Iron metabolism dictates NK cell function

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Jasmin Grählert

aus Deutschland

Basel, 2020

Genehmigt von der Philosophischen-Naturwissenschaftlichen Fakultät auf Antrag von

Prof. Christoph Hess

Dr. Martin Stern

Prof. Werner Held

Basel, den 21.05.2019

Prof. Dr. Martin Spiess

Dekan

TABLE OF CONTENTS

1 ACKNOWLEDGEMENTS I

2 ABBREVIATIONS III

3 SUMMARY V

4 INTRODUCTION 1

4.1 NATURAL KILLER CELLS 1

4.2 IMMUNOMETABOLISM 7

4.3 NUTRIENTS SUPPORTING IMMUNOMETABOLISM AND EFFECTOR FUNCTION 11

5 AIM OF THE THESIS 14

6 RESULTS 15

7 DISCUSSION 39

8 MATERIALS AND METHODS 45

9 REFERENCES 52

1 ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Christoph Hess for accepting me into his team and trusting me with a great deal of independency and providing guidance in critical moments. Thanks for having constant faith in my results. I admire your talent to see the big picture, the courage to think differently and your brilliant scientific ideas. It goes without saying that this project would not be where it is now without your support. And in addition, thanks for reminding me how to spell the words p-a-t-i-e-n-c-e and l-i-s-t-e-n-i-n-g.

I would like to acknowledge Martin Stern and Werner Held for being the members of my PhD committee, for helpful scientific discussions and willingness to review my thesis. Thanks to Martin Stern for statistical help. I thank Jean Pieters for agreeing to chair the defense.

I would like to thank Glenn Bantug for guidance and helpful scientific advice throughout my studies. Further, I want to thank Bojana Müller-Durovic for guidance and support. Bojana, you are an excellent scientist, a fantastic colleague and friend.

Thanks to Bojana, Glenn, Ilija, Maria and Jonas for proofreading different sections of this thesis.

Next, I would like to acknowledge all the past and present member of the Hess lab: Anne-Valérie Burgener, Maria Balmer, Rebekah Steiner, Gunhild Unterstab, Jordan Löliger, Jonas Lötscher, Philippe Dehio, Nadine Assmann, Réka Belle, Raja Epple, Patrick Gubser, Sarah Dimeloe, Marco Fischer and Leyla Develioglu. Thank you for the great atmosphere in the lab and for all scientific and non-scientific discussions we have had over the years. And thanks for putting up with my endless singing. Special thanks goes to Nadine, Jordan, Jonas and Philippe, I value the time we have spent over after-work beer. Maria, thank you so much for all the support; scientific and non-scientific advice throughout my PhD. Gunni, I highly appreciate our discussions about home and life in general; you are a great person. A very special thanks to Anne for your friendship, roughly 600 shared Mohrenköpfe, nights filled with great discussions and endless support.

Further, I would like to express my gratitude to Stipan Jonjić and Ilija Brizić. Our collaboration made a very important contribution to this work. Thank you so much for offering me the unique opportunity to work at your institute, the warm welcome and your trust. Thanks to the entire lab team of Stipan Jonjić and Bojan Polić. I enjoyed the kind atmosphere during work and I am grateful for your help. Felix, you are one of the most brilliant scientists I have met. A special thanks to Eddi for your kind personality and lovely discussions we had. Božo, thanks for your invaluable help on days of large mouse experiments. And thank you Tihana, Enrico, and Ivana for your friendship during my time in Rijeka and beyond.

I am grateful to everyone who has been involved in this project and I had the pleasure to collaborate with. I would like to give my credits to Robert Ivanek for his help with the RNA sequencing analysis. My sincere thanks goes to Nitish Mittal and Mihaela Zavolan for helping with ribosome profiling in NK cells. Thanks to Suzanne Welten and Annette Oxenius for performing MCMV infection experiments. Thanks to Nidhi Meta and David Finlay for experiments with knock-out mice.

I would also like to thank the great people I have met during this time and who contributed in different ways to where I am now: Sophia, Gernot, Olli, Aleksei, Julia, Marc, Madeleine, Laurent, Maria Z., Timo, Olga, Tamara and Sjoerd.

Most importantly, I would like to thank my parents, Birgit and Frank, for their endless support and unconditional love. Thanks to Charlotte and Manuel for always making me feel at home at your house. Thanks to Tamara for choosing the exact same career path, being my companion and friend for 20 years and with whom I share so many good memories. Special thanks also to Laura, Simone and Patricia. It would not have been possible without your love, continued patience, and most importantly all the moments we spent together.

2 ABBREVIATIONS

2-DG	2-Deoxy-D-glucose
6AN	6-aminonicotinamide
ACLY	ATP citrate lyase
ActD	Actinomycin D
AML	Acute myeloid leukemia
ATP	Adenosine triphosphate
BIP	2,2′-Bipyridyl
CE NK cell	Cytokine-enhanced Natural Killer cell
CFSE	Carboxyfluorescein succinimidyl ester
CHX	Cycloheximide
CLP	Common lymphoid progenitor
CMS	Citrate-malate shuttle
DC	Dendritic cell
DPI	Days post-infection
ECAR	Extracellular acidification rate
eIF4E	Eukaryotic translation initiation factor 4E
EBV	Epstein-Barr virus
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FTH1	Ferritin heavy chain 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Glut1	Glucose transporter 1
GvL	Graft versus leukemia
GvT	Graft versus tumor
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HPG	L-Homopropargylglycine
HSC	Hematopoietic stem cell
IFN-γ	Interferon gamma
IRP	Iron regulatory protein
KIR	Killer immunoglobulin-like receptor

LAMP-1	Lysosomal-associated membrane protein 1
LAT1	L-type amino acid transporter 1
MCMV	Murine cytomegalovirus
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
MIC	MHC I chain-related molecules
mTOR	Mammalian target of rapamycin
NADH	Nicotinamide adenine dinucleotide
NK cell	Natural Killer cell
NV NK cell	Naïve Natural Killer cell
OXPHOS	Oxidative phosphorylation
PCA	Principal component analysis
PFU	Plaque forming unit
PID	Primary immunodeficiency
PBMCs	Peripheral blood mononuclear cells
PPP	Pentose phosphate pathway
SIREs	Searching for iron-responsive elements
SREBP	Sterol regulatory element-binding protein
TCA	Tricarboxylic acid cycle
TIBC	Total iron-binding capacity
UIBC	Unsaturated iron-binding capacity
ULBP	UL16-binding protein
WT	Wild type

3 SUMMARY

Background and rational: The ability to lyse tumor cells without prior sensitization endows NK cells with great therapeutic potential for adoptive cell therapy against various malignancies. Of particular interest are cytokine-enhanced – "trained" or "memory-like" – NK cells due to their enhanced responsiveness when reactivated. The molecular mechanisms underpinning augmented responses of cytokine-enhanced NK cells remain unknown. Cellular metabolism regulates cell proliferation and effector maturation alike, both critically important to effective and sustained adoptive cell therapy. Here we assessed how cellular metabolism relates to proliferation and effector maturation of naïve (NV) vs. cytokine-enhanced (CE) NK cells.

Results: Glycolysis was similarly induced and equally required for NV and CE NK cells to proliferate and acquire effector function. By contrast, upregulation of CD71 was a key discriminating factor between *in vitro* activated NV and CE NK cells, with distinctly higher cell surface expression on stimulated CE NK cells. Differential expression of CD71 translated into an increased capacity of CE NK cells to take up transferrin/iron, and was associated with higher proliferation rates. CD71-mediated iron uptake was a prerequisite for activation-induced NK cell proliferation also *in vivo*. In CE NK cells upregulation of the iron regulatory proteins 1 and 2 (IRP1/2) selectively created a pseudo iron deficient state. This cellular state enabled increased translation of CD71 and hence proliferation of activated CE NK cells.

Conclusions: Our data (i) identify CD71-mediated iron uptake as a metabolic checkpoint regulating NK cell proliferation, and (ii) assign a novel role to IRP1/2 through creating pseudo iron deficiency in a cell population-selective manner. Regulating CD71 in the context of pseudo iron deficiency enabled increased proliferation of CE NK cells – a concept with potentially broad relevance when aiming to improve proliferation of engineered immune cells.

4 INTRODUCTION

4.1 NATURAL KILLER CELLS

NK cells were classically defined as innate immune cells due to their ability to lyse tumor cells without prior sensitization[1,2]. However, recent studies in mice and humans have identified NK cell subsets possessing immunological memory features, defined as “adaptive” or “memory” NK cells[3-8]. It is now well accepted that NK cells have roles that span both innate and adaptive immune responses. NK cells are members of the innate lymphoid cell (ILC) family and, according to the current ILC nomenclature, belong to the group 1 ILC subset[9]. Cells in the ILC1 subset typically express the transcription factor T-bet and produce interferon gamma (IFN-γ) as their principal cytokine. However, unlike other group 1 ILC members, NK cells are unique in their expression of the transcription factor Eomes. More importantly, NK cells possess potent cytolytic function, which is in sharp contrast to other ILCs[10-12]. NK cells constitute 5-15% of circulating lymphocytes and they predominantly reside in blood, liver, spleen and bone marrow, and to a lesser extent in lymph nodes. NK cells are an important component of host protection, especially in anti-tumor and anti-viral immunity. They survey the host for infected or malignant cells and protect the host by directly targeting and eliminating these cells[13-15].

4.1.1 NK cell development and homeostasis

NK cells in mice and humans arise from bone marrow (BM) $CD34^+$ hematopoietic stem cells (HSCs). Initially, they were thought to develop exclusively in the BM, however, recent studies have indicated that they can also develop in tissues such as the thymus, secondary lymphoid tissue and the liver[16]. Maturation of NK cells is accompanied by the differential expression of lineage-specific surface markers that selectively identify developmental intermediates[17]. NK cell lineage commitment in mice and humans is characterized by the acquisition of the IL-15 receptor (CD122) and thus IL-15 responsiveness. Once committed towards the NK cell lineage, these cells require continuous IL-15 signaling, which promotes NK cell differentiation, functional maturation and survival[18,19]. In addition to IL-15, other common γ chain (γC) cytokines (i.e. IL-2, IL-4, IL-7, IL-9, and IL-21) regulate NK cell development and maintenance in the periphery[20,21]. The microenvironment strongly shapes NK cell development leading to the generation of diverse, tissue-specific subsets with unique functions. Recent studies have

revealed that NK cells are remarkably diverse, with up to 30'000 phenotypically distinct subsets in the peripheral blood of healthy individuals[22-24].

The best characterized human NK cell subsets are identified as immature $CD3^{-}CD56^{bright}CD16^{-}$ and mature $CD3^{-}CD56^{dim}CD16^{+}$ cells. $CD56^{bright}$ NK cells are more responsive to activation by cytokines (e.g. IL-2, IL-15 and IL-12) and rather have immunomodulatory roles. Activation of $CD56^{bright}$ NK cells primarily induces potent IFN-γ and TNF production, as well as induction of other cytokines and chemokines (e.g. GM-CSF, MCP-1, CCL3 (MIP1α), CCL4 (MIP1β), CCL5 (RANTES)). By contrast, $CD56^{dim}$ NK cells are more responsive following stimulation of activating receptors and are less responsive towards cytokines. $CD56^{dim}$ cells are considered to be the cytotoxic effector subset, due to preformed stores of granzymes and perforin that are able to quickly mediate potent cytotoxic function. It should be noted that upon activation, $CD56^{dim}$ NK cells can also produce abundant amounts of IFN-γ and other proinflammatory cytokines[25,26].

Similar to the CD56 classification in humans, expression levels of CD27 and CD11b provide a functional classification of murine NK cells. $CD11b^{-}CD27^{+}$ NK cells are the most immature subset, followed by the double positive $CD11b^{+}CD27^{+}$ subset, whereas $CD11b^{+}CD27^{-}$ NK cells represent the most mature one. Analogous to their human counterparts, murine NK cells also produce less cytokines but display enhanced cytotoxic function during peripheral maturation[27].

4.1.2 Activation of NK cells

NK cell responses (e.g. cytotoxicity, proliferation or cytokine secretion) triggered by virus-infected or malignant cells are often controlled by the integration of signals through multiple activating and inhibitory receptors[28]. In addition, cytokines released by immune cells, such as dendritic cells (DCs), macrophages and T cells are required to trigger optimal NK cell function[20].

Inhibitory and activating receptors

Inhibitory receptors engage mostly major histocompatibility class I (MHC class I) molecules, which are ubiquitously expressed on the surface of nucleated cells. This self-recognition is important to prevent NK cell auto-reactivity. Healthy cells expressing high levels of MHC class I sustain self-tolerance and are protected from NK cell killing (**Figure 1**). By contrast, downregulation of MHC class I molecules during viral infection or malignant transformation triggers NK cells activation by removing inhibitory signals ("missing self") (**Figure 1**). Prominent

inhibitory NK cell receptors in humans are the highly polymorphic killer immunoglobulin-like receptors (KIRs), whilst the murine counterparts of KIRs are the Ly49 receptors. The non-polymorphic inhibitory CD94/NKG2A receptor is expressed in both species[29-31].

Activating receptors recognize stress-induced ligands on virus-infected or malignant cells, e.g. MHC I chain-related molecules (MICA and MICB) or UL16-binding proteins (ULBPs). Expression of these stimulatory ligands on target cells can overcome constitutive inhibition delivered by inhibitory receptors and thus activate NK cells, a term called "induced-self recognition" (**Figure 1**)[31]. NK cells express several activating receptors, including natural cytotoxicity receptors (NCRs), such as NKp46, NKp44 and NKp30; NKG2D, CD94/NKG2C, DNAM-1, NKp80, 2B4 and the Fc receptor CD16[32]. There is no evidence for a dominant activating receptor on NK cells with the exception of CD16. Ligation of CD16 alone with monoclonal antibodies induces NK cell activation[33]. In general, activation is achieved by co-engagement of multiple activating receptors, which may serve as a safeguard to prevent unrestrained activation of NK cells.

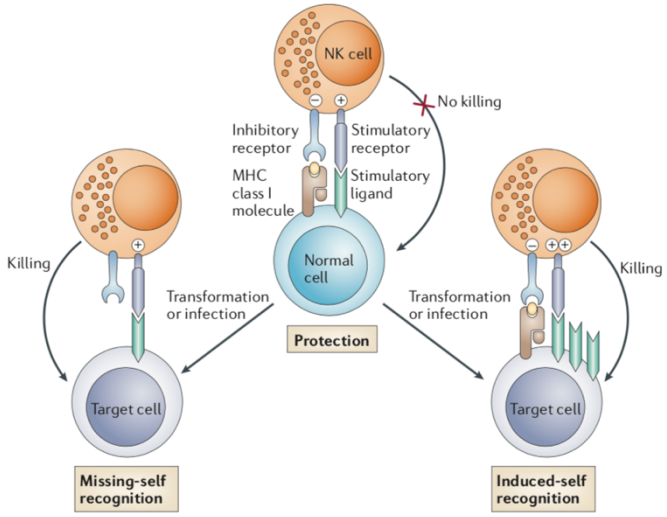

Figure 1: NK cell inhibition and activation through virus-infected or malignant cells.
Reprinted from Raulet and Vaunce, 2006[31].

