of 16 days. Data shown as average ± SEM, n=5. Data analysed with two-way ANOVA, with multiple comparison and Tukey's correction. (*P≤0.05, ***P*≤0.01)

Particular notice should be taken to day 1 of cell culture. This represents measurements of glucose uptake and lactic acid release immediately after 16 hours cytokine priming. There is a great glucose uptake in this phase, independently of culture condition, while no lactic acid was released (**Figure 28B** and **C, Appendix 2C** and **D**), suggesting high metabolism to meet cells' demand of increased energy. However, donors present high variation, independently of cytokine stimulation (**Appendix 2C** and **D**). Noteworthy is also the donor variation seen in lactic acid release and glucose uptake, independently of cytokine combinations used to stimulate NK cells (**Appendix 2A** and **B**), suggesting that not all NK cells are endowed with the same functions.

Taken together, these results how high metabolism because of long-term continuous cytokine stimulation. Condition 2 stimulation caused high lactic acid production during the initial phase of *in vitro* culture, whereas condition 3 caused high lactic acid production towards the end of *in vitro* culture. Within all conditions analysed, glucose uptake decreased after day 1, but dramatically increased again at day 13 and 16, which could indicate that it might be consumed differently by NK cell subpopulations.



Glucose
Lactic Acid
Day 4
Day 7
Day 10
Day 13
Day 16
Glucose rate [fmol/h/cell]
Lactic Acid rate [fmol/h/cell]
Fold expansion
Condition 1
r = -0.8906
*p = 0.0214
Condition 1
r = -0.9078
*p = 0.0166
Condition1
r = -0.995
****p = <0.0001
Condition 2
r = -0.9616
**p = 0.0045
Condition 3
r = -0.9099
*p = 0.0160
Condition 3
r = 0.8910
*p = 0.0212
Condition 2
Condition 3
Condition 12
Condition 13

Figure 29. Correlation analysis between metabolism and NK cell proliferation

Glucose and lactic acid expression was correlated with NK cell proliferation during the 16-day culture period. Data analysed with one-tail Pearson correlation, n=5.

A linear regression model was used to closely understand the relationship between metabolism and expansion of cytokine activated NK cells, throughout the 16-day culture period. A negative correlation was observed at day 4 (r= -0.8906, p=0.0214) and 10 (r= -0.9-78, p= -0.0166) of culture, between lactic acid production and expansion when NK cells were cultured with condition 1. condition 1 also induced a negative correlation between cell expansion and glucose uptake at day 13 (r= -0.995, p,0,0001), indicating that NK cells were actively proliferating (**Figure 29**), consistent with the observation that condition induced a later cell expansion (**Figure 24**). In contrast with previous days where no association was observed, condition 2 induced a negative correlation between glucose uptake and cell expansion on day 16 (r= -0.9616, p= 0.0045 **Figure 29**). Similarly, condition 3 did not induce a correlation between fold expansion and glucose uptake or lactic acid production between day 4 and 13 of culture. On day 16, however, condition 3 induced a negative correlation between cell expansion and glucose uptake (r= -0.9099, p= 0.0160) while simultaneously exhibiting a positive correlation with lactic acid production (r= 0.8910, p= 0.0212) (**Figure 29**). This indicates a high rate of NK cell proliferation, in line with the observed NK expansion with condition 3 (**Figure 24**) and consistent with glucose and lactic acid metabolic changes (**Figure 28B** and **C**). Surprisingly, condition 12 and 13 did not induce any correlation between NK fold expansion and glucose uptake or lactic acid production in the donors tested.

4.8 Discussion

Characterization and profiling of NK cells is essential to better understand their biology, so they can be modulated for future NK cell-based immunotherapy. This chapter analyses signalling pathways downstream of the cytokines IL-2, IL-12, IL-15, IL-18, IL-21 and investigates their metabolism profile after cytokine stimulation. Understanding cytokine signalling pathway is hindered by the effect of specific cytokines on multiple transcription factors, which then induce the expression of several target genes. This is further complicated by crosstalk between signalling pathways, as seen with IL-12 and IL-18 synergistic effect, suggesting crosstalk between the JAK-STAT and the NF-kB signalling mechanisms (88,111).

To further evaluate the effect of cytokines on NK cell proliferation, additional conditions were analysed. Out of the twelve conditions, the IL-21 primed regimens condition 3, 10 and 12 give the best proliferation overall, with condition 3, showing the strongest proliferation (**Figure 17B**). These results are in sharp contrast with previous studies (229,268), which showed that IL-21 antagonises IL-15 positive effect on NK proliferation. However, IL-21 is a cytokine with a strong pleiotropic role in NK cell biology, and the results observed here of synergy with IL-15 and hence of increased NK cell expansion are in line with other studies (63,77,252). As already discussed in section **3.6**, IL-21 benefits in NK cell cultures are many. Amongst these, IL-21 has been shown to induce STAT3 activation and signalling pathways. This leads to the downstream activation of c-Myc which regulates several cellular processes such as increasing NK cell proliferation, induction of glycolysis, mitochondrial biogenesis, and the cell cycle (84). More importantly, STAT3 is an important driver of the Warburg metabolic reprogramming, a hallmark of cancer cells. Indeed, NK cells with a high STAT3 expression as a result of IL-21 stimulation, present features of tumour cells, such as high and rapid proliferation, long telomeres and resistance to senescence (84). Additionally, NK cells with high STAT3 expression resulted from IL-21 stimulation show upregulation of glycolysis and downregulation of oxygen dependent pathway oxidative phosphorylation (123). In line with this, NK cells cultured with condition 3 showed a remarkable expression of STAT3 (**Figure 18C**). A partial activation of STAT3 was also observed with condition 2, where no IL-21 was present, but this was the result of the other cytokines, such as IL-15, being able to activate

STAT3, albeit not to the same extent of IL-21 (**Figure 18C**). Therefore IL-21 role in NK cell culture is fundamental, as it contributes to render NK cells more robust to survive the TME and hence enhance success of immunotherapy, especially in solid tumours. Several solid tumours are defined as “cold” due to the low number or lack of immune cells that favour disease progression, leading to a higher tumour burden as seen in HCC (286–289). STAT3 upregulation is also essential for the hypoxia inducible factor 1α (HIF-1α) expression, which mediates cellular adaptation to hypoxia (107). This occurs after IL-15 priming of NK cells and suggest the benefit of including both IL-21 and IL-15 in NK cell culture. However, effects of IL-21 on HIF-1α require further analysis.

Inhibition analysis of STAT3 when NK cells were cultured with condition 3 (IL-21 present) showed an increased proliferation compared to condition 2, where no IL-21 is present (**Figure 18A** and **B**). This indicates that IL-21 mediates its effect on proliferation not only via STAT3, but also via other pathways such as the PI3K-AKT and MAPK (80) and that these have an important role in proliferation. PI3K role within the JAK-STAT pathway makes it a key player in potentiating the priming effect of cytokines as it lowers the activation threshold of NK cells stimulated with common γ-chain cytokines (290). Downstream of the PI3K-AKT pathway are activated mTOR and NF-kB. mTOR importance in NK cell proliferation has been analysed in the context of IL-15 stimulation (99,125). It can be reasoned that IL-21 also mediated increased proliferation via mTOR, explaining the results observed with STAT3 inhibition (**Figure 18A** and **B**). Furthermore, PI3K/AKT/NF-kB signalling pathway is essential to mediate oncogenic transformation and sustain cell proliferation (291–293). As NK cells acquire adaptations reminiscent of tumour cells after IL-21 stimulation, this could also be observed with the PI3K/AKT/NF-kB axis effect on NK cell proliferation, contributing to explain the results observed with STAT3 inhibition (82) (**Figure 18A** and **B**). Interestingly, NF-kB inhibition caused a lower proliferation in the presence of IL-21, suggesting a novel role for NF-kB downstream of IL-21 with strong implication in NK cell proliferation. Taken together these results indicate that there is an interplay between IL-21 and IL-18 and their respective transcription factors, STAT3 and NF-kB. Here we show that IL-21 signals though STAT3, however, its action, mediated by NF-kB signalling plays a much more important role in proliferation, warranting further investigation. A limitation of the current work is the lack of

analysis of NF-KB expression, as well as of mTOR, PI3K and AKT upon cytokine stimulation before and after STAT3 and NF-KB inhibition, to better understand their implications in NK cell proliferation. However, the importance of IL-21 and NF-kB in mediating NK cell expansion is highlighted by a great decrease in NKG2A expression after NF-kB inhibition in the presence of IL-21 (condition 3, **Figure 19B**). This is in line with Kaulfuss et al (240), which showed that NK cell expansion is greater in NKG2A^{+} cells and that this was not dependent on STAT3 signalling pathway, despite NK cells were stimulated with membrane-bound IL-21. Conversely, high levels of NKG2A might impair NK cell activation and effector function as cells might need to overcome a higher activation threshold. Therefore, it is vital to understand how to manipulate the fine balance between high proliferation versus high anti-tumoural function properties. The trade-off between NK cell proliferation and antitumour function is seen between condition 3 and 12. Although condition 12 induces a lower expansion than condition 3, this is not statistically significant. The lower cell yield is compensated by increased antitumour function, mediated by CD107a and IFN-γ expression (**Figure 21A** and **B**).

A major challenge to overcome in the development of NK cell-based therapy is NK cell expansion to obtain large numbers of healthy and functional cells to give to the patient. Vital for this are the cytokines used, in what sequence and within what timeframe (294). Selection of the correct cytokines to implement in NK culture systems is hindered by an elementary understanding of signalling pathways downstream of cytokines. Further complicated by different cytokines sharing the same signalling pathways and by synergism or antagonism as they induce transcription of target genes. Moreover, NK cells obtained from different donors can respond differently to the same combination of cytokines. Therefore, relating a combination of cytokines to a specific NK cell response such as proliferation across donors can be challenging. We address this challenge by developing a predictive framework that can predict fold changes in NK cell population in different cytokine combinations. The *in silico* model strongly predicted condition 3 to generate the highest NK cell expansion, which was matched by the experimental data (**Appendix 1**). Synergism between STATs and NF-kB drive NK cell proliferation, confirming the crosstalk between different signalling pathways, discussed above. STAT3, STAT4, STAT5 synergise with NF-kB (**Appendix 1D**), however it is not known to what extent and how much each transcription factor contributes, therefore further

analysis to better understand these mechanisms are needed. This model was based on the inhibition of STAT3, and NF-kB of NK cells expanded with condition 2 and condition 3. As both transcription factors were important for NK expansion, the constraint that both STAT3 and NF-kB were present in the combination matrices generated by the model was applied during the analysis. It would be interesting, to investigate the role of STAT4 and STAT5 to the *in silico* analysis and evaluate how this compared with the results obtained here. The strong model developed here can be used to predict the outcome of other cytokines combinations on NK cell proliferation, which can be validated experimentally. However, the model does not come without limitations; these are small data set and huge data spread. Testing more cytokine combinations will improve the model framework and prediction ability. Data spread is the result of great donor variation, and it might not be possible to normalise for this as different donors respond differently to cytokine stimulations, which might influence clinical outcomes. Therefore, it is important to characterise biomarkers indicative of high antitumour functions as well as proliferation (284,295–297).