Cytokine-induced NK cell activation

Cytokines, including IL-2, IL-12, IL-15 and IL-18 are known for their role in regulating maturation, activation and/or survival of NK cells. IL-2, produced by T cells and DCs, has been described to augment NK cell function. IL-2 promotes survival through the induction of an anti-apoptotic program; and induces IFN-γ production, proliferation and cytotoxicity[34-37]. IL-15, produced by DCs, macrophages and stromal cells, shares many properties with IL-2. IL15

augments proliferation, induces cytotoxicity, supports homeostatic proliferation and promotes survival of mature NK cells; in addition to its critical role in NK cell development[38].

IL-12 is produced by monocytes, macrophages and DCs and is able to augment IFN-γ production, cytotoxicity and proliferation of NK cells[37,39-42]. In addition, IL-12 has been implicated in promoting the differentiation of NK cells *in vitro* and is indispensable for the generation of MCMV-specific memory NK cells[43,44]. The proinflammatory cytokine IL-18, produced by DCs and macrophages, is also capable of directly activating NK cells and triggers cytokine production and cytotoxicity[45-50].

Importantly, the combined activation of NK cells with multiple cytokines or cytokine stimulation in combination with activating receptors results in a robust response[51,52].

4.1.3 NK cell effector function

NK cells are endowed with the inherent capacity to recognize and kill foreign, infected, and malignant cells but also have important immunomodulatory roles. Secretion of cytokines and chemokines by NK cells can regulate T cell proliferation, DC maturation and innate immune control[53-56].

Cytotoxicity

Recognition of an aberrant target cell, e.g. virus-infected or malignant cell, triggers cytotoxic NK cell responses that can be subdivided into four steps. First, the lytic function of NK cells is triggered following formation of an immunological synapse (IS) between NK cells and target cells. Next, IS-induced activation of NK cells results in a cytoskeletal polarization towards the target cell membrane. This is followed by polarization of the microtubule organizing center (MTOC) and recruitment of secretory lysosomes that contain cytotoxic molecules towards the IS. Secretory lysosomes pass through the cortical actin network *en route* to the plasma membrane. Third, these secretory lysosomes dock with the plasma membrane and; lastly, secretory lysosomes fuse with the cell membrane and release cytotoxic molecules towards the target cell[57,58].

During NK cell degranulation, lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) and -2 (LAMP-2 or CD107b) transiently appear on the surface of NK cells. The expression of LAMP-1 on the NK cell surface has been used as a surrogate for NK cell cytotoxic function[59]. Major cytotoxic granule proteins found in secretory lysosomes include perforin, a membrane

disrupting protein; and granzymes, a family of serine proteases with various substrate specificities[60]. Perforin released onto the target cell polymerizes and forms membrane-spanning pores that facilitate the entry of granzymes into the target cell[61,62]. Eleven granzymes have been described (A–H, K, M and N) five of these are expressed in humans (A, B, H, K and M) and ten are found in mice (A–G, K, M and N). Granzyme A and B are the most abundant and best-studied granzymes[63].

Cytokine production

Besides their ability to kill target cells, NK cells also have critical immunomodulatory roles through secretion of cytokines and chemokines. Among the most prominent cytokines produced by NK cells are IFN-γ and TNF that regulate functions of DCs, T cells and B cells. IFN-γ plays also a crucial role in anti-viral, anti-bacterial and anti-tumor activities mediated by NK cells. TNF is important to mediate apoptosis of NK cells and neighboring cells to delimit local immune response[56,64]. Chemokines produced by NK cells, i.e. CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), and CXCL8 (IL-8), attract effector lymphocytes and myeloid cells to inflamed tissues[65-67]. In contrast to activating local immune cells, NK cells can also dampen the inflammatory response via production of the immunosuppressive cytokine IL-10[68].

4.1.4 NK cells in viral clearance

NK cells are thought to contribute to the control of many viral infections, including human immunodeficiency viruses (HIV), influenza, hepatitis C virus (HCV), and most importantly in the control of herpesviruses (i.e. cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV))[69]. Several studies have demonstrated increased susceptibility and resistance to murine cytomegalovirus (MCMV) infection in mice after NK cell depletion and NK cell adoptive transfer, respectively[70,71]. Defects in NK cell effector functions, specifically in cytotoxicity and IFN-γ production, also increase susceptibility to MCMV infection[55,72]. Individuals with dysfunctional NK cells have an increased susceptibility to viral infections; particularly, herpesvirus susceptibility is a unifying feature of human NK cell deficiencies[73-75].

To control virus infection, NK cells can dampen viral replication, directly kill virus-infected cells and influence the ensuing $CD8^+$ T cell response[76]. The activating receptor Ly49H, from C57BL/6 mice, was the first NK cell receptor described to be a viral resistance gene (*cmv1*). Ly49H recognizes the MCMV m157 viral protein, which triggers release of cytotoxic granules,

cytokines, and chemokines, as well as a robust proliferative response in NK cells[77-79]. To date, there is no Ly49H analogue described in humans.

4.1.5 NK cells in tumor surveillance

As described above, NK cells are able to control malignant cells that downregulate MHC class I molecules and upregulate stress-induced ligands. NK cells can dampen tumor growth by direct cytotoxic action on tumor cells or by secretion of cytokines that orchestrates the anti-tumor immune responses. Several studies have shown, that the direct tumor cell lysis is primarily perforin-dependent[80-83]. Remarkably, NK cell-deficient mice display profoundly impaired tumor cell killing; and low NK cell activity in humans is associated with an increased cancer risk[84,85]. Since NK cells are remarkably efficient effectors against malignant cells, they hold great therapeutic potential for adoptive cell therapy against various malignancies.

4.1.6 NK cells in immunotherapy

Several attributes of NK cells make them ideal candidates for adoptive cell therapy. In addition to being highly cytotoxic effectors, NK cells are not restricted by antigen specificity, and they rapidly produce proinflammatory cytokines that potentiate adaptive immune responses[86].

NK cells from patients with cancer are often dysfunctional, displaying reduced rates of proliferation, decreased responses to cytokine stimulation and reduced effector function[87-89]. Thus, early immunotherapeutic strategies aimed to enhance or restore functions of endogenous NK cells. These strategies involved IL-2-induced activation of autologous NK cells *ex vivo,* followed by reinfusion into the patient together with combined IL-2 treatment during the course of therapy. Unfortunately, IL-2-activated NK cells did not impact tumor growth and the treatment regimen had severe side effects[90]. The use of allogeneic NK cells for the treatment of cancer patients is more promising, since allogeneic NK cells are fully functional, as compared to patients NK cells. In addition, allogeneic NK cells have graft-versus-leukemia/tumor (GvL/GvT) effects without causing graft-versus-host disease (GvHD), thus cause less immunopathology[91-93].

Clinically relevant responses have been achieved, particularly in the treatment of hematologic malignancies, such as acute myeloid lymphoma (AML) and non-Hodgkin lymphoma (NHL)[94-96]. However, the activity of NK cells alone is often insufficient to fully control tumor growth; and the treatment of solid tumors is particularly challenging due to the restrictive tumor

microenvironment[97-99]. Thus, strategies to enhance NK cell function have been investigated extensively. One approach to enhance NK cell anti-tumor activity is the utilization of cytokines. Various cytokines have been used (IL-2, IL-12, IL-15, IL-18, IL-21 and type I interferons) for *in vitro* expansion and activation of NK cells prior to adoptive transfer[100-103].

Cytokine-enhanced NK cells

One promising cytokine combination to maximize NK cell function is the combinatorial use of IL-12, IL-18 and IL-15. Stimulation with this combination induces a population of NK cells with "memory-like" features, such as prolonged survival and enhanced effector functions[104,105]. Pre-clinal studies have shown that cytokine-enhanced (CE) NK cells have substantial potential as anti-leukemia cellular therapy. In *in vivo* tumor models of lymphoma or melanoma CE NK cells had enhanced effector function (IFN-γ production and cytotoxicity)[106-108]. Also, following adoptive transfer into immunodeficient NOD-SCID-$\gamma_c^{-/-}$ mice (NSG), IL-2-enhanced CE NK cells persisted longer compared to control NK cells[109]. Finally, in AML xenografted NSG mice, CE NK cells substantially reduced AML burden and improved overall survival[110].

Molecular mechanisms driving increased effector functions of CE NK cells are currently unknown. For T cells, it is well established that the function of a certain subset, e.g. naïve $CD8^+$ T cells vs. memory $CD8^+$ T cell, is linked to different mechanism of metabolic regulation[111-113]. Thus, altered metabolic patterns in CE NK cells could support the enhanced function. Elucidating the molecular mechanisms that underpin differentiation of CE NK cells and their superior effector functionality is an important prerequisite to improve clinical efficacy.

4.2 IMMUNOMETABOLISM

Immune cells travers multiple tissues, thus facing diverse conditions of nutrients and oxygen availability. Thus, lymphocytes need to be able to readily change, adapt and integrate their cellular metabolism within the extracellular environment[114]. In addition, immune responses involve rapid changes in immune cell function and activities that rely on dynamic metabolic reprogramming and distinct metabolic activities to support cellular demands[112,113].

Metabolism can be broadly divided into anabolic, energy-consuming, biosynthetic processes; and energy-generating, catabolic processes. Cells require the energy-carrying molecules adenosine triphosphate (ATP) for most cellular processes, which is generated via glycolysis and/or oxidative phosphorylation (OXPHOS). Glycolysis converts glucose to pyruvate through

a series of enzymatic steps that occur in the cytosol that concomitantly generates two molecules of ATP. Depending on the cellular activity, pyruvate can be converted into lactate and secreted, or fully oxidized to CO_2 in the mitochondria. In the mitochondria, pyruvate is converted to acetyl-CoA, which is metabolized via the tricarboxylic acid (TCA) cycle. Breakdown of acetyl-CoA by the TCA generates the reducing equivalents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) that donate electrons to the electron transport chain to fuel OXPHOS, resulting in the generation of up to 34 ATP per molecule of glucose[115].

4.2.1 Distinct metabolic configurations to match immune cell function

Immune cell subsets can adopt distinct metabolic configurations to meet diverse cellular demands, such as energy production and cellular biosynthesis. In general, quiescent, anti-inflammatory and long-lived immune cells have low metabolic requirements. They predominantly depend on oxidative metabolism to efficiently produce energy required for homeostatic needs and immune surveillance (**Figure 2A**)[116,117].

In contrast, proinflammatory immune cells have increased metabolic demands, due to their increased rate of proliferation and effector molecule production. Activated T cells upregulate aerobic glycolysis, which is the conversion of glucose into lactate, despite the presence of oxygen, known as Warburg effect (**Figure 2B**)[112,118-120]. Secretion of lactate allows continuous glucose flux and enables glycolysis to rapidly keep up with ATP demands. Aerobic glycolysis also regenerates NAD^+, which is used as a cofactor by numerous enzymes; and also promotes the build-up of biochemical intermediates that are necessary for nucleotide, amino acid and fatty acid synthesis[121]. In addition to supporting cellular function energetically, aerobic glycolysis has also been described to support cytokine production[122,123].

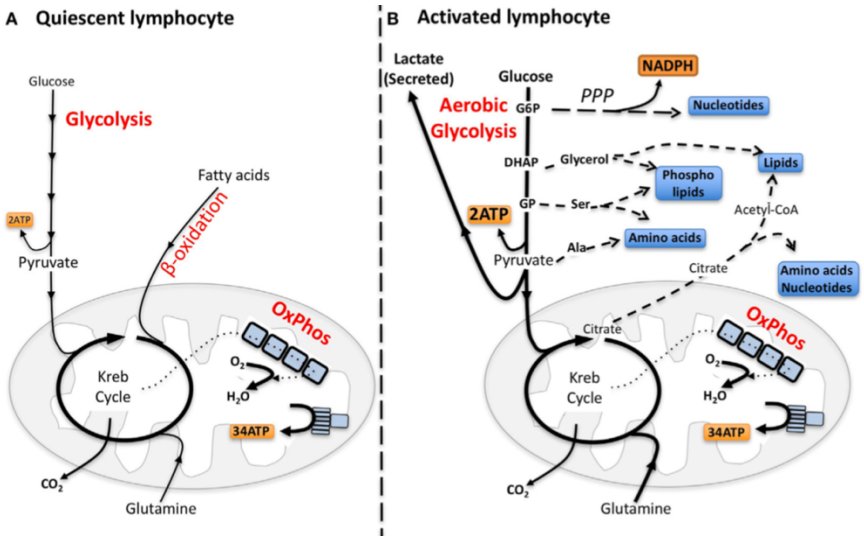

Figure 2: Metabolic phenotypes of quiescent and activated lymphocytes.
Reprinted from Gardiner and Finlay, 2017[116].

4.2.2 NK cell metabolism

Similar to quiescent T cells, resting NK cells have relatively low rates of glycolysis and OXPHOS[124,125]. IFN-γ production upon short-term stimulation with cytokines or via receptor crosslinking is independent of increased rates of glycolysis or OXPHOS[124]. However, stimulation of NK cells for sustained periods using IL-2, IL-12, IL-15 and IL-18 (or combinations thereof) strongly upregulates both glycolysis and OXPHOS[123,125-127].

In activated murine NK cells, a novel metabolic configuration driving OXPHOS has recently been revealed. NK cells stimulated with IL-2 and IL-12 metabolize glucose primarily to pyruvate and lactate, whilst low TCA cycle activity is observed. Remarkably, some pyruvate enters the mitochondria but is not metabolized via the TCA cycle as in other lymphocytes. Instead, pyruvate is converted to citrate which is further metabolized by the citrate-malate shuttle (CMS) (**Figure 3**). The CMS provides an alternative mode of generating NADH in the mitochondria to fuel OXPHOS and ATP synthesis via the export of mitochondrial citrate in exchange for cytosolic malate. In addition, cytosolic NAD^+ is also generated following the 2-step conversion of citrate to malate, which is an important cofactor for GAPDH, thus facilitating increased rates of glycolysis[128].

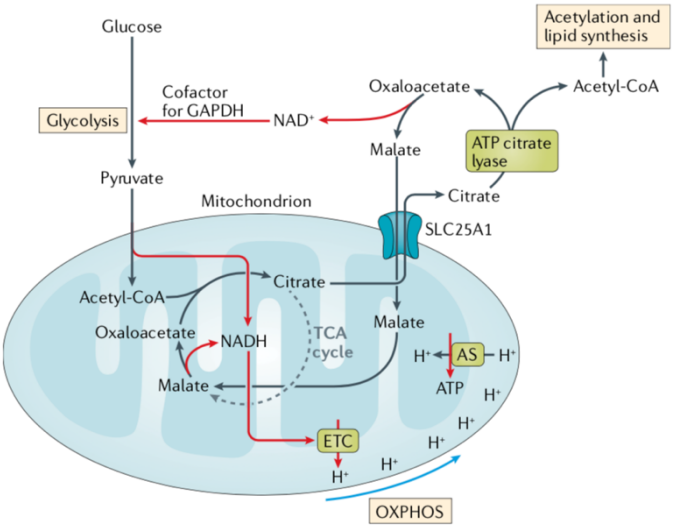

Figure 3: The citrate-malate shuttle.
Reprinted from O'Brien and Finlay, 2019[129].

Key regulators of NK cell metabolism

The mammalian target of rapamycin complex 1 (mTORC1) has been described as a key regulator of metabolism in cytokine-stimulated human and murine NK cells[123,125,126,130]. Increased mTORC1 activity in NK cells regulates increased expression of nutrient transporters and glycolytic enzymes, increased mitochondrial mass and production of effector molecules, including IFN-γ and granzyme B[123,125,126]. In addition, infection of mice with MCMV activates mTORC1 also *in vivo*, which regulates NK cell proliferation, IFN-γ production and cytotoxicity *in vivo*[131].

NK cells further depend on the activity of the transcription factor c-Myc, which regulates the transcription of genes related to the metabolic machinery, e.g. glucose transporters and glycolytic enzymes. Activity of c-Myc also supports high rates of OXPHOS by regulating mitogenesis to provide increased mitochondrial mass. Interestingly, IL-2 and IL-12-stimulated NK cells initially induce c-Myc expression in an mTORC1-dependent manner. However, sustained expression of c-Myc is facilitated by mTORC1-independent mechanisms[132].

Another transcription factor crucial for metabolic processes in cytokine-activated NK cells is sterol regulatory- element binding protein (SREBP). The canonical function of SREBP is the transcriptional regulation of genes involved in biosynthesis and uptake of lipids[133]. In cytokine-activated NK cells, however, SREBP is assigned a non-canonical function. It regulates the expression of the citrate-malate antiporter SLC25A1 and ATP Citrate Lyase (ACLY), both being

important for the CMS. SREBP thereby promotes increased rates of glycolysis and OXPHOS and thus is important for NK cell effector functions[128].

Most of these studies were performed on murine NK cells, and metabolism of resting and activated human NK cells has not been well characterized to date.

4.3 NUTRIENTS SUPPORTING IMMUNOMETABOLISM AND EFFECTOR FUNCTION

Major metabolic fuels that sustain lymphocytes are glucose, fatty acids and amino acids[134]. The utilization of these fuels supports distinct metabolic pathways important for lymphocyte function. In addition to macronutrients, which are important for fueling lymphocyte metabolism, micronutrients such as iron also support optimal lymphocyte function[135]. Several studies have reported that nutrient receptors, such as the high affinity glucose transporter (Glut1), the L-amino acid transporter LAT1/CD98 and the transferrin receptor (CD71) are expressed and upregulated on activated NK cells[123,125,126,136,137].

4.3.1 Fueling immunometabolism: glucose

Glucose is a key cellular fuel that serves as a substrate for ATP production and generation of biomolecules[138,139]. Importantly, glucose also fuels the pentose phosphate pathway (PPP) to generate NADPH and ribose 5-phosphate; a reducing equivalent and precursor for nucleotide synthesis, respectively[140]. As described, cytokine-mediated or MCMV-induced activation of NK cells is a key signal for glycolytic reprogramming that support the elaboration of effector functions in NK cells. NK cells take up glucose via Glut1 and glucose is then metabolized by glycolysis to generate pyruvate. Pyruvate is either metabolized to lactate which is secreted by the cell, or transported into mitochondria where it is metabolized mostly through the CMS[128].

4.3.2 Fueling immunometabolism: amino acids

Amino acids can serve as both a fuel source and a pool of biosynthetic precursors for protein and nucleic acid biosynthesis[141]. The heterodimeric amino acid transporter LAT1/CD98, with LAT1 being the transport competent unit, is a critical transporter of essential amino acids into activated lymphocytes. LAT1 preferentially imports large neutral amino acids (e.g. leucine, tyrosine, tryptophan) in exchange for the efflux of intracellular glutamine[142-144]. LAT1 is the predominant amino acid transporter in cytokine-activated NK cells. Amino acid uptake mediated by LAT1 is essential for sustaining c-Myc protein levels and thus, regulating

metabolic and functional responses of NK cells[132]. The importance of LAT1 in regulating NK cell function has been further demonstrated in a recent study: IL-18-activated NK cells induced dramatic metabolic changes via leucine-driven mTORC1 activation, which is necessary to drive proliferation of NK cells (**Figure 4**)[136]. Since LAT1-mediated amino acid uptake strictly depends on glutamine, glutamine uptake is indispensable for the above-described mechanisms to support NK cell function.

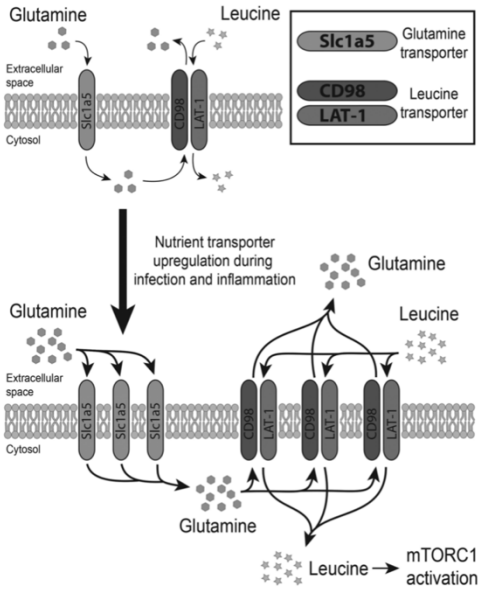

Figure 4: Leucine-driven mTORC1 activation in NK cells.
Reprinted from Almutairi et al. 2019[136].

4.3.3 Supporting lymphocyte function: iron

Highly proliferating cells strictly depend on iron to support basic processes, such as energy metabolism/respiration, DNA synthesis and repair, and cell cycle control[145-150]. The high amount of iron needed by proliferating cells, including lymphocytes, is provided by transferrin, which is taken up via the cell surface receptor CD71[135,151-153]. CD71 is commonly used as a lymphocyte activation marker and is expressed on activated NK cells[123,126,136,154].

Only few studies to date have investigated the importance of iron metabolism on lymphocyte function. A mutation in the CD71 receptor (*TFRC*$^{Y20H/Y20H}$) has recently been shown to impair T and B cell function due to impaired proliferation[135]. Reduced iron levels have been proposed to compromise NK cell cytotoxicity and dysfunctional NK cells and, in this setting, may contribute to cancer development in rats[155,156]. In addition, low serum ferritin levels have been associated with reduced NK cell activity in humans[157]. However, the specific impact of iron on NK cell-mediated immunity remains elusive.

Cellular iron homeostasis: IRP/IRE regulatory system

Cellular iron homeostasis is a tightly regulated process that involves the coordination of iron uptake, utilization and storage. It is mainly regulated at the post-transcriptional level by the iron regulatory protein/iron-responsive element (IRP/IRE) regulatory system. IRP1 and IRP2 are RNA binding proteins that recognize IREs in distinct mRNAs, thereby controlling their stability and translation to protein[158,159]. The activities of IRP1 and IRP2 are regulated in response to cellular iron levels[160-165]. Canonical IREs are present in the 5'UTR or the 3'UTR of mRNAs encoding for iron acquisition, iron storage, iron utilization, ATP production and iron export. Under iron-deficient conditions, the IRP activity is high and IRPs bind to IREs in the 5' or 3'UTR of the corresponding mRNAs. Depending on the localization of the IRE, IRPs can differently impact protein expression. Translation of mRNAs harboring an IRE in the 5'UTR (e.g. *FTH1* mRNA, ferritin light chain 1) is inhibited by IRP binding. In contrast, binding of IRPs to the 3'UTR IREs (e.g. *TFRC* mRNA, CD71) stabilizes mRNA and results in enhanced translation (**Figure 5**)[166-168]. Thus, the IRP/IRE regulatory network coordinates cellular iron homeostasis by selectively regulating the translation of certain mRNAs relative to the cellular iron status.

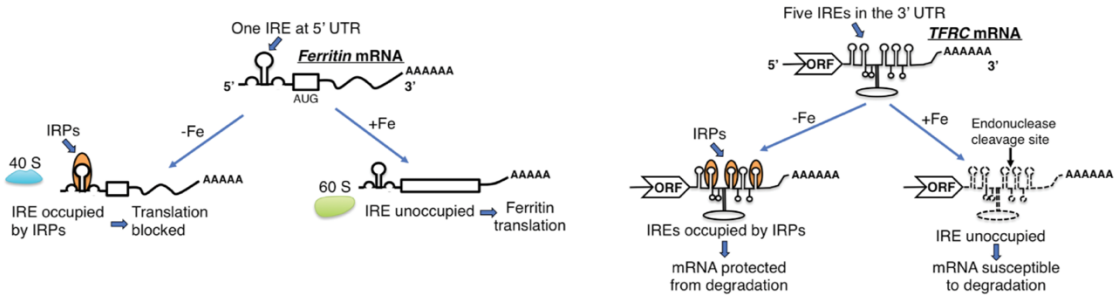

Figure 5: IRP/IRE regulatory system.
Adapted from Costain et al. 2019[169].

5 AIM OF THE THESIS

When used as adoptive cell therapeutics, cytokine-enhanced (CE) NK cells, compared to naïve (NV) NK cells, have superior efficacy *in vivo*, both in tumor models of lymphoma and melanoma, as well as in patients with acute myeloid leukemia. The aim of this thesis was to investigate how cellular metabolism relates to proliferation and effector maturation of NV and CE NK cells.

6 RESULTS

Naive and cytokine-enhanced NK cells similarly rely on glycolysis for IFN-γ production

Enhanced recall responses of cytokine-enhanced (CE) NK cells reflect a promising feature for immune cell therapy against cancer[106,107,110]. If and how CE NK cell metabolism underpins cytokine production, target cell clearance and proliferation remains unknown. To elucidate these key features of CE NK cells we used an established *in vitro* CE NK cell model that allowed us to compare naïve (NV) vs. CE NK cells[104]. Briefly, we primed freshly isolated human NK cells with IL-12 and IL-18 (IL-12/ IL-18) for 16 h, followed by a rest period in low dose IL-15 (IL-15 LD) to support survival. After 7 days of rest features of NV vs CE NK cells upon stimulation were compared (**Figure 6A**). In line with previous data priming of NK cells with IL-12/ IL-18 augmented their capacity to produce IFN-γ upon re-stimulation (**Figure 6B**). Of note, NK cells were similarly activated upon stimulation, as indicated by CD69 expression (**Figure 6C**). To explore how cellular metabolism relates to the function of NV vs. CE NK cells at the transcriptional level, RNA sequencing (RNA-seq) was performed using unstimulated and cytokine-stimulated cells. Both unstimulated as well as activated NV and CE NK cells clustered separately in the principal component analysis (PCA). Activation was, however, a much stronger overall discriminating factor, indicating a relative similarity between the transcriptomes of NV and CE NK cells (**Figure 6D**).

Rapid upregulation of aerobic glycolysis is a metabolic hallmark of activated lymphocytes, including NK cells[118,123,126]. Unexpectedly, both NV and CE NK cells similarly upregulated gene transcripts encoding for glycolytic enzymes upon stimulation, with the exception of *HK2* which was higher in CE than in NV NK cells (**Figure 6E**). In line with the transcriptome data, metabolic flux assays showed increased basal and maximal glycolytic rates of activated compared to unstimulated cells, yet no difference between NV and CE NK cells was observed (**Figure 6F**). Likewise, uptake of the glucose analogue 2-NBDG was not different between unstimulated and activated NV and CE NK cells (**Figure 6G**). To assess whether increased glycolytic metabolism was linked to the capacity of NV and CE NK cells to produce IFN-γ, we stimulated NV and CE NK cells with IL-12/ IL-18 in the presence of the hexokinase inhibitor 2-deoxy-d-glucose (2-DG). Inhibition of glycolysis during cytokine stimulation similarly reduced *IFNG* mRNA abundance and IFN-γ secretion in NV and CE NK cells (**Figure 6H and 6I**, left panel). Likewise, culturing NK cells in low glucose reduced production of IFN-γ in both subsets (**Figure 6I**, right panel).

Together these data identified a similar increase in basal and maximal glycolytic activity upon activation of NV and CE NK cells, which was in both subsets required for efficient production of the key inflammatory cytokine IFN-γ.

Figure 6

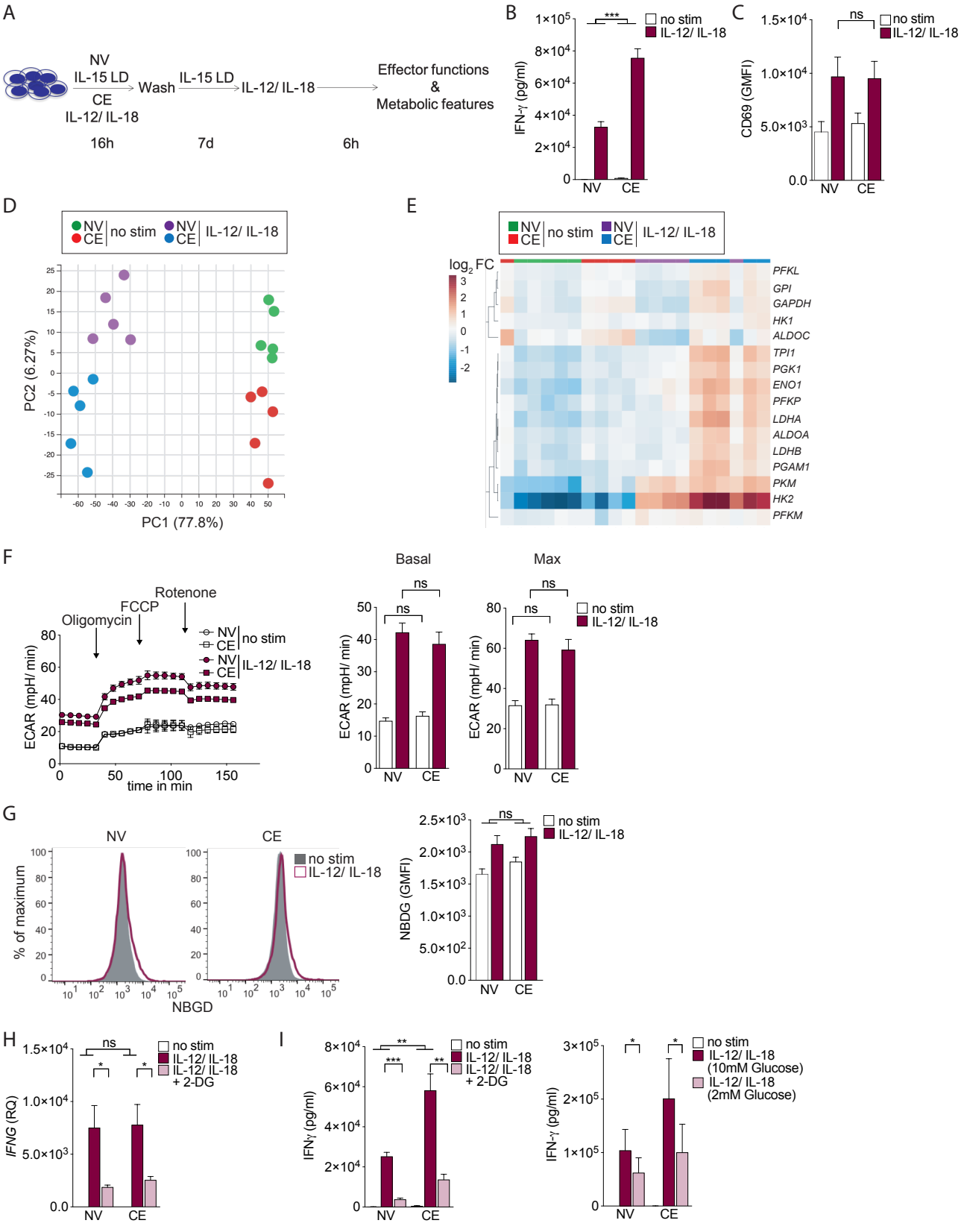
A
NV
IL-15 LD
CE
IL-12/ IL-18
16h
Wash
IL-15 LD
7d
IL-12/ IL-18
6h
Effector functions
&
Metabolic features
B
IFN-γ (pg/ml)
no stim
IL-12/ IL-18
NV
CE
C
CD69 (GMFI)
ns
D
PC2 (6.27%)
PC1 (77.8%)
E
log2 FC
PFKL
GPI
GAPDH
HK1
ALDOC
TPI1
PGK1
ENO1
PFKP
LDHA
ALDOA
LDHB
PGAM1
PKM
HK2
PFKM
F
Basal
Max
Rotenone
FCCP
Oligomycin
ECAR (mpH/ min)
time in min
G
NV
CE
% of maximum
NBDG
NBDG (GMFI)
H
IFNG (RQ)
IL-12/ IL-18
+ 2-DG
I
IFNγ (pg/ml)
IL-12/ IL-18 (10mM Glucose)
IL-12/ IL-18 (2mM Glucose)