An important factor that determines NK cell proliferation and effector function is cell maturation, which is affected by cytokine stimuli from the surrounding. Therefore, establishing the correct cytokine combination is indispensable. Alongside increased proliferation, IL-21 causes a fast expansion of cells as observed with condition 3 and 12 between day 7 and day 9 (**Figure 23B** and **Figure 24**). Therefore, it is important to better understand proliferation kinetics by measuring expression of the proliferation marker Ki-67 (298–301), as well as the marker Annexin V to detect apoptotic cells (302). It is interesting to see, however, that NK cells might not follow a rigid liner maturation path (**Figure 23B** and **D, Figure25**), and that all possible expression patterns of transitioning receptors might be possible with many phenotypically and functionally distinct subsets emerging, in line with the work of Björkström et al (32). CD16 expression illustrates this very well. Both CD16+ and CD16- subpopulations are present at any one time in the culture, however, CD16- (mainly CD16-CD117+CD57-) subpopulation decreased from day 5 to day 7 of the culture (**Figure 23D**), when more mature, CD16+ NK cells emerge (CD16+CD117+CD57-). The presence of more CD16- NK cells on day 9 suggests that the more mature NK cells subpopulations have undergone senescence or apoptosis, and less mature CD16- subpopulation reappear (**Figure**

23D). Considering this, I hypothesise that NK maturation occurs in waves of alternating mature/immature status. CD57 is a marker used to indicate poor proliferative capacity and possible cell senescence, gradually accumulating throughout cell maturation with the highest expression at the end of cells' lifetime (2,32). CD57 acquisition and appearance is not a reversable event *in vitro* or *in vivo* (32), suggesting that cytokine stimulated NK cells present differentially expressed receptors forming distinct subpopulations. Thus, I hypothesise that the $CD57^+$ subpopulations seen at day 5, stop proliferating, and $CD57^-$ cells take over, comprising most of the NK cell population. There is a small reappearance of $CD57^+$ subpopulation by day 9, especially with condition 1, caused by other subsets of NK cells that upregulate CD57 expression and undergo maturation later (**Figure 23D**). Valuable for a deeper understanding of NK cell maturation and effector function is the analysis of KIR expression as well as of NK cell education.

NK cell function can be amplified by understanding the expression of checkpoint inhibitors such as PD-1. PD-1 expression was initially characterised on T cells, where it is responsible for reducing excessive immune activation leading to serious damage. PD-1 expression in NK cells has been detected either on a background of several tumours or because of viral infections such as cytomegalovirus or HIV (303). Traditionally believed to induce exhaustion, recent studies have indicated that PD-1 expression on NK cells might be a sign of activation, albeit of less mature NK cells (283,304–306), explaining why it is expressed on stimulated NK cells. In contrast to the observations of *Quatrini et al* (283), high PD-1 expression was seen with all the cytokine combination analysed without the presence of glucocorticoids. Furthermore, in their study *Quatrini et al*, reported no upregulation of PD-1 after two days of NK stimulation, irrespective of glucocorticoid presence. However, in the current work, PD-1 upregulation was significantly seen after 6 days. This suggests that specific molecular mechanisms control the expression of PD-1 in a timely manner, explaining why we observed PD-1 upregulation after 10 days of cytokine stimulation even without glucocorticoids supplementation. PD-1 expression and upregulation has been reported on NK cells in many tumor, such as multiple myeloma, renal cell carcinoma, Kaposi sarcoma, ovarian carcinoma, Hodgkin's lymphoma, digestive cancers, such hepatocellular carcinoma, non-small cell lung cancer, primary and metastatic lung cancer. PD-1 expression on NK cells resulted in dysfunctional NK cell and a

higher tumor burden, which could be salvaged by interrupting PD-1/PD-L1 interaction (303). Although we were able to show that cytokine-activated PD-1^+ NK are not exhausted and still active against hepatocellular carcinoma, it is crucial to further understand the molecular mechanism and timing of PD-1 expression on healthy NK cells cultured *ex vivo*, to make full use of NK cells' antitumor function.

Important for the use of cytokine activated NK cells as a novel immunotherapy for hepatocellular carcinoma, is the CXCR6 on peripheral blood derived NK cells, which have been activated by cytokines (**Figure 26C**). In agreement with other analyses (307–310), we found that CXCR6$^+$ NK cells phenotypically resemble the tissue resident CXCR6$^+$ NK from the liver and the spleen. These are CD57^{low}, NKG2C^{low} and NKG2A^{high}. Cytokine-activated CXCR6$^+$ NK show an immature phenotype, highlighted by CD117 expression, but still retained high cytotoxicity against hepatocellular carcinoma, in line with other studies (307). Infusion of cytokine-activated-CXCR6$^+$ NK cells might induce "homing" to the liver, recruited by the CXCR6 ligand CXCL16. The high phenotypic and functional plasticity of the cytokine-activated-CXCR6$^+$ NK cells could regulate tumor burden, therefore, making it an efficient treatment for hepatocellular carcinoma. However, further work needs to be done to understand the expression of transcriptional factors specific to CXCR6 presence, such as Eomes and T-bet, and how those regulate NK cells. Additionally, further research is needed to elucidate the impact of the liver microenvironment on the mature and functionally active CXCR6$^+$ NK that have migrated from the peripheral blood.

Understanding how cytokine stimulation affects NK cells metabolism is important to fine tune their effector functions and optimise NK-based immunotherapies. In line with other studies (99,117,120,121), a sustained overnight and strong cytokine stimulation, independent of cytokine combination, causes a high increase in glucose uptake, indicating high cells metabolism required to accommodate increased cellular processes (**Figure 28C**). This is in part highlighted by the regression model which analyses the correlation between expansion and glucose uptake from the culture media or lactic acid produced by proliferating NK cells. The correlations observed on day 16 under condition 3 indicate high NK cell proliferation. Interestingly, however, this was not detected in the earlier days of the culture, despite

condition 3 was observed to induce an early NK cell expansion (**Figure 17B, Figure 24**). Cytokine-activated NK cells might utilize glucose to undergo cell growth, maturation, or differentiation, therefore rendering the detection of direct correlation between metabolism and NK cell proliferation rather complex, particularly when relying only on the methods employed in the current study. This warrants further detailed analysis of cytokine-activated NK cells metabolism.

Furthermore, high donor variation (**Appendix 2**) hindered the analysis of how cytokines modulate NK cell metabolism. Donor L is the one that represents the most variation, especially after 16 hours cytokine priming (cycle 1, **Appendix 2D**) with all conditions. Donor J also showed a marked difference when stimulated with condition 12, compared to the other donors. Due the relatively small sample size (n=5), it is difficult to confirm whether these donors are simply outliers or representative of a proportion of human population which present different NK cells metabolism and phenotype, which results in a stochastic outcome after cytokine stimulation. It has recently been shown that there is a broad variation in the functional capacity of NK cells derived by different individuals (284,295,311). While the incredible heterogeneity of NK cells has been well studied, analysis of donor variation and what is at the root of it is surprisingly scarce in the literature, resulting in a plethora of studies which assume that the differences seen in NK cell responses was due to their inherent heterogeneous nature. Therefore, it is worth investing in the identification of donors which might present stronger NK anti-tumour activity and desirable phenotype for the development of efficacious “off the shelf” NK-based tumour immunotherapy. It is important to investigate biomarkers indicative of greater NK cells persistency and potency, to standardize donor selection criteria and improve clinical efficacy.

Although challenging to neatly link metabolism with NK cells effector function of CD107a and IFN-γ secretion, cells upregulated their metabolism after a sharp sustained cytokine stimulation. Metabolism is reduced during a potential rest period of cells, and glucose uptake increases again towards the later phase of in vitro culture. Therefore, this reinforces the hypothesis proposed earlier of NK cells subsets within the general NK cells population which

upregulate their metabolism at different time points, generating a wave-like metabolic profile with peaks and troughs, driving NK population proliferation and function forward. This, however, needs further studies requiring a long-term analysis of NK cells, beyond 16 days of in vitro culture.

Furthermore, to better understand NK metabolism additional analysis are required. These include and are not limited to the evaluation of the expression of metabolite transporters, such as glucose transporter GLUT1, and amino acid transporter CD98; analysis of key transcription factors involved in metabolic regulation, such as MYC and SREBP. Lastly, the results observed here could be due to artifacts and data processing issues related with NMR technology, therefore this method needs to be further optimised.

To conclude, IL-21 is a potent modulation of NK cell maturation, proliferation, and antitumour-function, albeit only when used in combination with other cytokines such as IL-15, IL-18, IL-12. Its main signalling mode is via STAT3, although we were able to show that NF-kB might play an important role in mediating IL-21 function, which warrants further investigation.

Chapter 5 Development of CAR-NK and application to HCC cell lines

5.1 Introduction

The previous chapters indicated the optimal culture conditions required to increase NK cell proliferation *in vitro*, highlighting the importance of IL-21 in proliferation, cell maturation and cytotoxicity. In this final result chapter, my aim was to apply these findings and to explore the use of cytokine-activated NK cells to generate chimeric antigen receptor (CAR)-NK, targeting a specific antigen on hepatocellular carcinoma HCC cells.

HCC is the most common form of liver cancer and a global health challenge, being the second leading cause of cancer-related death worldwide. Traditional treatment for HCC is delivery of systemic therapies such as the tyrosine kinase inhibitor (TKI) sorafenib or lenvatinib, however these are not very effective, especially in more advanced stages of the disease (147–150,312,313). In recent years, immunotherapy developments have achieved exciting results in tumours such as melanoma and non-small cell lung cancer, and the benefits are seen in HCC too. Immune-checkpoint inhibitors (ICI) have significantly improved and revolutionized HCC treatments, and now more attention has been given to adoptive cell transfer (ACT) therapy, specifically for the development of chimeric antigen receptor (CAR)-engineered immune cells. NK cell-based therapy is an emerging ACT immunotherapy, with enhanced benefit seen with cytokine-induced memory-like (ML) NK cells. Thus, the combination of ML-NK cells with CAR-engineering (144), to generate ML-CAR-NK, can provide an interesting and novel treatment for HCC.

Essential for the de the development of an ACT therapy, is the need to have enough *ex vivo* expanded cells. As I demonstrated the increased expansion of NK cell with condition 3 (memory-like-NK inducing cytokines + IL-21), I hypothesized that *ex vivo* culture of NK cells with condition 3 would show increased cell expansion, consequently increasing the

proportion of CAR positive NK cells and hence increasing cytotoxicity of CAR-NK against HCC cells. Additionally, I was interested in comparing proliferation and cytotoxicity when CAR-NK cells were cultured with the cytokine cocktail (IL-12+15+18) shown to prime NK cells to generate memory-like NK cells (35,95), here named as condition 2 (no IL-21 present). As memory-like NK cells show an increased cytotoxicity (35), I hypothesised that this effect would be enhanced by the addition of a chimeric antigen receptor. Three different scFv were selected to test out this proof-of-concept analysis, which was performed in the research laboratories of Miltenyi Biotec in Bergisch Gladbach, Germany.

5.2 Selecting glypican-3 as a target for CAR-NK

For the development of a successful tumour-specific ACT therapy it is vital to carefully choose the specific tumour target antigen expressed by the tumour cells. This target antigen is used to design the extracellular single-chain variable fragment (scFv) of the CAR, which determines successful antigen binding and aids recognition of cancer cells by CAR-NK (314). Upon binding, CAR-NK cells receive stimulatory signals causing them to activate and become cytotoxic towards cancer cells. Identification of specific tumour antigens is key to successful immunotherapy development. Requirements for the selection of an optimal target antigen are immunogenicity, expression pattern, with prevalence in tumour tissue, and biological significance which elicits a response (315).

Following a literature search, a number of antigens were considered for the CAR-NK generation (listed in **Table 7**). The ideal target antigen for efficient CAR therapy should match all or most of the following criteria:

- The antigen is expressed only on tumour cells and not on healthy cells, to avoid on-target off-tumour binding.
- The antigen is expressed with a high frequency on tumour cells, to obtain greater killing and tumour regression.
- The antigen is expressed on the cell surface as a whole protein, not digested intracellularly and then presented by major histocompatibility (MHC) complexes.
- Ideally, the antigen has not previously been used as a target for immunotherapy, to ensure the novelty of the current research.

Antigen	Expression in HCC	Expression in healthy tissue	Expression on cell surface	Previous target for CAR therapy	Expressed in other cancers	Expressed on MHC complex
GPC-3	Yes (~70)(316–318)	No (317,318)	Yes (316)	Yes. CAR-T/-NK (212,319)	Yes	No
Annexin-A2	Yes (320–322)	Yes(323,324)	Yes - entire protein (323,324)	Yes	Yes (320–322)	No
hTERT	Yes (~70%) (325)	Low expression (326)	Yes (327–329)	Yes	Yes (326,329)	Yes (327–329)
AFP	Yes (~70-80%) (330–332)	No	Yes (332)	Yes. CAR-T (330)	Rare	Yes
MAGE-C1	Yes (333)	In testis (333)	Yes		Yes(333)	Yes
MAGE-A9	Yes – high (334,335)	In testis (334,335)	Yes		Yes	Yes
MAGE-C2	Yes (336–338)	In testis	Yes		Yes (336–338)	Yes
CD147	Yes (339,340)	Yes (339,341)	Yes (339,341)	Yes. CAR-T (340)	Yes (339,341)	No
c-MET	Yes - product of proto-oncogene MET (342) (343)	Low expression (342)	Yes (342,343)	Yes. CAR-T/-NK (344,345)	Yes (342,343)	No
NY-ESO-1	Yes (346)	In testis (346)	Yes (346)		Yes (346)	Yes
EpCam	Yes (347,348)	Low expression (347,348)	Yes		Yes (347,348)	No

Table 7. HCC antigens and criteria considered for the selection of the CAR-NK target.

HCC antigens listed represent targets considered for the development of CAR-NK.

The antigens annexin-2, MAGE (C1, A9, C2), CD147, c-MET, NY-ESO-1, EpCam were not considered for the development of CAR-NK, as they are also expressed on healthy tissues. This would lead to a high on-target off-tumour responses causing severe side effects such as toxicity. Although the antigen alpha-fetoprotein (AFP) is not expressed on healthy tissues (**Table 7**), it was not selected as the target either. This is because it is expressed in a small peptide form on the MHC complex, rather than as an intact cell surface antigen.

From the list of antigens compiled, glypican-3 (GPC-3) matched the defined criteria and was therefore selected for the construction of CAR-NK. GPC-3 is an oncofoetal antigen highly expressed in HCC but not in corresponding healthy liver tissues in adults, and it was not detected in other healthy tissue (317,318), making it a good tumour antigen candidate to target for the development of HCC treatments. Important for cell growth, differentiation and migration during foetal development, the protein is found in foetal liver but not in adult liver (316,317). GPC-3 has also been detected in the placenta, foetal lung, and kidneys and its overexpression does not seem to correlate with HBV or HCV infection (349). Additionally, GPC-3 expression in adults has been associated with worse prognosis and higher risk of death in patients with HCC, especially in the advanced stages, where it showed increased expression (350).

There have already been some preliminary investigations using GPC-3 to generate both CAR-NK and CAR-T cells (212,319). However, our study differs from previous investigations as it is using primary NK cells in free-cytokine culture with the aim of generating CAR-ML-NK cells. GPC-3 has also been used as a target to generate antibodies for the development of other types of immunotherapies. This can be an advantage, as the sequence of the variable fragment (Fv) can be used to create the scFv domain of CAR.

GPC-3 targeting antibodies were used to generate the new CAR-engineered NK cells analysed in this study. Three scFv from different antibodies were selected due to their high specificity and affinity to GPC-3, which increases cytotoxicity. These bind to different epitopes on GPC-3 and to evaluate the importance of the effect of GPC-3 epitope binding in CAR-NK, the antibodies GC33 (319,351), YP7 (352) and 9F2 (353), were compared. The antigen recognition region of these antibodies was cloned into a lentiviral vector, also encoding the other CAR domains, to construct CAR-NK and to be tested *in vitro* against HCC cell lines PLC and HepG2. The *in silico* design of CAR was performed by scientists at Miltenyi Biotech. GPC-3 targeting CAR-NK cells were generated using the protocols and platforms provided by Miltenyi Biotech.

5.3 Generation of GPC-3 specific chimeric antigen receptor

Purified NK cells from PBMC of healthy donors were cultured with condition 2 (IL-12+15+18 priming for 16hrs, then IL-2 only) or condition 3 (I12+15+18+21 priming for 16hrs, then IL-2 only). On day 2 from purification, cells were transduced with a Baboon envelope (BaEV)-pseudotyped lentiviral vector (LV), carrying a GPC-3-specific CAR. The construct consisted of one of the antigen-specific scFv described in section **5.2**, either GC33, YP7 or 9F2, a CD8 hinge and transmembrane (TM) domains, the intracellular (IC) 4-1BB costimulatory domain and CD3ζ activating domain (**Figure 30A**). As cells were already primed and cytokines (IL-12+15+18 and/or IL-21) were removed prior to lentiviral transduction, NK cells were cultured using IL-2 only after transduction. On day 12, the expression of the different CARs in the transduced NK cells was tested by using human glypican-3 protein tagged with the fluorophore APC (Acro Biosystems) and analysed by flow cytometry. GC33 and 9F2 showed a similar transduction efficiency of ~76% GPC-3-CAR, whereas YP7 showed a transduction efficiency of ~8% (**Figure 30B**), independent of culture condition.

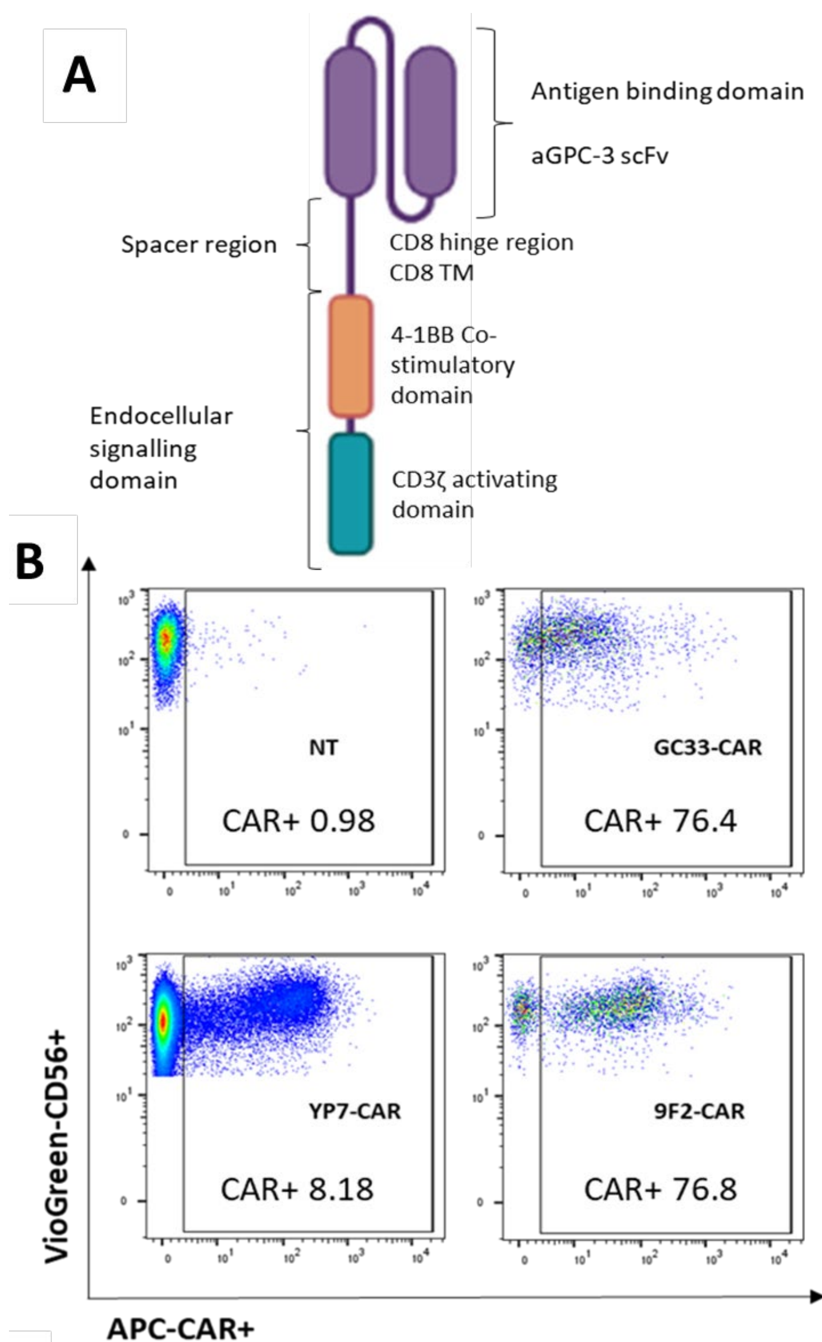


Figure 30. GPC-3 CAR-NK design and expression

A. schematic representation of the second-generation GPC3-targeted CAR generated in this study. Image realised with BioRender. **B**. surface expression of GPC3-targeted CAR, with the different scFv. Expression was analysed by flow cytometry after 12 days from lentiviral transduction into primary NK. NT = non-transduced (mock transduction).

5.4 Proliferation of GPC-3 targeted CAR-NK cells and cytokine production

CAR-transduced NK cells cultured with condition 2 and condition 3, where expanded *ex vivo* for a 15 days period (**Figure 31**). No severe impairment of expansion was seen during the culture, with any of the CARs tested, compared to the non-transduced cells (labelled as NT), with both condition 2 and condition 3 (**Figure 31A** and **B**). Non-transduced NK cells and CAR-NK cell proliferation increased overtime, to a 10^2-fold expansion at day 13. There was a trend for cell cultured under condition 3 to show an increased expansion, compared to cells cultured with condition 2, however this was not statistically significant (**Figure 31A**). Closer analysis of cell expansion at each culture day, revealed that non-transduced NK cells cultured with condition 3 show a statistically significant increased expansion compared to non-transduced NK cells cultured with condition 2 at day 7 of culture. This was also observed at day 10, with YP7 CAR-NK, condition 3 cultured NK cells show a statistically significant higher fold expansion than condition 2 (**Figure 31B**).