Figure 6: Naïve and cytokine-enhanced NK cells similarly rely on glycolysis for IFN-γ production

(**A**) Schematic of the experiment used to generate CE NK cells. (**B**) IFN-γ production by NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (mean ± SEM, n=18 donors). (**C**) GMFI of CD69 expression on NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (mean ± SEM, n=13 donors). (**D**) PCA of the transcriptome data, depicting the group relationships in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18. The proportion of component variance is indicated as percentage (n=5 donors). (**E**) Heatmap of relative expression of mRNA encoding for glycolysis genes from NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 of the transcriptome data (n=5 donors). (**F**) *Left panel:* Representative mitochondrial perturbation assay of NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18. Glycolysis (extracellular acidification rate - ECAR) was measured ''in Seahorse'' after injection of oligomycin, FCCP, and rotenone. *Right panel:* Basal and maximal rate of ECAR in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 analyzed by mitochondrial perturbation assay (mean ± SEM, n=12 donors). (**G**) *Left panel:* Representative histogram of NBDG uptake in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18. *Right panel:* GMFI of NBDG uptake in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (mean ± SEM, n=15 donors). (**H**) Expression of *IFNG* mRNA in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18, IL-12/ IL-18 + 2-DG. Transcript levels were determined relative to *18S* mRNA levels and normalized to unstimulated (no stim) NV NK cells (mean ± SEM, n=6 donors). (**I**) *Left panel:* IFN-γ production by NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18, IL-12/ IL-18 + 2-DG (mean ± SEM, n=6 donors). *Right panel:* IFN-γ production by NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 in 10mM glucose and in 2mM glucose (mean ± SEM, n=5 donors). Statistical significance was assessed by paired two-tailed Student's t-test (C, F, H, I) or linear-regression analysis (B, G, H, I). *p < 0.05, **p < 0.01, *** p < 0.001, ns, not significant.

Activated CE NK cells are characterized by high levels of cell-surface CD71 and rapid cell proliferation

To further characterize the metabolic profile of NV vs. CE NK cells, we analyzed surface expression of the nutrient transporters CD98 and CD71 reported to be upregulated on activated NK cells[123,126,130,154]. Upon stimulation, a slight and comparable increase in CD98 expression on both NK cell subsets was observed (Figure 7A). In contrast, upregulation of the transferrin receptor CD71 was much greater on CE vs. NV NK cells, both when expressed as GMFI and percentage of positive cells (Figure 7B). Increased cell surface expression of CD71 was reflected by an overall greater cellular abundance of CD71 protein as assessed by immunoblot analysis of whole cell lysates (Figure 7C). To test whether differential cell surface expression of CD71 could also be driven by NK cell stimulation via activating receptors, both subsets were stimulated with HLA-deficient target cells (K562 cell line). Similar to cytokine stimulation, upregulation of CD71 was more prominent on K562-exposed CE than NV NK cells (Figure 7D). To assess the functional capacity of increased CD71 expression, we used fluorescently labeled transferrin to monitor transferrin uptake in NV and CE NK cells. These experiments revealed increased transferrin uptake in activated CE as compared to NV NK cells (Figure 7E).

Expression of CD71 and rates of proliferation have previously been linked in various cell systems[170-172]. To test whether this association also applied to NK cells, proliferation of NV and CE NK cells was monitored using CFSE dilution assays. Under both steady-state conditions and upon stimulation, CE NK cells proliferated to a greater extent than their NV counterparts (Figure 7F). In line with differential proliferation rates, stimulated CE NK cells clustered when testing abundance of transcripts encoding for cell-cycle progression genes (Figure 7G). To elucidate if increased transferrin uptake was linked to increased cell proliferation, we used the intracellular iron chelator 2,2′-bipyridyl (BIP)[173]. These experiments revealed that BIP inhibited NK cell proliferation in a dose-dependent manner in both NV and CE NK cells (Figure 7H). Of note, BIP had minimal effects on cell viability (data not shown), and no effect on NK cell activation as assessed by CD69 expression (Figure 7I).

The pentose phosphate pathway (PPP), providing ribose 5-phosphate and NADPH for nucleotide synthesis and reducing equivalents, respectively, supports cell proliferation[140]. In line with the increased proliferation observed in CE NK cells, cytokine stimulation increased

mRNA abundance of several PPP-related genes more prominently in CE than NV NK cells (**Figure 7J**, left panel). The PPP inhibitor 6-aminonicotinamide (6AN) prevented expansion of cytokine-stimulated NK cells, further supporting the relevance of the PPP in promoting proliferation of both NV and CE NK cells (**Figure 7J**, right panel)[174,175]. Together, these experiments identified (i) preferential upregulation of CD71 on activated CE vs. NV NK cells, and (ii) increased proliferation of activated CE over NV NK cells, which relied on PPP activity.

Figure 7

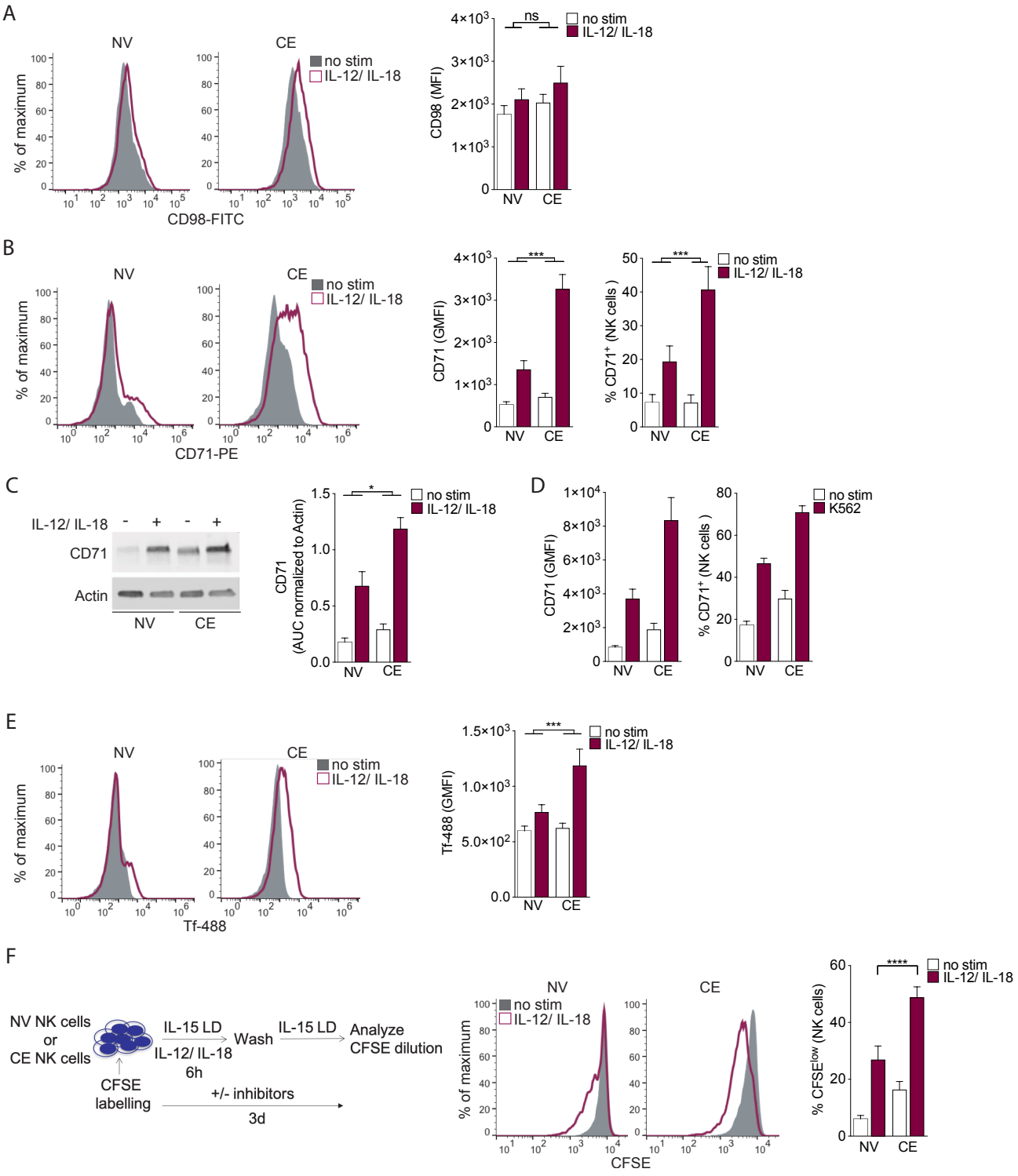
A
NV
CE
no stim
IL-12/ IL-18
% of maximum
CD98-FITC
CD98 (MFI)
ns
B
CD71-PE
CD71 (GMFI)
% CD71+ (NK cells)
***
C
IL-12/ IL-18
CD71
Actin
CD71 (AUC normalized to Actin)
*
D
K562
E
Tf-488
Tf-488 (GMFI)
F
NV NK cells
or
CE NK cells
CFSE
labelling
IL-15 LD
IL-12/ IL-18
6h
Wash
IL-15 LD
Analyze
CFSE dilution
+/- inhibitors
3d
% CFSElow (NK cells)
****

Figure 7

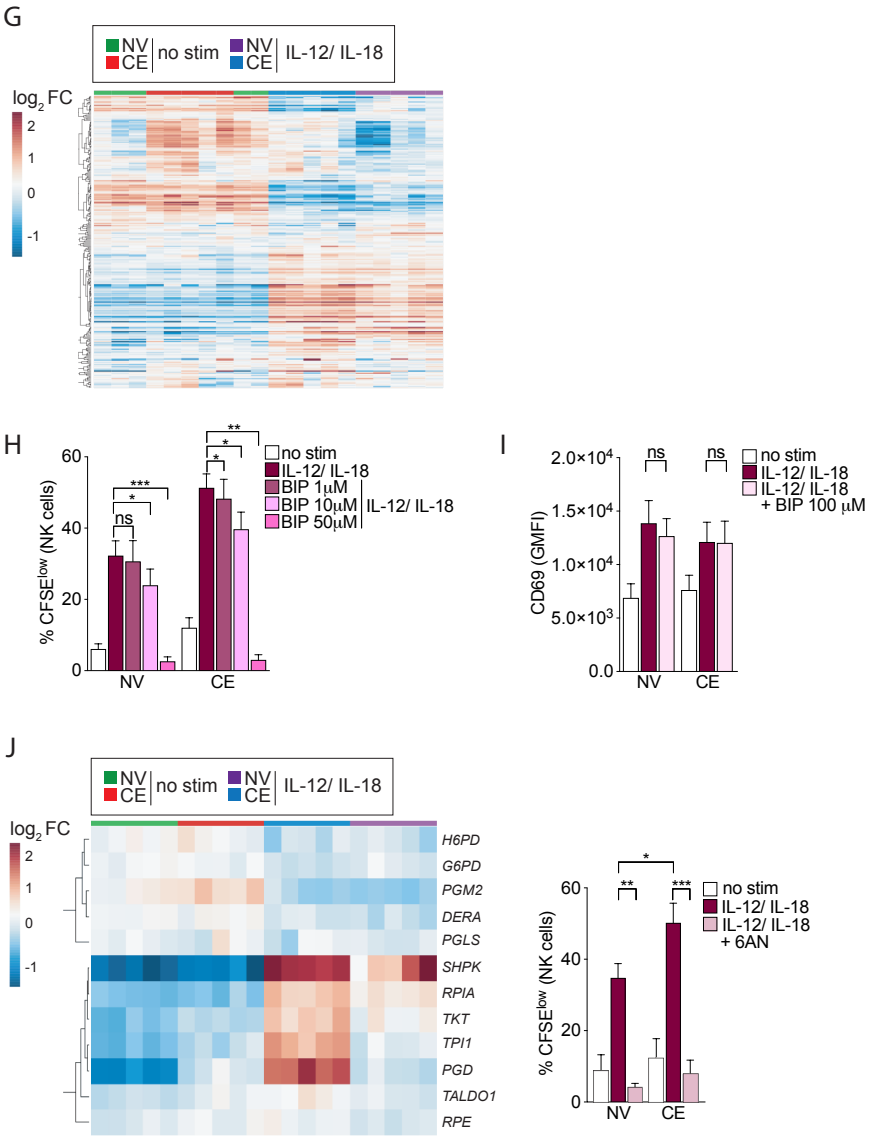
G
NV CE no stim
NV CE IL-12/ IL-18
log2 FC
2
1
0
-1
H
% CFSE low (NK cells)
60
40
20
0
NV
CE
ns
*
***
**
no stim
IL-12/ IL-18
BIP 1μM
BIP 10μM
BIP 50μM
IL-12/ IL-18
I
CD69 (GMFI)
2.0×10^4
1.5×10^4
1.0×10^4
5.0×10^3
0.0
ns
no stim
IL-12/ IL-18
IL-12/ IL-18
+ BIP 100 μM
J
log2 FC
H6PD
G6PD
PGM2
DERA
PGLS
SHPK
RPIA
TKT
TPI1
PGD
TALDO1
RPE
% CFSE low (NK cells)
no stim
IL-12/ IL-18
IL-12/ IL-18
+ 6AN

Figure 7: Activated CE NK cells are characterized by high levels of cell-surface CD71 and rapid cell proliferation

(**A**) *Left panel:* Representative histogram of CD98 expression on NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18. *Right panel:* MFI of CD98 expression on NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (mean ± SEM, n=8 donors). (**B**) *Left panel:* Representative histogram of CD71 expression on NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18. *Right panel:* GMFI and percentage of CD71 expression on NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (mean ± SEM, n=14 donors). (**C**) *Left panel:* Representative Western blot of total CD71 expression in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18. *Right panel*: Total CD71 expression normalized to actin in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (mean ± SEM, n=13 donors). (**D**) *Left panel:* GMFI of CD71 expression on NV and CE NK cells unstimulated (no stim) or stimulated with K562 (mean ± SEM, n=6). *Right panel:* Percentage of $CD71^+$ NK cells on NV and CE NK cells unstimulated (no stim) or stimulated with K562 (mean ± SEM, n=6 donors). (**E**) *Left panel:* Representative histogram of Tf-488 uptake in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18. *Right panel:* GMFI of Tf-488 uptake in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (mean ± SEM, n=10 donors). (**F**) *Left panel:* Schematic of the experiment used to analyze CFSE dilution in NV and CE NK cells. *Middle panel:* Representative histogram of CFSE dilution in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18. *Right panel:* Percentage of proliferated NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 analyzed by CFSE dilution (mean ± SEM, n=13 donors). (**G**) Heatmap of relative expression of mRNA encoding for cell cycle genes (GO:0006098) in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (n=5 donors). (**H**) Percentage of proliferated NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18, IL-12/ IL-18 + BIP (1, 10 and 50 μM) analyzed by CFSE dilution (mean ± SEM, n=11 donors for unstimulated, IL-12/ IL-18 and IL-12/ IL-18 + BIP 10 μM stimulation, n=8 donors for IL-12/ IL-18 + BIP 1 μM stimulation, n=3 donors IL-12/ IL-18 + BIP 50 μM stimulation). (**I**) GMFI of CD69 expression on NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18, IL-12/ IL-18 + BIP 100 μM (mean ± SEM, n=5 donors). (**J**) *Left panel:* Heatmap of relative expression of mRNA encoding for PPP genes (GO:0006098) in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (n=5 donors). *Right panel:* Percentage of proliferated cells in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18, IL-12/ IL-18 + 6AN 50 μM analyzed by CFSE dilution (mean ± SEM, n=6 donors). Statistical significance was assessed by paired two-tailed Student's t-test (F, H, I, J) or linear-regression analysis (A, B, C, E). *p < 0.05, **p < 0.01, *** p < 0.001, ns, not significant.