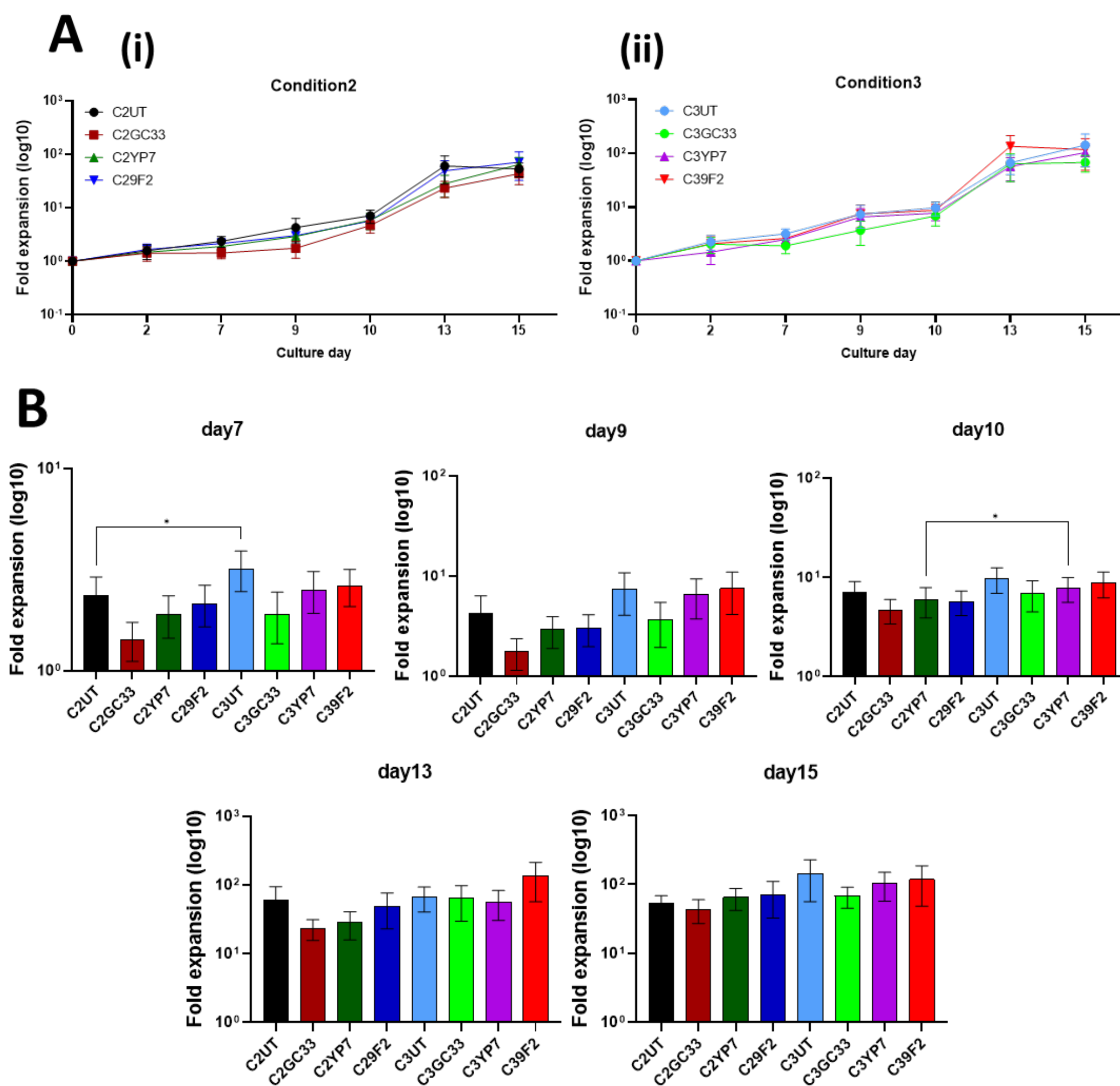


Figure 31. Proliferation of CAR-NK cells

Expansion of NK cells transduced with the different GPC3-targeting-CARs (GC33, YP7, 9F2) and non-transduced (NT)-NK cells, cultured with condition 2 or condition 3. **A.** Time lapsed expansion of CAR-NK and NT-NT. **B.** Expansion of CAR-NK and NT-NK at each culture day analysed. Data is expressed as mean ± SEM (n=9), analysed by repeated measure one-way ANOVA, followed by Tukey's multiple comparison (*P≤0.05). C2 = condition 2, C3 = condition 3, NT = non-transduced (mock transduction).

For functional *in vitro* analysis, the hepatocellular carcinoma cell lines HepG2 and PLC were chosen as the targets. The surface expression of GPC-3 expression was determined by flow cytometry. The cell lines expressed different levels of the target antigen GCP-3, with HepG2 expressing 40% more GCP-3 than PLC (**Figure 32**).

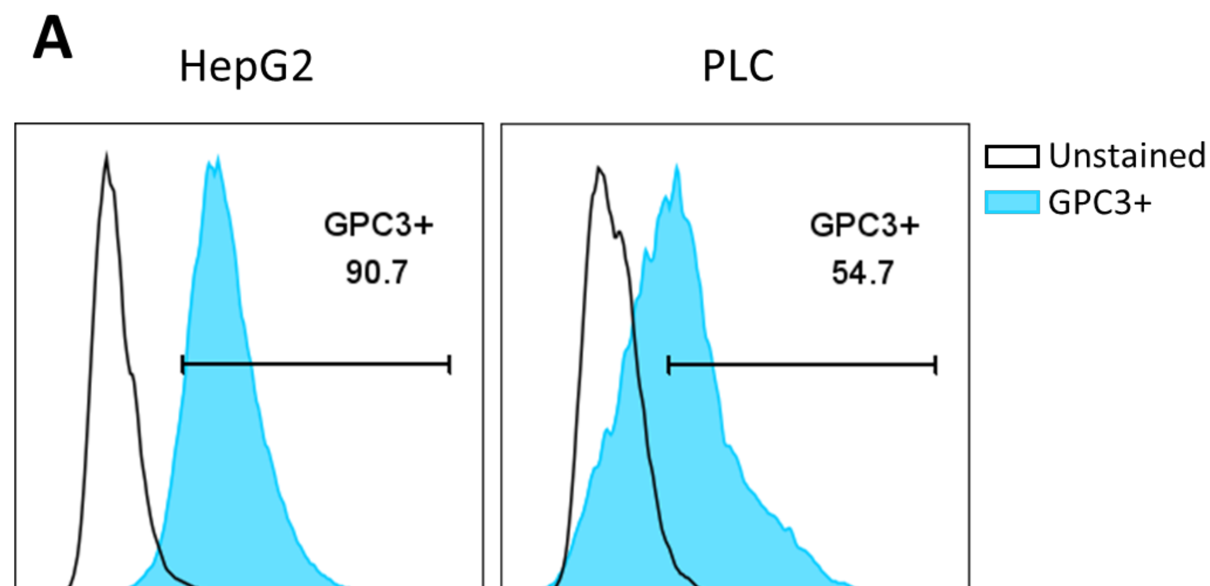


Figure 32. Expression of GPC-3 on HCC cell lines

Surface expression of GPC-3 on the hepatocellular carcinoma cell lines selected, HepG2 and PLC. This was done by flow cytometry, using the GPC-3 targeting antibody from Abcam.

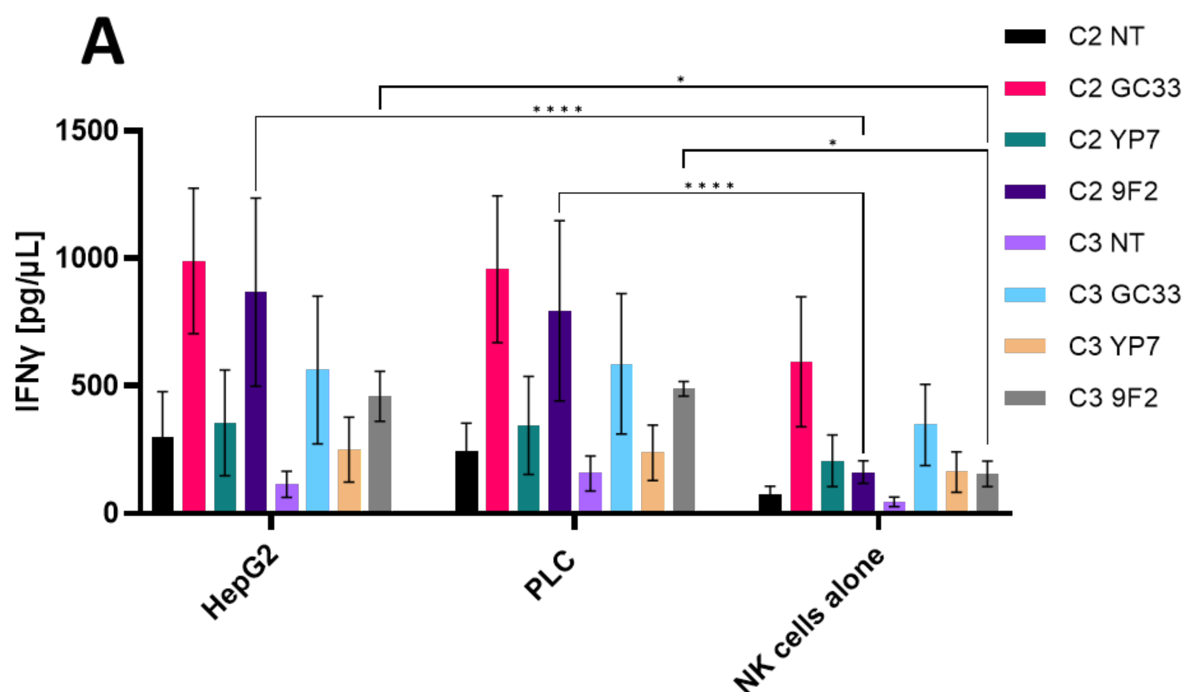


Figure 33. IFN-γ secreted by CAR-NK co-cultured with HCC cell lines

IFN-γ secretion by the indicated genetically modified NK cells, expressing the different CAR constructs, co-cultured with the HCC cell lines HepG2 and PLC for 24 hours. E:T 1:1, average of 5 independent experiments carried out with 5 different donors, in triplicates. Data expressed at mean ± SEM, analysed with repeated measures two-way ANOVA, followed by Dunnett's multiple comparison test. C2 = condition 2, C3 = condition 3, NT = non-transduced (mock transduction) (*P≤0.05, ****P≤0.0001).

The levels of IFN-γ secreted by CAR-NK were tested after 24 hours co-culture with the HCC cell lines HepG2 and PLC (**Figure 33**). Despite the difference in antigen expression between the cell lines, there was no difference in IFN-γ secretion when NK cells were incubated with either PLC or HepG2. However, the 9F2 CAR construct induced the highest IFN-γ release when NK cells were cultured with condition 2, compared to NK cells incubated with no target cell line (NK cells alone, **Figure 33**). The 9F2 construct also induced a high IFN-γ release, when NK cells were cultured with condition 3, compared to NK cells incubated with no target cell line, however this was lower than when cells were cultured with condition 2. Interestingly, a high release of IFN-γ was also seen with the GC33 CAR-NK with condition 2, against both HepG2 and PLC. However, a high secretion was also seen when NK cells were incubated alone (**Figure 33**), indicating that the NK activation is not sustained by the CAR- stimulation alone and hence IFN-γ release was mediated by other mechanisms such as cytokine stimulation or engagement of NK cells' endogenous activating receptor.

5.5 Cytokine-activated CAR-NK show strong *in vitro* cytotoxicity against GPC3+ HCC cell lines

To determine whether CAR-NK cells targeting GPC-3 could specifically recognize and kill GPC-3 positive hepatocellular carcinoma cell lines, and to compare cytolytic capabilities of the different CARs (GC33, YP7, 9F2), cytotoxic assays were performed. CAR-NK cells were co-cultured with each HCC cell line for 4 hours at 2:1 and 1:1 effector: target ratio (**Figure 34**). Overall, CAR-NK cells induced a higher lysis of HepG2 cells compared to PLC (**Figure 34A (i)** and **(ii)**). 9F2 CAR-NK cells cultured with condition 2, showed the highest lytic activity with HepG2 cell line, which was about 35% at the E:T ratio of 2:1, which decreased to about 30% at 1:1 E:T ratio (**Figure 34A (i)**). 9F2 CAR-NK cells cultured with condition 3, showed the second highest lytic activity with HepG2 cell line, which was about 30% at the E:T ratio of 2:1, and which decreased to about 25% at 1:1 E:T ratio. GC33 and YP7 CAR-NK cultured with condition 2, showed a very similar killing of the target cell line HepG2, which was lower than 9F2 CAR-NK cells, cultured with condition 3. GC33 and YP7 CAR-NK cultured with condition 3 showed the lowest cell killing, which was the same as or lower than NT-NK cells (**Figure 34A (i)**).

NT-NK cells cultured with condition 2, which showed the lowest HepG2 cell lysis, which was about 15% at 2:1 E:T ratio and 12% at 1:1 E:T ratio. Interestingly, NT-NK cells cultured with condition 3, showed a higher cell lysis which was about 22% at 2:1 E:T ratio and 17% at 1:1 E:T ratio (**Figure 34A (i)**).

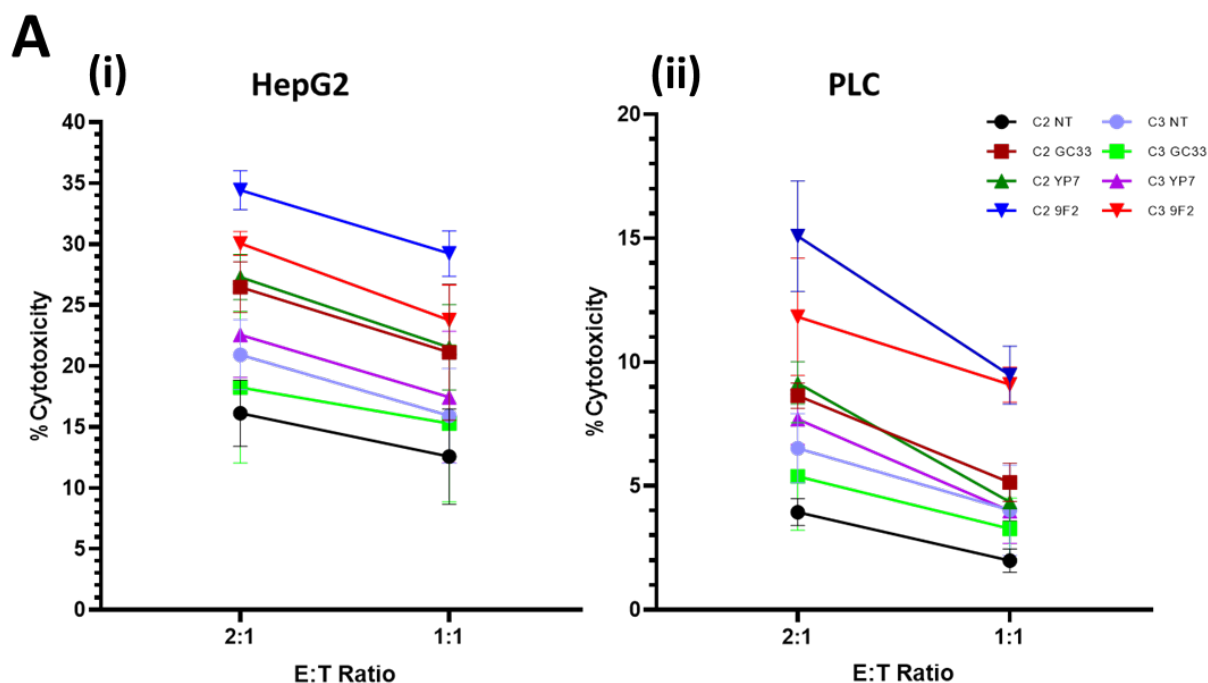


Figure 34. GPC-3-CAR-NK cells show *in vitro* cytotoxicity against GPC-3 positive HCC cells, with varying activation from the different CARs analysed

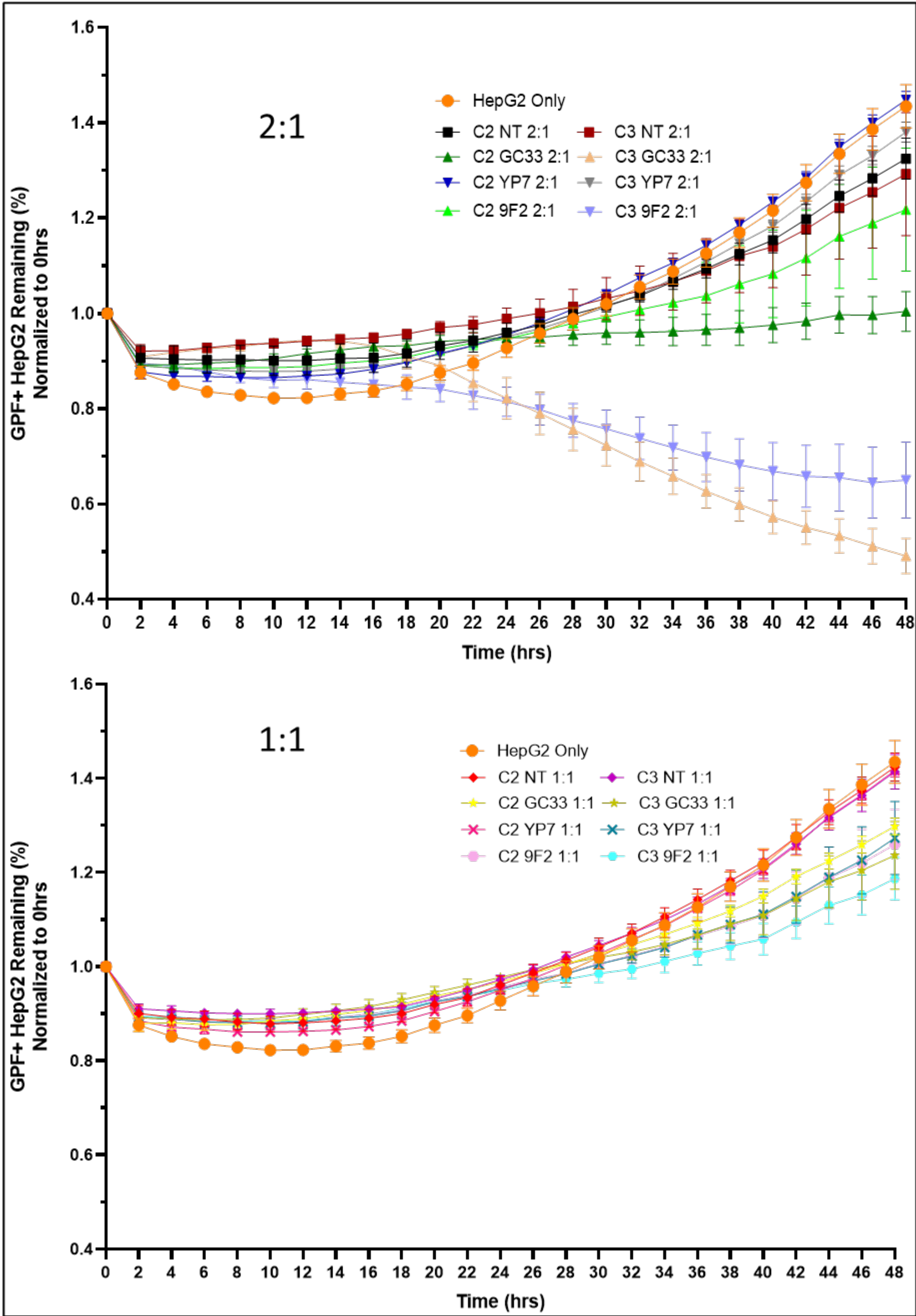
Cytolytic activity of CAR-transduced NK cells from healthy donors, cultured either with condition 2 or condition 3 after 4 hours incubation with HepG2 **(i)** or PLC **(ii)** cells, at the shown E:T ratio. Co-cultures were incubated at 37 ˚C and 5% CO_2. Data expressed at mean ± SEM, n = 4 with HepG2 cell line, n=3 with PLC cell line. C2 = condition 2, C3 = condition 3, NT = non-transduced (mock transduction).

All CAR-transduced NK cells, cultured with either condition 2 or condition 3, were less effective in killing PLC cells in the 4 hours co-culture period (**Figure 34A (i)**). As seen with HepG2, 9F2 CAR-NK cells cultured with condition 2, showed the highest lytic activity with PLC cell line too, which was about 15% at the E:T ratio of 2:1, which decreased to about 10% at 1:1 E:T ratio (**Figure 34A (ii)**). 9F2 CAR-NK cells cultured with condition 3, showed the second highest lytic activity with PLC cell line, which was about 12% at the E:T ratio of 2:1, decreasing to about 10% at 1:1 E:T ratio, which is the same percentage of lysis seen with 9F2 cultured with condition 2. The other CAR-NK cells (GC33 and YP7) showed a similar cell killing as NT-NK cells, or lower, when NK cells were cultured with condition 2 or condition 3 (**Figure 34A (ii)**).

As seen with HepG2, NT-NK cells cultured with condition 2, which showed the lowest PLC cell lysis, which was about 4% at 2:1 E:T ratio and 3% at 1:1 E:T ratio. NT-NK cells cultured with condition 3, showed a higher cell lysis which was about 7% at 2:1 E:T ratio and 5% at 1:1 E:T ratio (**Figure 34A (ii)**).

When the cytotoxic activity was evaluated by dynamic motoring over a longer time frame, using IncuCyte, CAR-NK cells exhibited HCC control at best, albeit only with the higher effector-to-target ratio of 2:1 (**Figure 35A** and **B**). Interestingly, a longer incubation period with tumour cells, resulted in different CAR constructs in mediating tumour cell death (**Figure 35A** and **B**). After 10 hours co-culture, 9F2 CAR-NK cultured with condition 3, showed a higher killing of the HepG2 cell lines than 9F2 CAR-NK cultured with condition 2 at a 2:1 E:T ratio, causing a decline in tumour cell growth which plateaued close to 40 hours co-culture (**Figure 35A**). However, after 14 hours co-culture, GC33 CAR-NK cultured with condition 3 at a 2:1 E:T ratio, caused a steady and rapid decline in tumour cell growth which was lower than 9F2 CAR-NK cultured with condition 2, after 24 hours co-culture (**Figure 35A**). The difference in CAR efficacies in killing tumour cells, were seen at an earlier time point with PLC, 2:1 E:T ratio (**Figure 35B**). After 8 hours co-incubation, 9F2 CAR-NK cultured with condition 3, showed a higher killing of PLC cells; after 14 hours co-culture, YP7 CAR-NK cultured with condition 3, matched killing of the 9F2 CAR-NK cultured with condition 3 (**Figure 35B**). GC33 CAR-NK cultured with condition 2 also caused a decrease in PLC cell growth, but to a lesser extent than 9F2 and YP7 CAR-NK cultured with condition 3 (**Figure 35B**). The increased cytotoxic effects of IL-21 were significantly seen in a long-term co-culture, as shown in **Figure 35**.