CD71-mediated iron uptake and dietary iron availability impact NK cell function

Recently, a mutation in the *TFRC* gene (*TFRC*$^{Y20H/Y20H}$) has been shown to impair B and T cell function, causing a primary immunodeficiency (PID). The mutation affects receptor-mediated endocytosis and compromises CD71-mediated iron uptake both in human cells and when introduced into mice. NK cell numbers in patients harboring this mutation are normal, however, functional properties have not been previously assessed[135]. To test whether CD71 function and NK cell proliferation are linked, we assessed CFSE dilution in IL-15 LD and IL-12/ IL-18-stimulated wild type (WT) and *Tfrc*$^{Y20H/Y20H}$ murine NK cells, *ex vivo*. These experiments revealed a striking lack of IL-15 LD and IL-12/ IL-18-induced proliferation among NK cells harboring the *Tfrc* mutation (**Figure 8A**).

Given this strong phenotype we wondered whether mild iron deficiency might be sufficient to cause NK cell dysfunction. To explore this notion, we first established systemic iron deficiency in a mouse model (**Figure 8B**, left panel). As expected, mice maintained on an iron-deficient diet for 6 weeks displayed reduced iron, ferritin and hematocrit levels in peripheral blood, while the unsaturated iron-binding capacity (UIBC) and the total iron-binding capacity (TIBC) increased as compared to mice kept on control diet (**Figure 8B**, right panel). While splenic T and B cell numbers were normal in mice with iron deficiency, NK cell numbers tended to be lower, possibly indicating selective sensitivity of these cells to systemic iron abundance (**Figure 8C**). No impact of iron deficiency on the NK cell maturation phenotype was observed (**Figure 8D**). However, upon MCMV infection, splenic NK cell-mediated viral control and IFN-γ production by NK cells tended to be reduced in mice maintained on iron-deficient diet, indicating impaired NK cell function (**Figure 8E**, left panel, **and 8F**). Noteworthy, replication of Δm157 MCMV evading NK cell-mediated control was unaffected by reduced iron levels (**Figure 8E**, right panel)[176]. Together, these data established that CD71-mediated iron uptake had an important role in regulating NK cell proliferation. Further, reduced systemic iron levels significantly impaired immune control of MCMV infection *in vivo,* possibly by reducing NK-cell function. It remains to be elucidated, whether impaired NK cell-mediated immunity resulted from NK cell intrinsic or extrinsic factors.

Figure 8

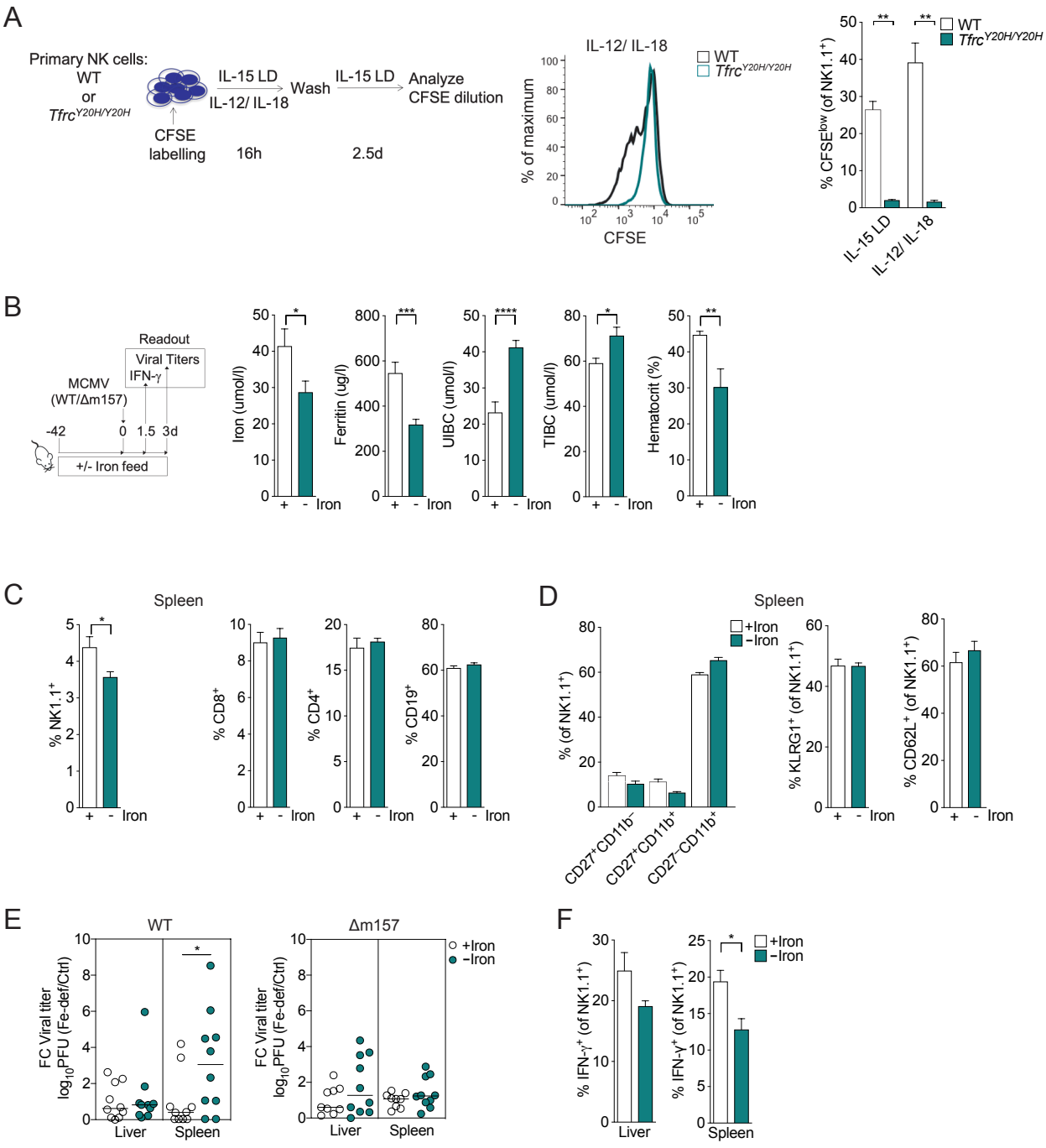
A
Primary NK cells:
WT
or
Tfrc Y20H/Y20H
CFSE labelling
IL-15 LD
IL-12/ IL-18
16h
Wash
IL-15 LD
2.5d
Analyze
CFSE dilution
IL-12/ IL-18
WT
Tfrc Y20H/Y20H
% of maximum
CFSE
% CFSE low (of NK1.1+)
IL-15 LD
IL-12/ IL-18
B
Readout
Viral Titers
IFN-γ
MCMV
(WT/Δm157)
-42
0
1.5
3d
+/- Iron feed
Iron (umol/l)
Ferritin (ug/l)
UIBC (umol/l)
TIBC (umol/l)
Hematocrit (%)
Iron
C
Spleen
% NK1.1+
% CD8+
% CD4+
% CD19+
Iron
D
Spleen
+Iron
-Iron
% (of NK1.1+)
CD27+CD11b-
CD27+CD11b+
CD27-CD11b+
% KLRG1+ (of NK1.1+)
% CD62L+ (of NK1.1+)
E
WT
Δm157
+Iron
-Iron
FC Viral titer
log10PFU (Fe-def/Ctrl)
Liver
Spleen
F
+Iron
-Iron
% IFN-γ+ (of NK1.1+)
Liver
Spleen

Figure 8: CD71-mediated iron uptake and dietary iron availability impact NK cell function

(**A**) *Left panel:* Schematic of the experiment used to analyze CFSE dilution in WT and *Tfrc*$^{Y20H/Y20H}$ NK cells from spleen. *Middle panel:* Representative histogram of CFSE dilution in WT and *Tfrc*$^{Y20H/Y20H}$ NK1.1^{+} NK cells from spleen with IL-12/ IL-18 stimulation. *Right panel:* Percentage of proliferated WT and *Tfrc*$^{Y20H/Y20H}$ NK1.1^{+} NK cells from spleen in IL-15 LD or stimulated with IL-12/ IL-18 analyzed by CFSE dilution (mean ± SEM, n=5 for WT NK cells, n=6 for *Tfrc*$^{Y20H/Y20H}$ NK cells). (**B**) *Left panel:* Schematic of MCMV infection experiment of mice fed +/– iron feed for 6 weeks. *Right panel:* Serum levels of iron, ferritin, UIBC, TIBC; and hematocrit from mice fed +/– iron feed for 6 weeks (mean ± SEM, n=8-18 for iron, ferritin, UIBC and TIBC and n=3 for hematocrit). (**C**) *Left panel:* Percentage of NK1.1^{+} NK cells in spleen of mice fed +/– iron feed for 6 weeks (mean ± SEM, n=5). *Right panel:* Percentage of CD8^{+}, CD4^{+}, CD19^{+} cells in spleen of mice fed +/– iron feed for 6 weeks (mean ± SEM, n=5). (**D**) *Left panel:* Percentage of CD27^{+}CD11b$^{–}$, CD27^{+}CD11b^{+}, CD27$^{–}$CD11b^{+} on NK1.1^{+} NK cells in spleen of mice fed +/– iron feed for 6 weeks (mean ± SEM, n=5). *Right panel:* Percentage of KLRG1^{+} and CD62L^{+} on NK1.1^{+} NK cells in spleen of mice fed +/– iron feed for 6 weeks (mean ± SEM, n=5). (**E**) *Left panel:* Viral titer in liver and spleen of WT MCMV-infected mice fed +/– iron feed for 6 weeks 3 dpi (each dot represents data from cells isolated from one mouse, data displayed as fold change difference normalized to mice fed + iron feed, horizontal line indicates median, n=10). *Right panel:* Viral titer in liver and spleen of *Δm157* MCMV infected-mice fed an +/– iron feed for 6 weeks 3 dpi. (each dot represents data from cells isolated from one mouse, data displayed as fold change difference normalized to mice fed + iron feed, horizontal line indicates median, n=9-10). (**F**) Percentage of IFN-γ^{+} in NK1.1^{+} NK cells in liver and spleen of WT MCMV-infected mice fed +/– feed for 6 weeks 1.5 dpi (mean ± SEM, n=4-5). Statistical significance was assessed by unpaired two-tailed Student's t-test (A, B, C, D, E, F). *p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001, ns, not significant.

CD71 supports NK cell proliferation and optimal effector function during viral infection

In order to test the functional importance of CD71-mediated iron uptake for NK cells, we generated mice specifically lacking CD71 in NK cells, by crossing *Ncr1Cre* mice with *Tfrc*$^{fl/fl}$ mice (*Tfrc*$^{fl/fl}$*Ncr1Cre*)[177]. Under homeostatic conditions, percentage and absolute numbers of NK cells in *Tfrc*$^{fl/fl}$*Ncr1Cre* mice were slightly reduced in both liver and spleen as compared to *Tfrc*$^{fl/fl}$ littermate controls (**Figure 9A**). Percentage and absolute numbers of CD8^{+} and CD4^{+} T cells, as well as CD19^{+} B cells, were unaffected by NK cell specific deletion of CD71 (**Figure 9B and 9C**). Further, expression of terminal NK cell maturation markers was comparable between *Tfrc*$^{fl/fl}$*Ncr1Cre* and *Tfrc*$^{fl/fl}$ mice (CD27, CD11b, KLRG1, CD62L and Ly6C) (**Figure 9D and 9E**). Likewise, the NK cell activating receptor, Ly49H, which is important for controlling MCMV infection, was equally expressed on *Tfrc*$^{fl/fl}$*Ncr1Cre* and *Tfrc*$^{fl/fl}$ NK cells (**Figure 9F**)[79].

NK cell activation by MCMV drives proliferation of Ly49H^{+} NK cells[3]. To examine whether deletion of CD71 affects antigen-specific NK cell expansion *in vivo*, we co-transferred congenic Ly49H^{+} WT and *Tfrc*$^{fl/fl}$*Ncr1Cre* NK cells into Ly49H-deficient (*Klra8*$^{-/-}$) recipients (**Figure 9G**, left panel). We then infected recipient mice with MCMV and tracked expansion of transferred NK cells. WT NK cells robustly expanded in liver, spleen, lung and blood, constituting 80-90 % of the Ly49H^{+} NK cell pool at 7 and 30 days post-infection (dpi) when compared to *Tfrc*$^{fl/fl}$*Ncr1Cre* NK cells (**Figure 9G**, right panel). We next addressed whether expansion of NK cells in lymphopenic hosts, which is driven by the availability of common-γ-chain-dependent cytokines, was also dependent on CD71[21,178]. To this end we transferred WT and *Tfrc*$^{fl/fl}$*Ncr1Cre* NK cells at equal ratios into *Rag2*$^{-/-}$*IL2rg*$^{-/-}$ recipient mice (**Figure 9H,** left panel)[179,180]. Similar to the infection experiment, at 6 dpi frequencies of *Tfrc*$^{fl/fl}$*Ncr1Cre* NK cells were much lower than those of WT cells (**Figure 9H**, right panel). Reduced numbers of *Tfrc*$^{fl/fl}$*Ncr1Cre* NK cells in both adoptive transfer experiments could have resulted from either a lack of expansion, increased cell death or a combination of both. To assess how deletion of CD71 in NK cells related to their proliferation *in vivo*, WT and *Tfrc*$^{fl/fl}$*Ncr1Cre* NK cells were labeled with CFSE and transferred into recipient mice at equal ratios (**Figure 9I**, left panel). Recipients were then infected with MCMV and donor cells harvested at 3.5 dpi. CFSE dilution, and hence proliferation, of adoptively transferred *Tfrc*$^{fl/fl}$*Ncr1Cre* NK cells in liver and spleen was significantly lower than that of WT cells (**Figure 9I**, right panel). These findings were further confirmed in *in vitro*

proliferation studies, in which IL-15 LD and IL-12/ IL-18-stimulated *Tfrc*$^{fl/fl}$*Ncr1Cre* NK cells showed reduced proliferation compared to control cells (**Figure 9J**).

To address whether deletion of CD71 affects NK cell-mediated viral control, we challenged *Tfrc*$^{fl/fl}$ and *Tfrc*$^{fl/fl}$*Ncr1Cre* mice with MCMV (**Figure 9K**, left panel). In line with the competitive transfer assays described above, a significant reduction in both percentage and absolute numbers of NK cells at 3.5 and 5.5 dpi was observed in both liver and spleen of *Tfrc*$^{fl/fl}$*Ncr1Cre* mice (**Figure 9K**, middle and right panel). Insufficient expansion of NK cells was associated with higher splenic viral titers among *Tfrc*$^{fl/fl}$*Ncr1Cre* mice at 3.5 dpi, with a similar trend observed in the liver (**Figure 9L**). In addition, IFN-γ production of splenic and liver infiltrating *Tfrc*$^{fl/fl}$*Ncr1Cre* NK cells was reduced upon MCMV infection (**Figure 9M**). Of note, despite poor expansion and reduced effector capacity, CD71 deficiency did not impair terminal maturation of MCMV-challenged CD71 deficient NK cells, as indicated by CD27, CD11b and KLRG1 expression (**Figure 9N**). Altogether, these data indicated a critical role of CD71 in NK cell proliferation both during infection and in a lymphopenic environment.

Figure 9

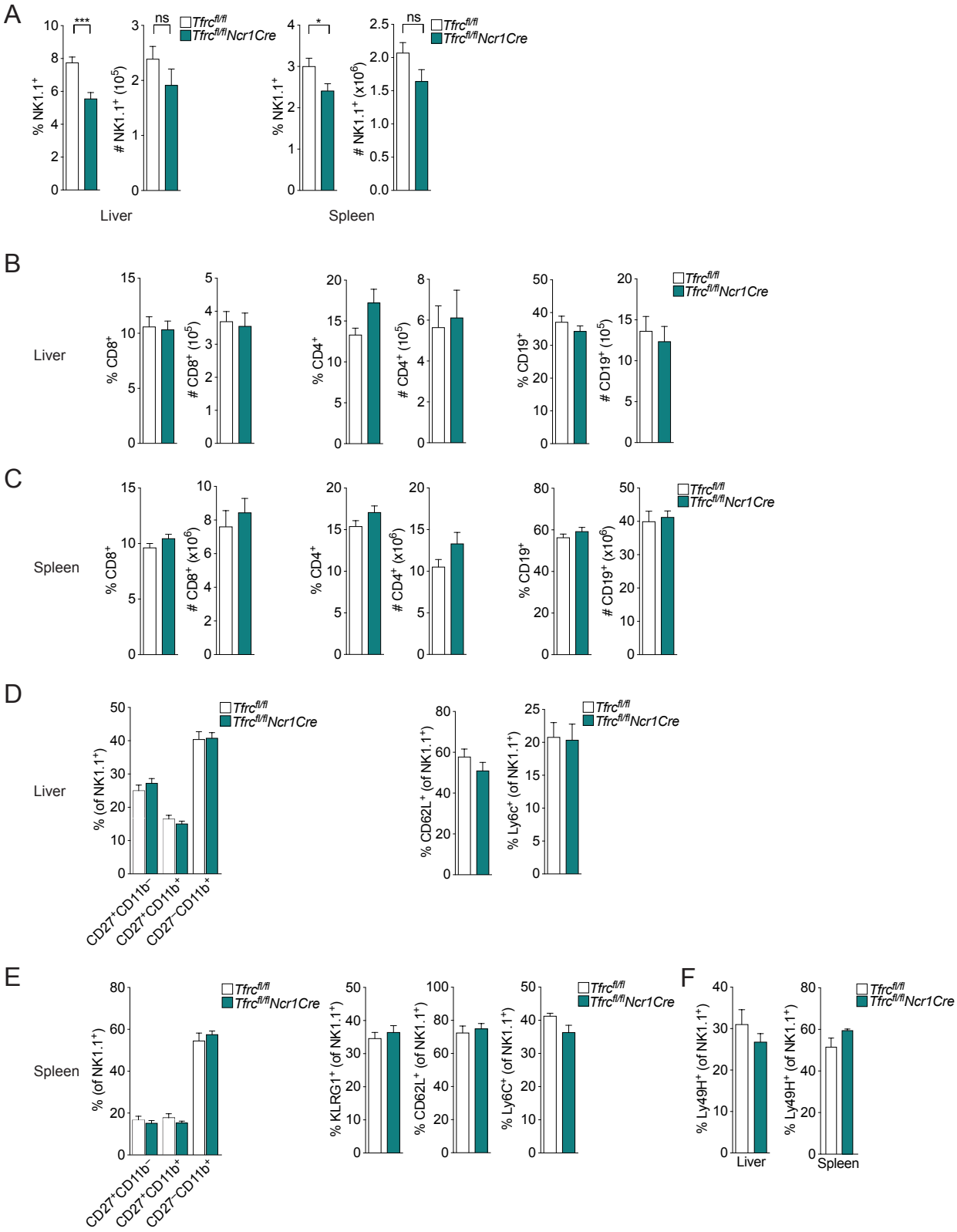
A
B
C
D
E
F
Liver
Spleen
Tfrc fl/fl
Tfrc fl/fl Ncr1Cre
% NK1.1+
# NK1.1+ (10^5)
# NK1.1+ (x10^6)
% CD8+
# CD8+ (10^5)
# CD8+ (x10^6)
% CD4+
# CD4+ (10^5)
# CD4+ (x10^6)
% CD19+
# CD19+ (10^5)
# CD19+ (x10^6)
% (of NK1.1+)
% CD62L+ (of NK1.1+)
% Ly6c+ (of NK1.1+)
% KLRG1+ (of NK1.1+)
% Ly6C+ (of NK1.1+)
% Ly49H+ (of NK1.1+)
CD27+CD11b−
CD27+CD11b+
CD27−CD11b+
***
*
ns

Figure 9

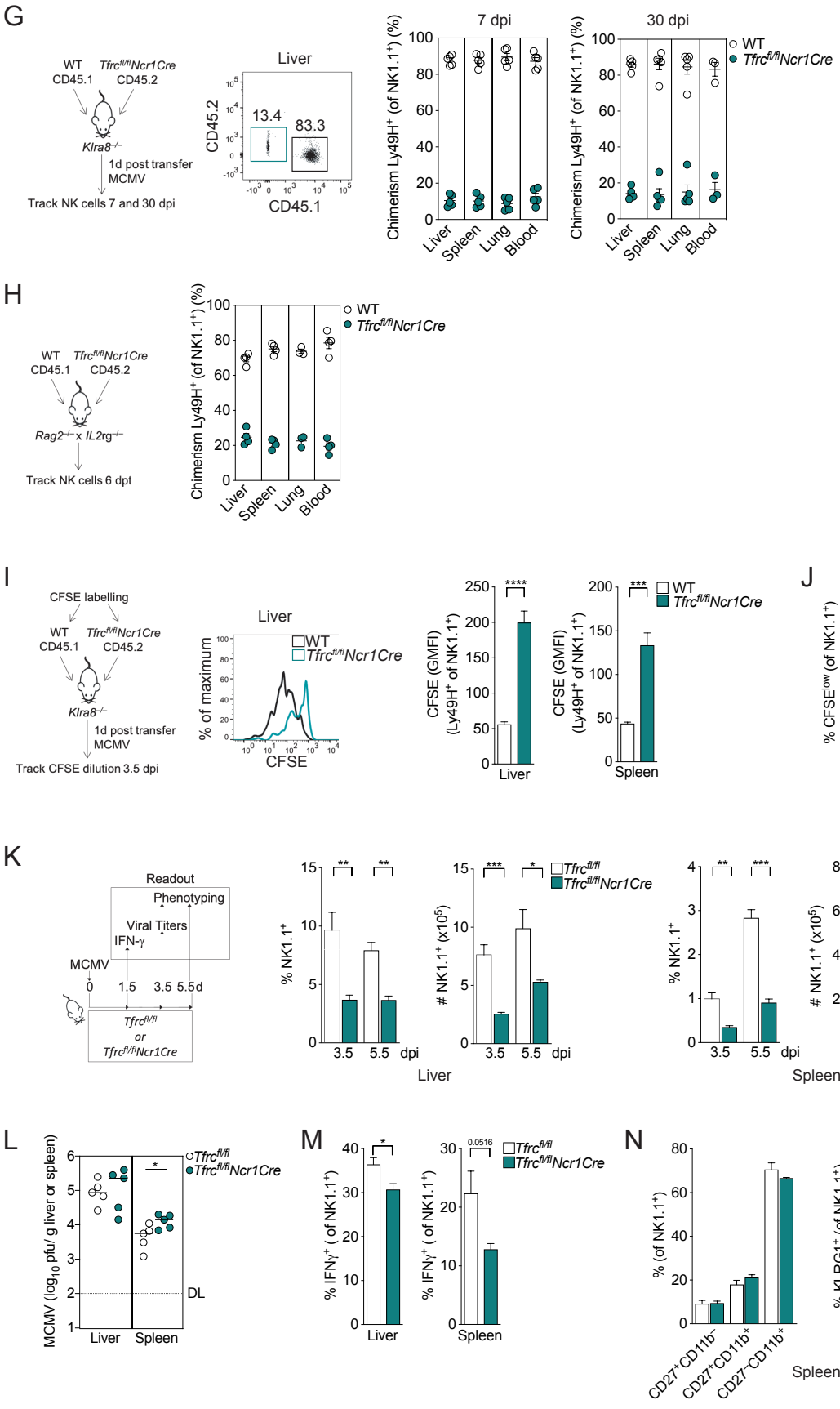
G
WT CD45.1
Tfrc fl/fl Ncr1Cre CD45.2
Klra8 −/−
1d post transfer MCMV
Track NK cells 7 and 30 dpi
Liver
CD45.2
CD45.1
13.4
83.3
7 dpi
30 dpi
Chimerism Ly49H+ (of NK1.1+) (%)
WT
Tfrc fl/fl Ncr1Cre
Liver
Spleen
Lung
Blood
H
WT CD45.1
Tfrc fl/fl Ncr1Cre CD45.2
Rag2 −/− x IL2rg −/−
Track NK cells 6 dpt
Chimerism Ly49H+ (of NK1.1+) (%)
I
CFSE labelling
WT CD45.1
Tfrc fl/fl Ncr1Cre CD45.2
Klra8 −/−
1d post transfer MCMV
Track CFSE dilution 3.5 dpi
Liver
WT
Tfrc fl/fl Ncr1Cre
% of maximum
CFSE
CFSE (GMFI) (Ly49H+ of NK1.1+)
Liver
Spleen
J
% CFSElow (of NK1.1+)
IL-15 LD
IL-12/ IL-18
K
Readout
Phenotyping
Viral Titers
IFN-γ
MCMV
0
1.5
3.5
5.5d
Tfrc fl/fl or Tfrc fl/fl Ncr1Cre
% NK1.1+
# NK1.1+ (x10^5)
3.5
5.5
dpi
Liver
Spleen
L
MCMV (log10 pfu/ g liver or spleen)
DL
M
% IFNγ+ (of NK1.1+)
0.0516
N
% (of NK1.1+)
CD27+CD11b−
CD27+CD11b+
CD27−CD11b+
% KLRG1+ (of NK1.1+)

Figure 9: CD71 supports NK cell proliferation and optimal effector function during viral infection

(**A**) *Left panel:* Percentage and absolute numbers of NK1.1$^+$ NK cells in liver of *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice (mean ± SEM, n=10). *Right panel:* Percentage and absolute numbers of NK1.1$^+$ NK cells in spleen of *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice (mean ± SEM, n=17-22). (**B**) *Left panel:* Percentage and absolute numbers of CD8$^+$ cells in liver of *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice (mean ± SEM, n=10). *Middle panel:* Percentage and absolute numbers of CD4$^+$ cells in liver of *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice (mean ± SEM, n=4). *Right panel:* Percentage and absolute numbers of CD19$^+$ cells in liver of *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice (mean ± SEM, n=10). (**C**) *Left panel:* Percentage and absolute numbers of CD8$^+$ cells in spleen of *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice (mean ± SEM, n=9-19). *Middle panel:* Percentage and absolute numbers of CD4$^+$ cells in spleen of *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice (mean ± SEM, n=11-22). *Right panel:* Percentage and absolute numbers of CD19$^+$ cells in spleen of *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice (mean ± SEM, n=12-22). (**D**) *Left panel:* Percentage of CD27$^+$CD11b$^-$, CD27$^+$CD11b$^+$, CD27$^-$CD11b$^+$ on NK1.1$^+$ NK cells in liver of *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice (mean ± SEM, n=10). *Right panel:* Percentage of CD62L$^+$ and Ly6C$^+$ on NK1.1$^+$ NK cells in liver of *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice (mean ± SEM, n=6). (**E**) *Left panel:* Percentage of CD27$^+$CD11b$^-$, CD27$^+$CD11b$^+$, CD27$^-$CD11b$^+$ on NK1.1$^+$ NK cells in spleen of *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice (mean ± SEM, n=5). *Right panel:* Percentage of KLRG1$^+$, CD62L$^+$ and Ly6C$^+$ on NK1.1$^+$ NK cells in spleen of *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice (mean ± SEM, n=5). (**F**) Percentage of Ly49H$^+$ on NK1.1$^+$ NK cells in liver and spleen of *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice (mean ± SEM, n=5-6). (**G**) *Left panel*: Schematic of adoptive transfer experiment into *Klra8$^{-/-}$* recipients to track expansion of WT and *Tfrc$^{fl/fl}$* NK cells upon MCMV infection. *Middle panel*: Representative flow plot gated on adoptively transferred CD45.1$^+$ and CD45.2$^+$ (Ly49H$^+$NK1.1$^+$) NK cells in liver of WT MCMV-infected recipients 7 dpi. *Right panel*: Percentage of adoptively transferred WT (Ly49H$^+$NK1.1$^+$CD45.1$^+$) and *Tfrc$^{fl/fl}$Ncr1Cre* (Ly49H$^+$NK1.1$^+$CD45.2$^+$) NK cells in liver, spleen, lung and blood of WT MCMV-infected recipients 7 and 30 dpi (each dot represents data from cells isolated from one mouse, bars indicate ± SEM, two independent experiments, 1st n= 5 for 7 dpi and n=3-5 for 30 dpi, 2nd n=4 for 7 dpi and n=2 for 30 dpi). (**H**) *Left panel:* Schematic of adoptive transfer experiment into *Rag2$^{-/-}$IL2rg$^{-/-}$* recipients to track expansion of WT and *Tfrc$^{fl/fl}$* NK cells. *Right panel:* Percentage of adoptively transferred WT (Ly49H$^+$NK1.1$^+$CD45.1$^+$) and *Tfrc$^{fl/fl}$Ncr1Cre* (Ly49H$^+$NK1.1$^+$CD45.2$^+$) NK cells in liver, spleen, lung and blood 6 dpt (each dot represents data from cells isolated from one mouse, bars indicate ± SEM, n= 3-4). (**I**) *Left panel:* Schematic of adoptive transfer experiment into *Klra8$^{-/-}$* recipients to analyze CFSE dilution in WT and *Tfrc$^{fl/fl}$* NK cells upon WT MCMV infection. *Middle panel:* Representative histogram of CFSE dilution in adoptively transferred WT and *Tfrc$^{fl/fl}$Ncr1Cre* NK1.1$^+$ NK cells in liver of WT MCMV-infected recipients 3.5 dpi. *Right panel*: GMFI of CFSE of adoptively transferred WT (Ly49H$^+$NK1.1$^+$CD45.1$^+$) and *Tfrc$^{fl/fl}$Ncr1Cre* (Ly49H$^+$NK1.1$^+$CD45.2$^+$) NK cells in liver and spleen of WT MCMV-infected recipients 3.5 dpi (mean ± SEM, two independent experiments, 1st n=5, 2nd n=4). (**J**) Percentage of proliferated *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* NK1.1$^+$ NK cells from spleen in IL-15 LD or stimulated with IL-12/ IL-18 analyzed by CFSE dilution (mean ± SEM, n=3-4). (**K**) *Left panel:* Schematic of MCMV infection experiment of *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1cre* mice. *Middle panel:* Percentage and absolute number of NK1.1$^+$ NK cells in liver of WT MCMV-infected *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1cre* mice 3.5 and 5.5 dpi (mean ± SEM, n=4-6). *Right panel:* Percentage and absolute number of NK1.1$^+$ NK cells in spleen of WT MCMV-infected *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1cre* mice 3.5 and 5.5 dpi (mean ± SEM, n=3-6). (**L**) Viral titer in liver and spleen of WT MCMV-infected *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1cre* mice 3.5 dpi (each dot represents data from cells isolated from one mouse, horizontal line indicates median, n=5). (**M**) Percentage of IFN-γ$^+$ in NK1.1$^+$ NK cells in liver and spleen of WT MCMV-infected *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1cre* mice 1.5 dpi (mean ± SEM, n=4). (**N**) *Left panel:* Percentage of CD27$^+$CD11b$^-$, CD27$^+$CD11b$^+$, CD27$^-$CD11b$^+$ on NK1.1$^+$ NK cells in spleen of WT MCMV-infected *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice 5.5 dpi (mean ± SEM, n=3-5). *Right panel*: Percentage of KLRG1$^+$ on NK1.1$^+$ NK cells in spleen of WT MCMV-infected *Tfrc$^{fl/fl}$* and *Tfrc$^{fl/fl}$Ncr1Cre* mice 5.5 dpi (mean ± SEM, n=3-5). Statistical significance was assessed by unpaired two-tailed Student's t-test (A, B, C, D, E, F, I, J, K, L, M, N, O). *p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001, ns, not significant.