The differences in CAR-NK mediated specific lysis indicated the importance of target-dependent specificity (**Figure 34**, **Figure 35**). Furthermore, the cytokine milieu plays an important part in pre-activating NK cells, supporting the difference in cell killing between CAR-NK cultured in condition 2 or condition 3 (**Figure 34**, **Figure 35**). Lastly, effector:target ratio was fundamental in the effective killing of tumour cells, indicating that a higher ratio of effector cells (CAR-NK) is needed, compared to the numbers of tumour cells (**Figure 34**, **Figure 35**).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Time (hrs)
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C2 NT 1:1
C3 NT 1:1
C2 GC33 1:1
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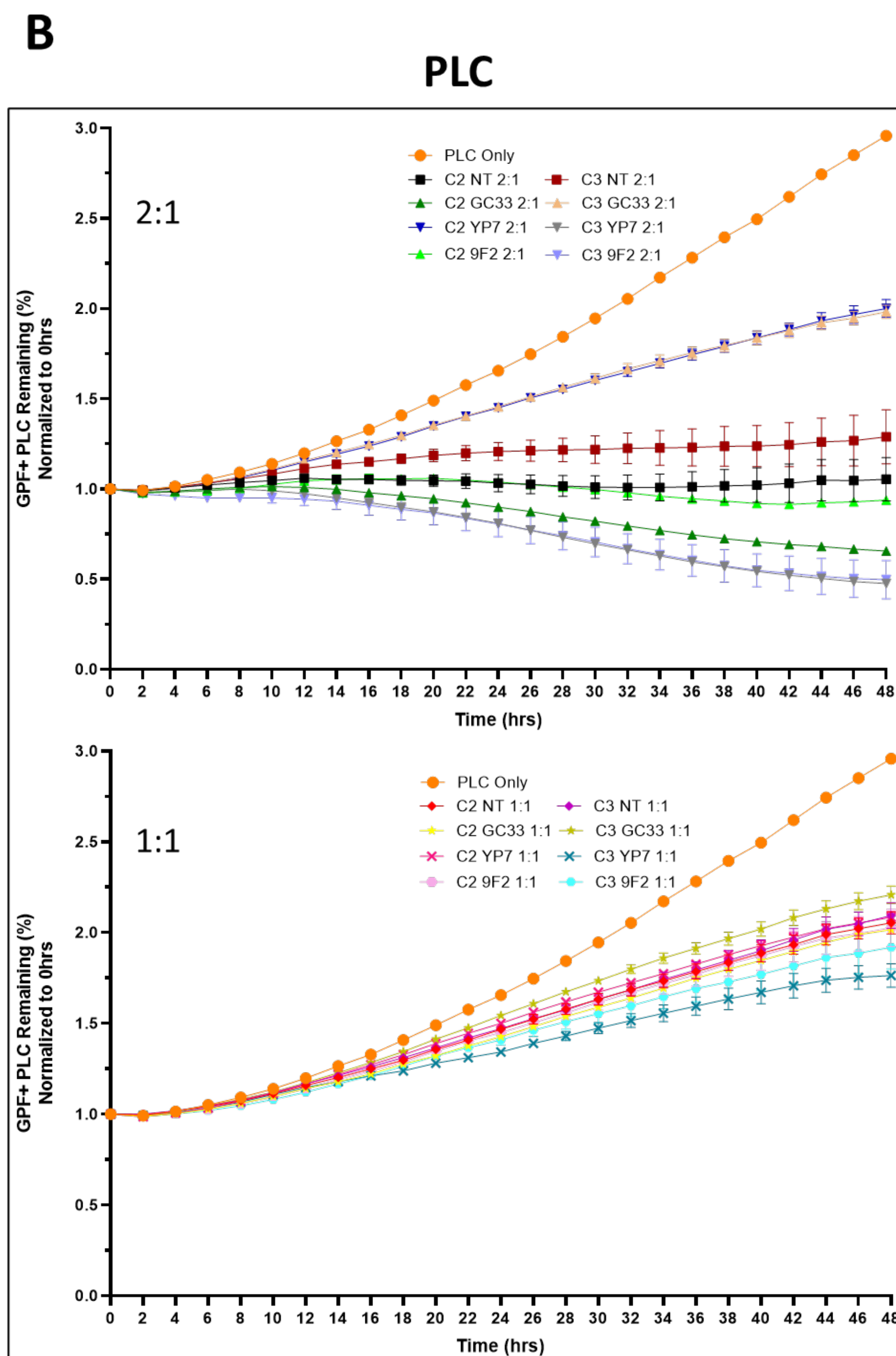


Figure 35. HCC cell lines are killed more effectively by CAR-NK during a long-term co-incubation

Dynamic monitoring of cytolytic activity of CAR-transduced NK cells, cultured either with condition 2 or condition 3. On day 12 after transduction, CAR-NK cells from a different set of healthy donors were co-cultured with GFP-positive **(A)** HepG2 or **(B)** PLC cell lines, at the shown E:T ratio. Decrease in fluorescence emission by tumour cells was determined using IncuCyte (Promega) S3 imaging platform, over 48 hours. Data expressed at mean ± SEM, n=2. C2 = condition 2, C3 = condition 3, NT = non-transduced (mock transduction).

5.6 Discussion

The field of CAR-engineered immune cells is rapidly evolving. A plethora of studies have demonstrated the efficacy and success of CAR-modified T cells for the treatment of blood tumours such as chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), which led to the approval of these drugs by the U.S. Food and Drugs Administration (FDA) and the European Medicine Agency (EMA) (143,354–359). However, the side effects tied with CAR-T cells therapies have prompted the search of alternative immune cells to use (360–362). Recent studies have shown that primary CAR-NK cells are safe to use and highly effective, though, further research on CAR-engineered NK cells is urgently required, to ensure the safety profile and their role in targeting solid tumours.

This chapter focused on the development of memory-like NK cells armed with CARs, to enhance their potential to kill hepatocellular carcinoma cells, as a proof-of-concept study. To achieve this, NK cells from peripheral blood of healthy individuals were primed either with condition 2 (IL-12+15+18) or condition 3 (IL-12+15+18+21) for 16 hours and on day 2 of culture were transduced with CAR constructs. This approach generated memory-like NK cells with stable expression of CAR, which expanded well in vitro (**Figure 31**). CAR-transduced NK cells did not show a lower expansion than NT-NK cells. Unlike what was hypothesised, however, addition of IL-21 did not benefit NK cell expansion, except at day 7 with the NT-NK cells and at day 10 with YP7 CAR-transduced NK cells (**Figure 31B**). Transduction is a highly demanding process which reduces proliferation; however, this is rescued by the stimulation of NK cell with cytokines at least two days prior to the transduction. Our results, though, are in contrast with previous studies regarding the optimization of CAR-NK manufacturing, implementing IL-21 in their protocols (249,363). However, autologous feeder cells were also included which elicit enhanced proliferation via cell surface receptor-ligand interaction, further enhanced by cytokines (363,364), such as IL-21.

These ML-CAR-NK induced a higher response against GCP-3 positive HCC target cell lines, with a prominent synergistic combination of memory-like and CAR-mediated killing (**Figure 34**). Overall, the results indicated that CAR-engineered NK cells mediate tumour cell killing by incorporating the use of their own endogenous receptors, cytokine-augmented effector functions through priming and CAR-driven cytotoxicity. As expected, successful tumour cell killing was seen with HepG2 cell line (**Figure 34**), as it expressed higher levels of the target antigen GCP-3 (**Figure 32**). A closer analysis revealed that the three CAR constructs investigated, GC33, YP7 and 9F2 showed notably different results, influenced not only by the condition used to culture CAR-NK cells, but also by co-culture time with the target cell lines. 9F2 CAR-engineered NK cells showed the highest killing of both HepG2 and PLC after 4 hours co-culture (**Figure 34**), and enhanced functional response measured as IFN-γ secretion (**Figure 33**). Together these results indicated that the increased anti-tumour functions are CAR-mediated and antigen specific. After 4 hours co-culture, addition of IL-21 (condition 3) did not seem to increase cytotoxicity, in line with what we observed in chapter 3 (**Figure 14**) and in chapter 4 (**Figure 21**).

Interestingly though, a longitudinal dynamic monitoring of CAR-NK co-culture with HCC cell lines, showed the marked benefits of including IL-21 in the protocol (**Figure 35**). Furthermore, long term co-culture induced a switch in the CAR construct involved in tumour cells killing. As an explanation for this, two hypotheses were put forward. The simpler one is that the tumour cells might have mutated the antigen and hence the epitope available for the CAR to bind, as a consequence of the stress induced by the co-culture and the CAR-NK killing. This is a known mechanism adopted by cancer cells, to escape treatment and induce therapy resistance (365). The other, more complex, hypothesis is serial killing by NK cells, which acts in concert with the CAR-mediated killing (366). NK cells can mediate cytotoxicity by release of lysosomal-enclosed granules perforin and granzyme. Upon contact with the target cell, the secreted perforins form pores on the target cell membranes, through which granzymes reach the cytosol and initiate apoptosis. This process is fast and happens as soon as the NK cells come in contact with the tumour cells, inducing rapid tumour cells death (367–369). In addition to granules release, NK cells can also express ligands such as TNF-related apoptosis-inducing ligand (TRAIL) which activate the receptive receptor on the surface of the target cells. This

leads to activation of the death-inducing signalling complex, recruiting other adaptor proteins, and ultimately leading to apoptosis. This, however, is a much slower and time-consuming process. After apoptosis, via granule or death receptor mechanism, the NK cells disengage from the tumour cell and subsequently mediate killing of neighbouring target cells, a process named serial killing (370–372). It is possible that the early and rapid granule-mediated killing coincided with the 4 hours co-culture. After this, the NK death-receptor endogenous killing mechanism commences and it heavily synergises with CAR-mediated killing, causing increased killing with the different CAR. It can also be speculated that the mechanism of death-receptor mediated killing, might be favoured and enhanced by IL-21, as more tumour cell death is seen with condition 3 after in the long-term co-culture (**Figure 35**). Furthermore, the combination of tumour antigen mutation, and synergy between CAR-mediated and death-receptor-mediated induced killing, might explain the change of CAR which brings about the highest tumour death. This might justify the generation of CAR containing different scFv and, therefore, ensuring an increased CAR-NK antitumour response sustained over time, but more research is needed for this.

The anti-GPC-3 CARs analysed in this chapter, have already been included in other studies, and interestingly there are strikingly differences in the results reported. Previous studies describe a much higher transduction efficacy and higher cytotoxicity against the tumour cell lines HepG2 and PLC than we observed (212,319,373–375). Our aim was to compare these single-chain variable fragments (scFv) side by side, under the same experimental conditions, to determine their behaviour towards the target antigen and to establish which one gives the best results. As such, assessment of CAR expression might vary depending on when after transduction it is carried out and what is the detection method used. Selection of the appropriate scFv significantly affects the therapeutic efficacy of CAR-engineered immune cells, therefore, this step is crucial in a successful therapy outcome. The importance of scFv design has recently been reported in studies that show different scFvs targeting the same antigen but yielding different CAR-mediated results (376,377). Hence this phenomenon is not unique to our investigation, offering an important point of reflection which warrants further research for CAR optimization. Furthermore, it has been shown that post-translational modification of proteins results in differently functional CARs being expressed in B and T cells

(376). Indeed, most of the studies involving anti-GPC-3 CAR have been done either on T cells or NK-92 cell lines (212,319,373), hence we speculate that the post translational modification in the primary NK cells used in our studies might have affected CAR expression and hence its efficacy against GPC-3 positive tumour cells. Optimization of CAR design requires a thorough analysis of the elements necessary for transcriptional regulation of CAR such as the promoter or repressor, as well as the signal peptide, which encodes information for the post-translational assembly of the recombinant protein, to obtain a satisfactory outcome in the immune cells investigated.

The binding strength and efficacy of the CAR is also determined by the orientation of the light and heavy chains (VL and VH, respectively) that make up the scFv (378). These can be arranged in a VH-VL or VL-VH orientation, leading to a more or less effective CAR-mediated response. Future studies can focus on the analysis of the three GCP-3 scFv included in this chapter in both orientations to determine which one would the most efficient. In recent years, computer-assisted three-dimensional structural modelling has been used to predict several scFv designs with stable binding with the target, thus ensuring scFv efficacy (379). This approach can also be used for the of future generation of improved GCP-3 targeting scFv, which can be used to advance the affinity of CAR recombinant proteins and to minimise the on target but off tumour response.