Glycolysis is required for induction of CD71 in activated NK cells

Our experiments established (i) iron uptake via CD71 as a critical metabolic checkpoint controlling NK cell proliferation; and (ii) highly preferential upregulation of CD71 on activated CE vs. NV NK cells. These findings prompted us to ask how CD71 *per se* was regulated in NV and CE NK cells. To address this question, we first assessed whether induction of CD71 relied on NK cell transcriptional activity. As in previous experiments, CD71 was induced to a greater extent in cytokine-stimulated CE than NV NK cells (**Figure 10A**). In both subsets inhibition of transcription (using Actinomcyin D) entirely prevented stimulation-induced upregulation of CD71, as did blocking of translation (using cycloheximide) (**Figure 10A**). Thus, transcription and translation were similarly required in both cell subsets. Glycolytic reprogramming has previously been demonstrated to drive transcription in activated NK cells[123]. As glycolysis was similarly triggered in activated NV and CE NK cells (**Figure 6F**), we examined the possibility that glycolytic metabolism may differentially impact *TFRC* (which encodes CD71) transcription between NV and CE NK cells. *TFRC* mRNA abundance was indeed higher in activated CE than NV NK cells, yet similarly reduced when inhibiting glycolysis with 2-DG (**Figure 10B**). Cell surface expression of CD71 and total CD71 levels followed the same pattern when exposing cells to 2-DG (**Figure 10C**). Dependence on glucose to induce CD71 expression was recapitulated upon activation of NK cells in low glucose medium and translated into reduced transferrin uptake in 2-DG treated NK cells (**Figure 10D and 10E**). Glycolysis thus enabled transcription and translation of CD71. However, no evidence was found that glycolysis regulated the differential abundance of CD71 in activated CE vs. NV NK cells.

c-Myc has been established as a key regulator of *TFRC* transcription in various immune cells[153,181]. Given the increased abundance of *TFRC* mRNA among activated CE over NV NK cells (**Figure 10B**), preferential c-Myc induction in CE NK cells could explain differential regulation of CD71 between activated CE and NV NK cells. Yet, c-Myc was robustly but equally induced in both NK cell subsets (**Figure 10F**). Together these data established a symmetric need in activated NV and CE NK cells for (i) continuous transcription and translation to support expression of CD71 and (ii) glycolytic reprogramming as a metabolic requirement for CD71 expression.

Figure 10

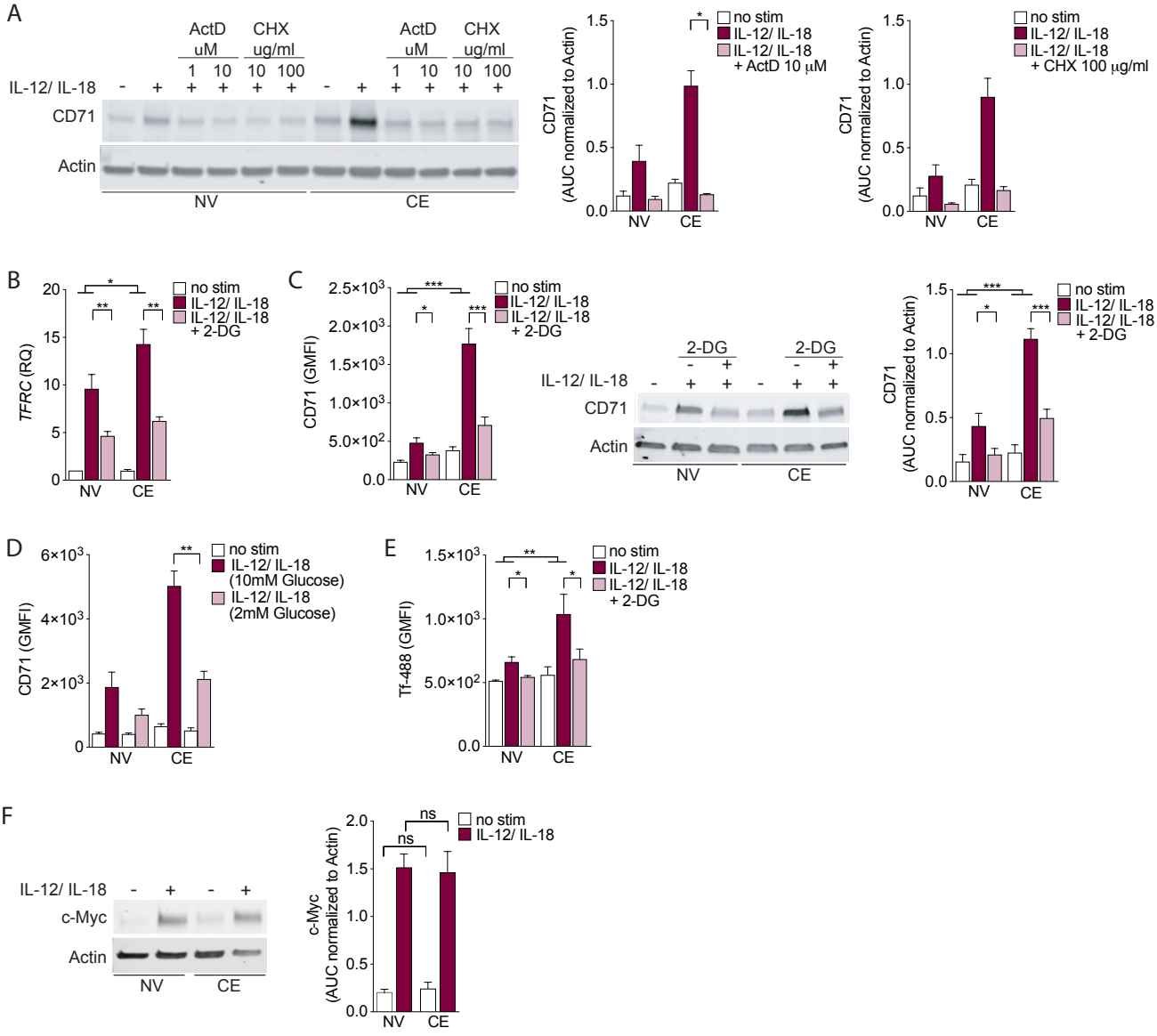
A
ActD uM
CHX ug/ml
1 10 10 100 1 10 10 100
IL-12/ IL-18 - + + + + + - + + + + +
CD71
Actin
NV
CE
CD71 (AUC normalized to Actin)
no stim
IL-12/ IL-18
IL-12/ IL-18 + ActD 10 μM
IL-12/ IL-18 + CHX 100 μg/ml
B
TFRC (RQ)
IL-12/ IL-18 + 2-DG
C
CD71 (GMFI)
2-DG
D
(10mM Glucose)
(2mM Glucose)
E
Tf-488 (GMFI)
F
c-Myc
ns

Figure 10: Glycolysis is required for induction of CD71 in activated NK cells

(**A**) *Left panel*: Representative Western blot of total CD71 expression in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18, IL-12/ IL-18 + ActD (1 and 10 μM) and IL-12/ IL-18 + CHX (10 and 100 μg/ ml). *Middle panel:* Total CD71 expression normalized to actin in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18, IL-12/ IL-18 + ActD 10 μM (mean ± SEM, n=3 donors). *Right panel:* Total CD71 expression normalized to actin in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18, IL-12/ IL-18 + CHX 100 μg/ml (mean ± SEM, n=2 donors). (**B**) Expression of *TFRC* mRNA in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18, IL-12/ IL-18 + 2-DG. Transcript levels were determined relative to *18S* mRNA levels and normalized to unstimulated (no stim) NV NK cells (mean ± SEM, n=6 donors). (**C**) *Left panel:* GMFI of CD71 expression on NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18, IL-12/ IL-18 + 2-DG (mean ± SEM, n=6 donors). *Middle panel:* Representative Western blot of total CD71 expression in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18, IL-12/ IL-18 + 2-DG. *Right panel:* Total CD71 expression normalized to actin in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18, IL-12/ IL-18 + 2DG (mean ± SEM, n=5 donors). (**D**) GMFI of CD71 expression on NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 in 10mM glucose and in 2mM glucose (mean ± SEM, n=5 donors). (**E**) GMFI of Tf-488 uptake in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18, IL-12/ IL-18 + 2-DG (mean ± SEM, n=5 donors). (**F**) *Left panel:* Representative Western blot of total c-Myc expression in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18. *Right panel:* Total c-Myc expression normalized to actin in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (mean ± SEM, n=6 donors). Statistical significance was assessed by paired two-tailed Student's t-test (A, B, C, D, E, F) or linear-regression analysis (B, C, E). *p < 0.05, **p < 0.01, *** p < 0.001, ns, not significant.

Cytokine priming induces the IRP/IRE regulatory system

Many genes involved in cellular iron homeostasis contain iron responsive elements (IREs) in the 5' or 3'UTR of their mRNA. Iron regulatory proteins 1 and 2 (IRP1 and IRP2) bind IREs, thereby controlling mRNA stability and translation[158,159]. *TFRC* mRNA contains 5 IREs in the 3'UTR; binding of IRPs stabilizes the mRNA and facilitates translation[166,167]. As described, this would occur under iron-deficient conditions[182,183]. Hence, we hypothesized that increased abundance of IRPs, selectively in CE NK cells, could be a possible mechanism regulating enhanced CD71 expression in activated CE NK cells. At the mRNA level, abundance of both IRP transcripts, *ACO1* and *IREB2*, was similar in NV and CE NK cells (**Figure 11A**, left panel). Protein abundance of IRP1 and IRP2 was, however, higher in quiescent and activated CE NK cells (**Figure 11A**, right panel). This finding was compatible with a novel role for IRPs, generating a *pseudo iron deficient state* in a cell subset-specific manner, thereby post-transcriptionally controlling abundance of a distinct set of proteins.

To extend this observation we analyzed transcript abundance of known IRE containing mRNAs expressed in NK cells (**Figure 11B**). We included the critical eukaryotic translation initiation factor 4E (eIF4E) in the list of IRE containing mRNAs, since *searching for iron-responsive elements* (SIRE) algorithm revealed an IRE-like motif in the 3'UTR of the *EIF4E* mRNA (**Figure 11C**)[184]. This analysis prompted us to assess the transcriptional and translational pattern for *EIF4E*. The pattern observed for CD71 was somewhat recapitulated, activated CE NK cells expressed more *EIF4E* transcript and clearly more eIF4E protein (**Figure 11D**). Yet, already quiescent CE NK cells expressed increased levels of eIF4E compared to NV NK cells. Of note, despite higher abundance of eIF4E, global protein translation was not discernibly different between activated NV and CE NK cells, as assessed by using L-homopropargylglycine (HPG) incorporation assays (**Figure 11E**). In addition, we noted increased *FTH1* mRNA abundance in activated CE NK cells (**Figure 11B**). *FTH1* mRNA contains an IRE in the 5'UTR and binding of IRPs to 5'UTRs IREs inhibits translation[168]. This constellation enabled us to test the hypothesis of a pseudo iron deficiency driven by selective increase in IRPs abundance in CE NK cells. Indeed, despite higher transcript levels, protein abundance of ferritin heavy chain 1 (the gene product of *FTH1*) was, if anything, lower in both unstimulated and activated CE NK cells (**Figure 11F**). This finding was highly suggestive of IRPs, with their CE and NV NK cell specific abundance, being involved in

regulating *FTH1* mRNA translation. Together these data established a regulatory axis, selectively induced in CE NK cells, in which pseudo iron deficiency enables increased translation of CD71 – and hence proliferation – of activated CE NK cells.