Another key step in a successful immunotherapy development is the identification of specific tumour antigens. However, this is not an easy process, as antigens need to meet certain requirements. These are immunogenicity, expression patter with prevalence in tumour tissue, and biological significance which elicits a response (315). Additionally, antigens can be divided in different categories: tumour-associated antigens (TAA) which are proteins over expressed in tumour cell but also expressed in normal cells at lower levels, and as tumour-specific antigens (TSA) which are only found on cancer cells and can be defined as neoantigens (380). TSA, of course would be the ideal candidates, limiting the selection to antigens risen as a result of somatic mutations, chromosomal translocations resulting in neo-antigens, or viral derived antigens. However, exome sequencing has recently shown that somatic mutation

patterns, especially in HCC (381), are extremely variable among individuals therefore increasing the complications of TSA choice. Therefore, a viable alternative is oncofoetal antigens, expressed during embryogenesis but usually not in adults. Glypican-3 falls within this category, justifying the selection of this antigen as the one targeted by CAR-NK. HCC arises on a background of hepatis-B (HBV), and it is very prevalent in eastern Asia, where it accounts for ~65% of cases. Western countries are low endemic areas for HBV, where HCC is mostly diagnosed in patients with non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH) (147,315) and chronic alcohol abuse. Therefore, the pathophysiology of HCC is rather complex, and its understanding is yet to be translated into the clinics. This elucidates the difficulty of good TSA selections and further supports the choice of the oncofoetal antigen GCP-3 as the target, which has fast become the most specific and attractive target in HCC (148).

The work outlined in this chapter does not come without limitations. Due to time restrictions, it was not possible to test more NK cell donors which would have made the results more robust. This would have also informed us on how different donors respond and, therefore, how to tackle donor variation. Secondly, these experiments would benefit from more optimization. The analysis would benefit from the inclusion of non-cancerous cell line to evaluate the off-tumour effect of GCP-3 CAR-NK on healthy tissue. Furthermore, it would be good to carry out more long-term dynamically monitored CAR-NK co-cultured with tumour cell lines, using IncuCyte imaging systems and for a longer period than 48 hours. Additionally, the flow cytometry based killing assay might not be the best method to evaluate killing of tumour cells by CAR-NK as the cell lines used are adherent which might skew results. A better method to calculate killing of tumour cells could be to use GFP-based viability assay which measures a reduction of the green fluorescent signal released by the tumour cells. Alternatively, quantification of luciferase expression by tumour cells might constitute another method. Both these methods necessitate the expression of GFP and/or luciferase by the tumour cells, which can be circumvented by insertion of a reporter gene.

In summary, this chapter demonstrates the proof of concept to progress the study of CAR-modified memory-like NK cells for HCC and translate it into the clinic. Memory-like NK cells show an enhanced anti-tumour activity, which is enhanced by the addition of CAR. Thus, ML-CAR-NK are a promising approach to cancer immunotherapy and warrant *in vivo* and early-phase clinical trials.

Chapter 6 Final discussion

NK cells are important lymphocytes needed for the defence against virally infected or tumour cells within the body, playing an essential role in immunity (4). Their unique innate ability to kill modified body cells can be exploited and applied to develop novel immunotherapies. Understanding how NK cells can be manipulated for immunotherapies will improve the treatment for several difficult to treat cancer types, such as HCC. Unfortunately, the treatments currently available for HCC are not effective and it remains a challenging disease to manage (382). Thus, the purpose of this study is to understand how NK cells can be manipulated *in vitro*, to increase their cytotoxicity and proliferation for later development of NK-based immunotherapy against HCC.

NK cells can be modulated by cytokines, to enhance their anti-tumour function. Specifically, cytokine concentration, exposure time and combination all have an impact. This was the aim of the current study, and to this end we were able to show the importance of including IL-21 in NK cells protocols and how it regulates NK cell expansion and phenotype. However, further studies on how cytokine stimulation affects specific signalling pathways, as well as their molecular effect need to be carried out. Addition of IL-21 to the combination of cytokines required to generate memory-like NK cells caused increased proliferation, and high expression of activating receptors such as NKG2D, CD16, NKp30, NKp46, NKG2C. However, it also caused a high expression of the inhibitory receptor NKG2A. I hypothesized that high NKG2A expression drives NK cell proliferation forward, in line with recent studies (240). This, however, does not come without a cost, as high NKG2A expression level caused lower anti-tumour activity of NK cells (**Figure 14B**, **Figure 21A** and **B**). Therefore, it is important to balance cell proliferation and cell anti-tumour function, where a lower proliferation will result in higher effector function. This was observed with condition 12, where IL-21 synergises with IL-15 in the pre activation phase, causing a marginally lower proliferation with the benefit of a much higher anti-tumoural function (**Figure 17B**, **Figure 21A** and **B**). Interestingly however, NKG2A expression after stimulation with condition 12 is not lower than the one observed with condition 3, however a longer IL-18 exposure (3 days post priming in condition 12, (**Figure**

17B), might be enough to lower the activation threshold to overcome to achieve NK anti-tumour functions. IL-18 signals via NF-kB and because NF-kB inhibition caused lower NKG2A expression, I hypothesised that longer IL-18 exposure (for 3 days post priming, in condition 12) contributed to increased anti-tumour functions. High NK proliferation driven by IL-21 stimulation, was mediated by STAT3 signalling (**Figure 18C**), however inhibition of STAT3 or NF-kB revealed a previously unexplored signalling pathway of IL-21 via NF-kB. This shows that IL-21 mediated by NF-kB signalling might play a much more important role in proliferation, warranting further studies.

IL-21 induced NK cells to have a phenotype canonical of both immature and mature NK cells, expressing CD117, NKG2A and CD16, but lacking CD57 (**Figure 13**, **Figure 23**). I proposed a model where NK cells population comprises several subgroups with different phenotype and function, in line with Björkström et al (32). I hypothesise that NK cell population maturation occurs in waves, where older CD57+ senescent NK cells die off and the highly prolific CD57- cells take over and form the largest part of the population. This indicated that NK cells' maturation is not a linear nor a cyclical process and several phenotypical combinations can be present at any one time. The wave-like behaviour of cytokine-stimulated NK cells is also observed in their metabolic profile, presenting peaks and troughs (**Figure 28**).

The field of NK cell-based immunotherapy has experienced remarkable growth over the last 20 years, and currently is a major area of innovation regarding more specific tumour targeting. Challenges encountered during the development of NK-based immunotherapy have involved optimization of *ex vivo* manipulation of NK cells, especially to increase their expansion, enhancement of NK cell cytotoxicity and subsequent persistence *in vivo.* A variety of methods have been employed to augment NK cells cytotoxicity and longevity such as cytokine-based cultures, feeder cell-based methods, genetic engineering with chimeric antigen receptors, cell-engager molecules, pairing with immune-checkpoint inhibitors or antibodies. Preclinical and clinical studies have shown the great potential of NK-based immunotherapies, especially in myeloid cancer. This has encouraged further research to optimise and validate early clinical results in lager cohort of patients, by investigating resistant tumours and by analysing the

impact of the immunosuppressive tumor microenvironment on NK cells to extend the use of NK-based immunotherapy to solid tumours (19,383,384).

Cytokine-induced memory-like NK cells (CIML NK), generated via brief exposure to IL-12, IL-15, IL-18, have a greater ability to recognise and control tumour cells, compared to conventional unstimulated NK cells. CIML NK cells show an upregulation of activating receptors, are not affected by inhibitory KIRs, and have enhanced persistence *in vivo* (64,66,385). The efficacy of CIML NK cells has already been investigated in ovarian cancer and melanoma (141,386). Additionally, CIML NK cell expanded safely without causing CRS or neurotoxicity and induced composite complete remission in 47% of all the patients affected with relapsed/refractory AML involved in the phase I study (142). Furthermore, there has been a general effort to improve CIML NK recognition of tumor cells as reflected by preclinical analysis on haematological tumours combining CIML NK with CAR genetic engineering. The knowledge gained from a plethora of studies done on T cells for the development of CAR-T cells therapies, is being applied on NK cells. Combining cytokine activation with CAR-engineering, provides a synergistic effect that will augment NK cells anti-tumour activity and create a novel and promising therapeutic approach for cancer immunotherapy.

Similarly, to CIML NK cells, CAR NK have shown reduced risk of GvHD, CRS and neurotoxicity. Recent proof-of-concept analysis on CAR CIML NK cells targeting resistant B-cell lymphomas and AML demonstrated significantly enhanced anti-tumour functions (143,144). These promising results warrant further preclinical investigation to encourage the start of phase I trials. A very recent preclinical study demonstrated that CAR CIML NK cells had enhanced anti-tumour functions against head and neck squamous cell carcinoma (HNSCC), establishing a rationale for early phase clinical trials for patients affected with HNSCC (387). The successful results obtained in these studies, highlight the potential of CAR CIML NK cell-based approaches for advancing cancer immunotherapies. Understanding how cytokines modulate NK cells behaviour is essential for the development and improvement of NK cell-based tumour immunotherapies. However, further studies need to be carried out to analyse biomarkers that aid the development of efficacious “off-the-shelf” NK cells therapies. Not all

NK are endowed with the same anti-tumour functions due to baseline differences presented by the different NK cells subgroups. Interestingly, variation among donors, plays an important role in determining NK cells' function and response to cytokines. Therefore, it is important to identify the factor(s) that might set certain individuals apart to become better candidates for the development of efficacious "off-the-shelf" NK cell therapies.

The effectiveness of NK-based therapies in solid tumours has been scarce compared to haematological malignancies. This might be due to the tumour cells expressing high levels of ligands to inhibitory KIR and NKG2A, or due to the suppressive action of the TME (129). Therefore, while necessary to enhance NK cell-based therapies, investigation of strategies to modulate the TME and improve immunotherapy success is crucial. TME effect is specifically seen in the liver and HCC immunotherapy development and success. While immune checkpoint inhibitor (ICI) is a now well-established active therapy in the advanced stages of HCC, there is still a need for the development for new therapies with an improved outcome (148). Proof-of-concept studies on CAR-NK targeting the HCC antigens GPC3 or AFP have shown high anti-tumour activity against HCC (212,388). CAR-NK against the HCC antigen CD147 has also showed increased antitumor activities (389). The effectiveness of CIML CAR NK cells against HCC has not been investigated yet, therefore the results shown here of cytokine-activated CAR-GCP3 NK of higher tumour-killing ability against HCC cell lines compared to CIML NK or CAR NK only are very encouraging (**Figure 34**, **Figure 35**). This warrants improvement of current protocols employed to generate CIML CAR-GCP3 NK as well as *in vivo* investigation into mouse models, with the potential of future early phase clinical trial tests.

Chapter 7 Future work

7.1 Identification of optimal in vitro culture condition and the influence of cytokines stimulation on expanded NK cell

Deeper evaluation of NK cells signalling mechanisms, downstream of cytokine stimulation is fundamental to better understand how to manipulate NK cells and enhance their effector functions. To this end, analysis of the NF-kB, mTOR, and PI3K/AKT pathways downstream of IL-21 are necessary. Additionally, investigation of inhibitory and activating KIR and NKG2A expression before and after cytokine stimulation, especially in the presence of IL-21, via flow cytometry, can provide insights into NK cell education potentially explaining their activation pattern. This would contribute to the evaluation of NK cells' activation threshold against cancers. Furthermore, it would be interesting to evaluate whether cytokine stimulation might be able to switch KIR/NKG2A expression pattern to enhance NK effector functions.

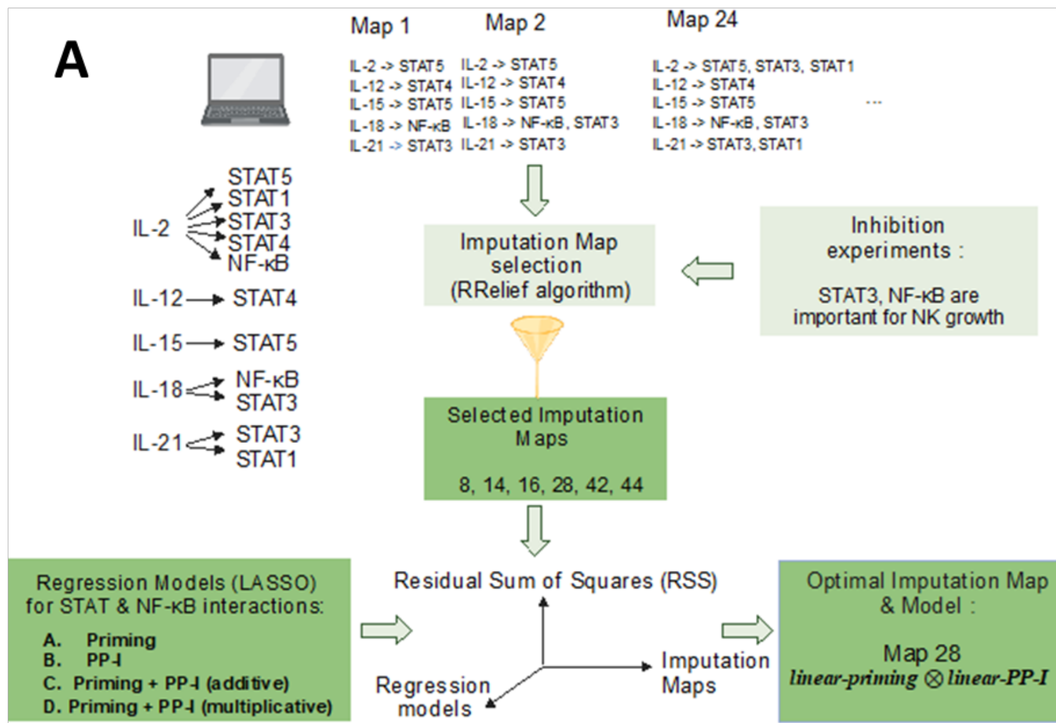
7.2 CAR-NK

Further research is needed to improve CAR design, specifically regarding the orientation of the VH-VL of the scFv region, surface expression of the recombinant protein and increase transduction efficiency.

A previous study from the Khakoo laboratory, shows that cytokine priming rescues antitumour functions from NK cells of HCC patients (65). Therefore, it would be interesting to combine this work with CAR-engineered primed NK cells and compare the response of peripheral blood NK cells from healthy as well as HCC patients. This would support the use of autologous CAR-NK generation, despite the individual being affected by HCC. Second to this, but certainly not less important is the analysis of the ability of CAR-NK generated using peripheral blood NK cells, to home to the liver. Essential in this is the expression of the receptor CXCR6, which mediates this process. Important for the success of a tumour immunotherapy is the effect of TME on the immune cells. As HCC has been described a "cold tumour" with remarkable tolerogenic and inhibiting abilities, it is fundamental to investigate how CAR-NK cells can be potentiated to circumvent the TME. Important to understand this

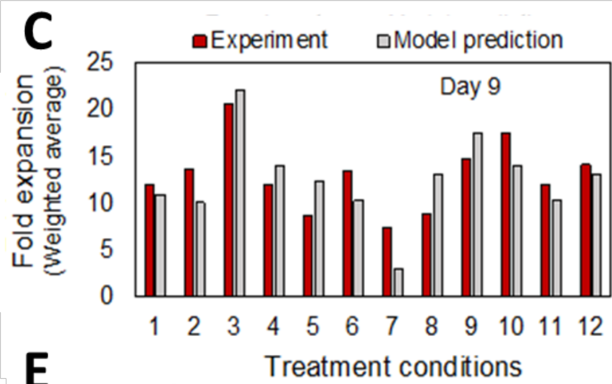
would be analysis of CAR-NK cells within HCC-generated organoids. A final and essential step in a proof-of-concept analysis to assess safety and efficacy of the therapy is to undertake *in vivo* studies in mouse models to evaluate the anti-tumour ability of CAR-NK cells.

Appendix A Additional results



B

Imputation Map 28
IL2 - STAT5, STAT4, STAT1
IL12 - STAT4
IL15 - STAT5
IL18 – NF-κB, STAT3
IL21 - STAT3, STAT1



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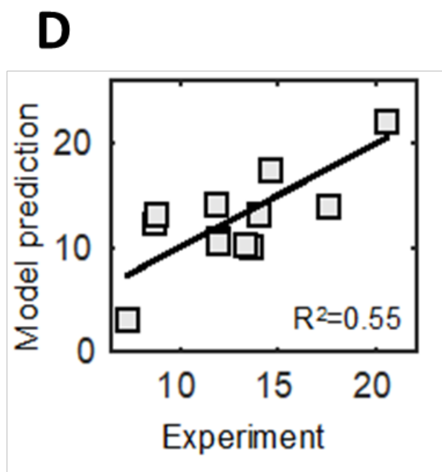
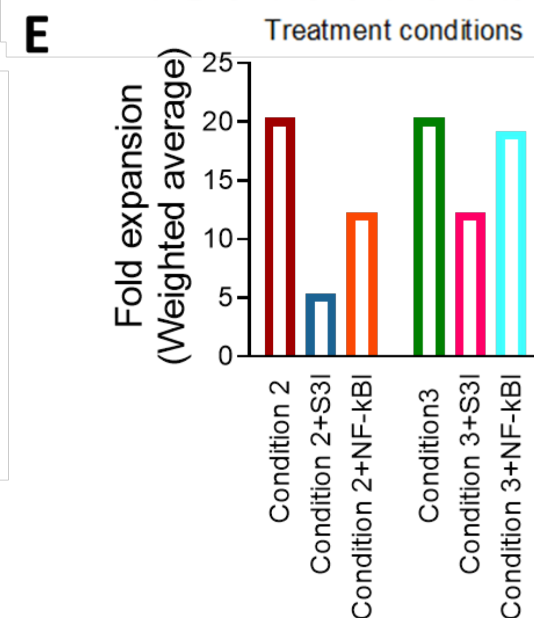
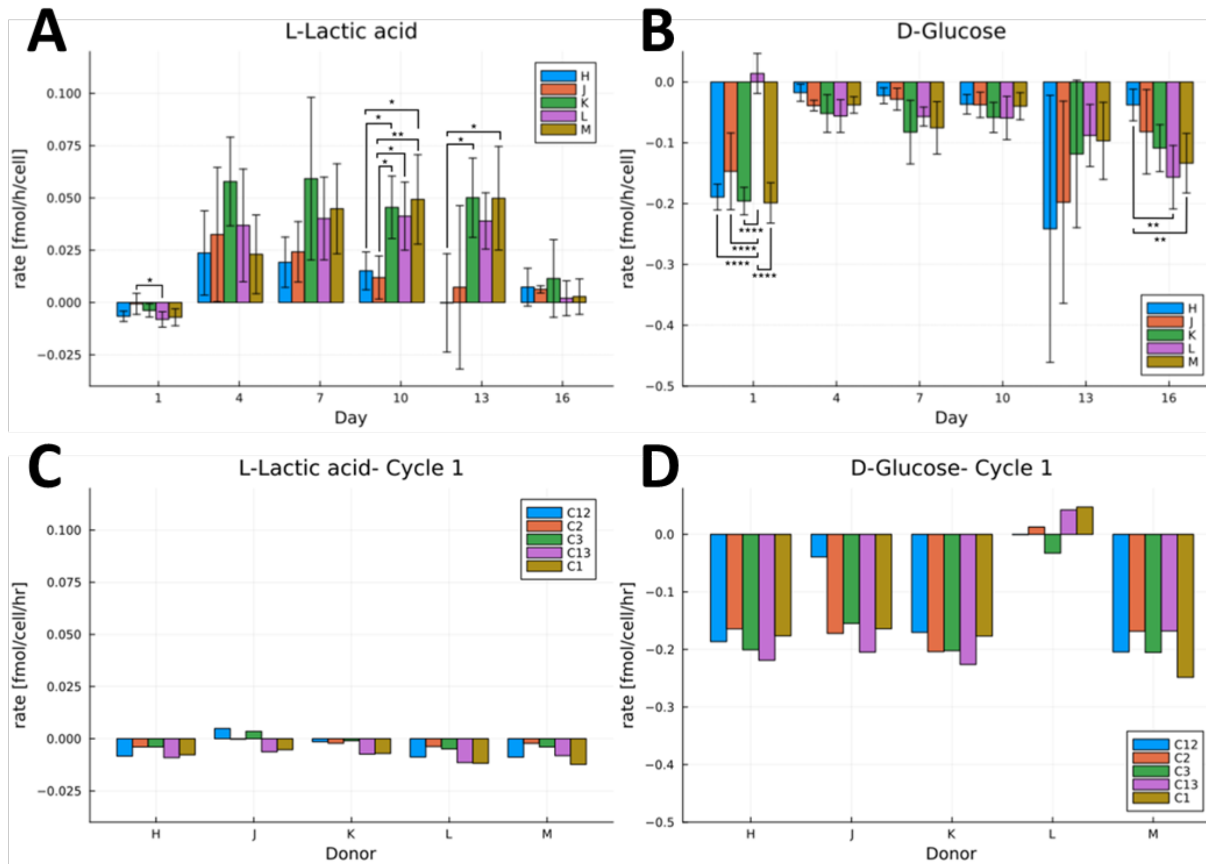




Appendix 1. Developing and testing a model for NK cell proliferation

A. Construction of the *in silico* predictive model for NK growth in terms fold change for various cytokine cocktails. **B.** STAT and NF-κB activation are shown for the optimal imputation map (Map 28) used in the *in silico* model at day 9. **C.** Comparison between the fold changes between experiment (maroon) and in silico model prediction (grey) are shown at day 9 for 12 cytokine cocktails using the optimal map and regression model. **D.** Overall prediction ability for 12 cytokine cocktail conditions using the optimal map and model is shown at day 9. Model prediction vs. experiment in fold change gives R-square = 0.55. **E.** Using optimal map and model, weighted average of fold expansion at day 9 are predicted for condition 2 and condition 3 with and without the presence of STAT3 and NF-κB inhibitors.



Appendix 2. Metabolism analysis of cytokine stimulated NK cells

A. L-Lactic acid produced by NK cells. **B.** D-Glucose taken up by NK cells. Metabolites in A. and B. were measured every three days for a total of 16 days. Data shown for each donor, independent of cytokines stimulation, as average ± SD, n=5. Data analysed with one-way ANOVA, with multiple comparison and Tukey's correction (*P≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001). **C.** L-Lactic acid produced by NK cells. **D.** D-Glucose taken up by NK cells. Metabolites in C. and D. were measured only for the first 16 hours of culture, priming stage. C1= condition 1, C2= condition 2, C3= condition 3, C12= condition 12, C13= condition 13 (IL-15 only, **2.10.6.1**).

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