Figure 11

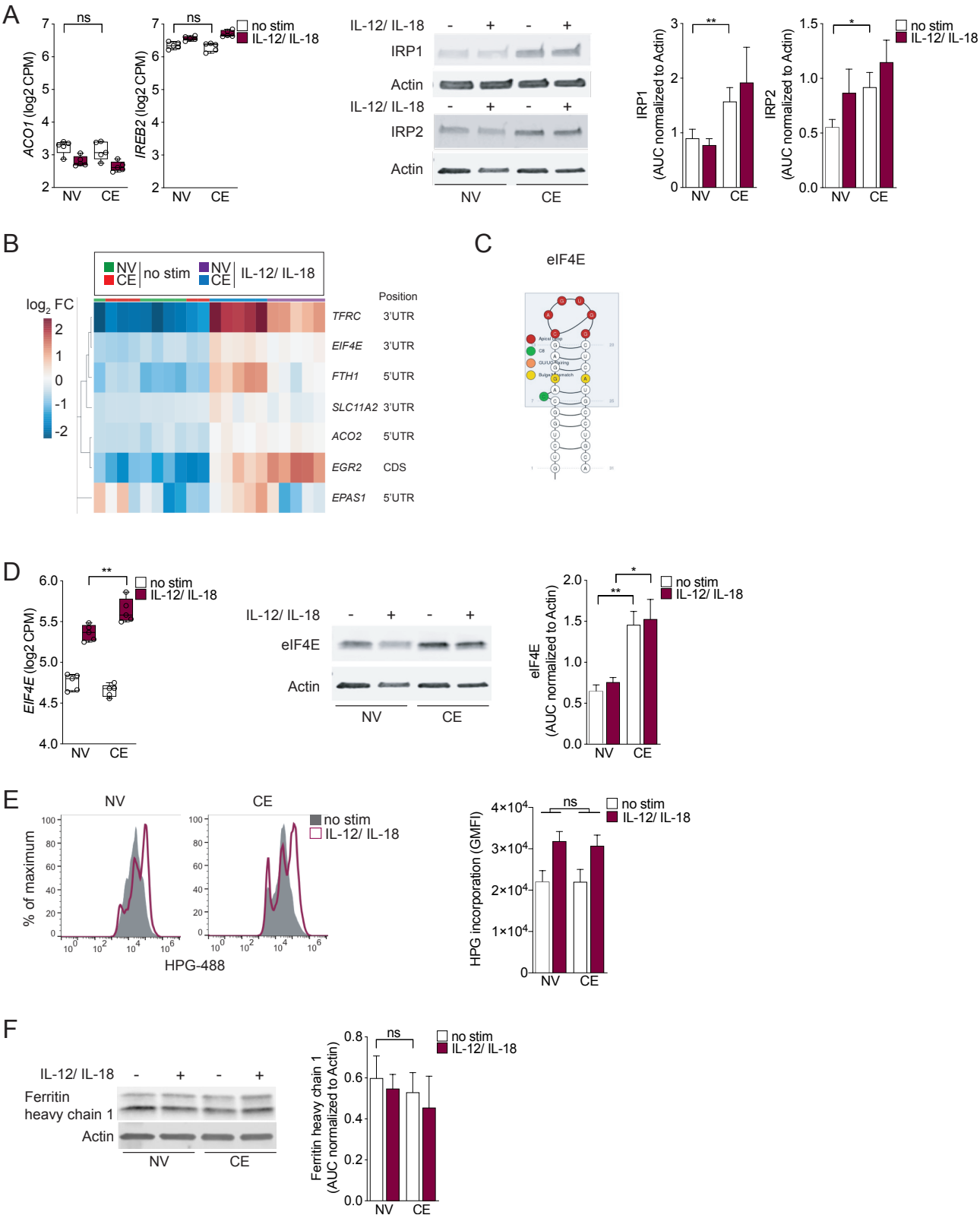
A
ns
ACO1 (log2 CPM)
IREB2 (log2 CPM)
NV
CE
no stim
IL-12/ IL-18
IL-12/ IL-18
IRP1
Actin
IRP2
IRP1 (AUC normalized to Actin)
IRP2 (AUC normalized to Actin)
B
log2 FC
Position
TFRC 3'UTR
EIF4E 3'UTR
FTH1 5'UTR
SLC11A2 3'UTR
ACO2 5'UTR
EGR2 CDS
EPAS1 5'UTR
C
eIF4E
D
EIF4E (log2 CPM)
eIF4E (AUC normalized to Actin)
E
% of maximum
HPG-488
HPG incorporation (GMFI)
F
Ferritin heavy chain 1
Ferritin heavy chain 1 (AUC normalized to Actin)

Figure 11: Cytokine priming induces the IRP/IRE regulatory system

(**A**) *Left panel:* Expression of *ACO1* and *IREB2* mRNA in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 of transcriptome data (n=5 donors). *Middle panel:* Representative Western blot of total IRP1 and IRP2 expression in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18. *Right panel:* Total IRP1 and IRP2 expression normalized to actin in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (mean ± SEM, n=7 donors for IRP1, n=6 donors for IRP2). (**B**) Heatmap of relative expression of mRNAs encoding for genes harboring IREs in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (n=5 donors). (**C**) Representation of the IRE-like motif in *EIF4E* mRNA. Squared region indicates the IRE core region predicted by SIREs software. (**D**) *Left panel:* Expression of *EIF4E* mRNA in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 of transcriptome data (n=5 donors). *Middle panel:* Representative Western blot of total eIF4E expression in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18. *Right panel:* Total eIF4E expression normalized to actin in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (mean ± SEM, n=6 donors). (**E**) *Left panel:* Representative histogram of HPG incorporation in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18. *Right panel:* GMFI of HPG incorporation in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (mean ± SEM, n=5 donors). (**F**) *Left panel:* Representative Western blot of total ferritin heavy chain 1 expression in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18. *Right panel:* Total ferritin heavy chain 1 expression normalized to actin in NV and CE NK cells unstimulated (no stim) or stimulated with IL-12/ IL-18 (mean ± SEM, n=4 donors). Statistical significance was assessed by paired two-tailed Student's t-test (A, D, F) or linear-regression analysis (E). *$p < 0.05$, **$p < 0.01$, ns, not significant.

7 DISCUSSION

The key findings of this study were (i) that CD71-mediated iron uptake was a prerequisite for activation-induced NK cell proliferation, and (ii) differential IRPs abundance between NV and CE NK cells, creating a pseudo iron deficient state in CE NK cells, which selectively supports CD71 translation and hence proliferation.

CD71 is widely used as an activation marker, with its cell surface expression induced on various activated immune cells including NK cells[123,125,126,136,154,181]. We confirmed upregulation of CD71 on IL-12/ IL-18-stimulated NK cells. However, the functional importance of CD71 in host defense, and specifically for NK cell function, has not been investigated in any detail so far. Iron deficiency has been proposed to compromise NK cell cytotoxicity; and dysfunctional NK cells in this setting may contribute to cancer development in rats[155,156]. In addition, low serum ferritin levels have been associated with reduced NK cell activity in humans[157]. These findings suggest a role for iron metabolism in NK cell-mediated immunity and tumor surveillance.

We found that reduced serum iron levels increased susceptibility to MCMV, presumably as a consequence of reduced NK cell function. This association was only observed in splenic NK cells but not in the liver, perhaps due to hepatic iron stores. Indeed, the liver functions as the major iron storage site in the body, serving as an iron buffer[185]. Many eukaryotic proteins require iron is as a cofactor, such as iron-sulfur cluster protein, heme-binding proteins and ribonucleotide reductases. These iron-dependent proteins support basic processes, such as energy metabolism/respiration, DNA synthesis and repair, and cell cycle control[145-150]. The high amount of iron needed by proliferating cells, including lymphocytes, is provided by transferrin. Transferrin, which – under physiologic circumstances – is iron saturated at around 30%, is taken up by proliferating cells via the cell surface receptor CD71[151,152]. Intriguingly, a mutation in the CD71 receptor (*TFRC*$^{Y20H/Y20H}$) has recently been shown to impair T and B cell function. Specifically, in affected individuals activated T and B cells fail to proliferate, causing a primary immunodeficiency (PID). Of note, in these patients NK cell numbers were reported normal, yet their functional properties were not assessed[135]. Our own data now demonstrate the functional importance of this CD71 mutation also in NK cell-mediated proliferation, adding complexity to this PID. Selective deletion of CD71 in NK cells also resulted in severe

proliferation deficits, both in response to viral infection as well as in the context of lymphopenia. Terminal maturation of NK cells lacking CD71 was not affected with Cre recombinase induced by the Ncr1 promotor, i.e. late during NK cell development. However, it is possible that earlier deletion may affect maturation of NK cells. T cells devoid of CD71, for example, arrest very early in their development[186]. NK cell numbers in liver and spleen of *Tfrc*$^{fl/fl}$*Ncr1Cre* mice were only slightly reduced; similar to patients carrying the above-described mutation in the *TFRC* gene who present largely normal lymphocyte counts, except for a reduction in memory B cell counts[135]. This suggested that, under steady state conditions, adequate iron acquisition was maintained in a CD71-independent manner. Several CD71-independet mechanisms for cellular iron acquisition have indeed been reported. In human erythroid precursor cells receptor-mediated ferritin uptake has been described, and CD71-deficient hamster ovary cells, nonetheless, acquire transferrin bound iron[187-190]. A variety of other cells; including hepatocytes, HeLa cells, fibroblasts, and mature erythrocytes, have been shown to take up non-transferrin bound iron directly[191-194]. Thus, it is plausible that the iron demand of NK cells under homeostatic conditions is met in a CD71-independent manner.

Our data emphasizes, however, that CD71 expression is a prerequisite for activation-induced NK cell proliferation. The ability of CE NK cells to rapidly upregulate CD71 was striking, and enhanced expression was linked to their capacity for increased proliferation when compared to naïve counterparts. While iron is indispensable for growth and survival, excess iron catalyzing the formation of reactive oxygen species can be toxic[195]. Therefore, iron import, export, and storage are all tightly regulated, which occurs in a highly dynamic manner and at the post-transcriptional level via the IRP/IRE regulatory system[151,196]. Iron availability regulates the RNA binding of IRPs to IREs harbored by a distinct set of mRNAs. Under iron-deplete conditions, the binding of IRP1 and IRP2 to the 3' *TFRC* IREs stabilizes *TFRC* mRNA, facilitating enhanced translation of CD71 and increased iron uptake[183,197]. Remarkably, quiescent and activated CE NK cells expressed higher levels of IRP1 and IRP2 when compared to NV NK cells. Both NK cell subsets were cultured under identical conditions with serum in the medium being rich in iron. *In vitro* iron-deficiency thus could be excluded as a cause for selectively increased IRP expression. By increasing the expression of IRP1 and IRP2 in CE NK cells only – despite comparable iron levels between both subsets – a pseudo iron deficient state was thus

established in a cell subset-specific manner, thereby post-transcriptionally controlling levels of CD71.

The activity of IRP1 vs. IRP2 is regulated in distinct ways. IRP1 contains an iron-sulfur cluster and under iron-replete conditions functions as cytosolic aconitase. When iron is scarce, the iron-sulfur cluster becomes devoid of iron and IRP1 changes its configuration, thus becoming able to bind to IREs of mRNAs[160-162]. The herein identified increased IRP1 protein abundance was, by contrast, suggestive of non-iron dependent regulation. Different than IRP1, IRP2 is rapidly degraded in relative iron excess by the ubiquitin proteasome system[163]. The transcription factor c-Myc is a direct transcriptional activator of IRP2[198]. Low abundance of c-Myc and equal transcript levels of *IREB2* in unstimulated NV and CE NK cells, however, argue against transcriptional upregulation (via c-Myc) of IRP2 in CE NK cells. The SKP1-CUL1-FBXL5 ubiquitin ligase protein catalyzes IRP2 ubiquitination and proteasomal degradation in the presence of iron. Under iron-deplete conditions, the adaptor protein FBXL5 is degraded leading to increased IRP2 levels[164,165]. Thus, the ubiquitin ligase functions as an iron sensor and a regulator of iron homeostasis. Future work could address whether IRP1 and IRP2 are equally important to stabilize *TFRC* mRNA in CE NK cells. Tissue specific variation in activities of IRP1 and IRP2 have been described, and IRP1 and IRP2 knockout mice have distinct phenotypes[199-202]. IRP1 and IRP2 are both overexpressed in breast cancer cells, but only IRP2 plays a growth promoting role[203,204]. Further, in prostate cancer cells, IRP2 plays the essential role in regulating cell growth[205]. These data suggest a dominant role for IRP2 in regulating cell proliferation. How enhanced IRP1 and IRP2 expression is regulated in CE NK cells, and if there is any specificity for either IRP1 or IRP2, remains to be defined. It also remains to be explored whether similar mechanisms operate among *in vivo* generated adaptive NK cells.

Intriguingly, our data further revealed that CE NK cells selectively upregulated the expression of eIF4E prior to stimulation. eIF4E is the major cap-binding protein, which has been assigned a rate limiting role within the EIF4F translation initiation complex[206]. In addition, eIF4E is involved in nuclear export of mRNAs involved in the cell cycle[207,208]. Increased expression of eIF4E has been demonstrated in various malignant tumors, contributing to increased cell proliferation[209-211]. Further, the translation of specific mRNA subsets, regulating growth and survival, is selectively sensitive to the activity and amount of eIF4E in non-lymphoid cells[212-214]. Thus, alongside the pseudo iron deficient state mediated by augmented IRPs abundance,

increased levels of eIF4E may as well contribute to transcript-specific translational regulation of *TFRC* mRNA. Remarkably, the search for iron responsive element algorithm (SIREs) on *EI4E* mRNA revealed an IRE-like motif in the 3'UTR. The IRP/IRE regulatory system and eIF4E may thus work in concert to allow efficient translation of specific mRNAs in CE NK cells.

In a mouse model of multiple sclerosis iron was reported to contribute to the production of GM-CFS and IL-2 in $CD4^+$ T cells. Specifically, iron abundance was shown to prevent degradation of an RNA binding protein, which in turn stabilizes proinflammatory cytokine mRNA, leading to enhanced translation and promotes inflammatory conditions[215]. It will be interesting to test whether, in addition to the striking proliferation phenotype described in our study, similar mechanisms operate in NK cells.

Several non-canonical roles for CD71 have further been described. For example, CD71 mediates signal transduction events in T cells by interacting with the ζ chain of the T cell receptor-CD3 complex and ZAP70 upon stimulation and presumably influences downstream signaling[216]. In addition, in IL-2-activated NK cells the inhibitory receptor KLRG1 forms disulfide-linked heteromers with CD71. In T cells such heteromers hinder KLRG1-mediated inhibition, suggesting a lower activation threshold of CD71(high) $KLRG1^+$ T cells[217]. Whether CD71 has further non-canonical roles in NK cell function remains to be determined.

Increased proliferation of CE NK cells was supported by a subset selective enhancement of PPP activity, aligning with increased mRNA levels of PPP-related genes. Cellular metabolism is intimately linked with cell fate by providing substrates or cofactors for epigenetic modifications, and therefore directly regulates DNA and histone (de-)methylation and histone acetylation[218-221]. Priming of naïve NK cells with IL-12/ IL-18 to generate CE NK cells, plausibly shaped the epigenetic landscape via activation-induced metabolic reprogramming. It is well-established that metabolic reprogramming towards glycolysis is a driver of NK cell effector functions[123,125,126,222]. Acetyl-CoA, a substrate for histone acetylation, is primarily derived from glucose oxidized in mitochondria[218,219]. Induction of glycolysis, and hence generation of acetyl-CoA, during priming could be a potential mechanism changing epigenetic marks in the promoters of specific genes. The TCA intermediate α-Ketoglutarate (α-KG) can alter DNA and histone demethylation, serving as a catalytic cofactor for JmjC domain-containing demethylases[221]. In addition, one carbon metabolism is functionally connected to histone and DNA methylation by providing the active methyl donor S-adenosyl-methionine (SAM)[220].

Epigenetic modifications can be maintained over cellular and organismal generations, and different cellular states or types are characterized by a distinct epigenome[223-225]. Increased mRNA abundance of PPP-related genes could thus well be imprinted by epigenetic marks supporting increased transcription. Future studies will be needed to assess the role of epigenetic remodeling during the NK cell priming-phase.

Metabolic reprogramming towards glycolysis upon stimulation was key for IFN-γ production by NV and CE NK cells, in line with previous findings[123,125,126,222]. Our observations suggested that glycolysis regulated IFN-γ production at the transcriptional level, one possible mechanism could be epigenetic alterations in the *IFNG* promotor region during stimulation. What specifically drives enhanced IFN-γ production in CE NK cells remains unknown. In adaptive human memory-like NK cells, enhanced IFN-γ production relies on epigenetic changes in the conserved noncoding sequence (CNS) 1 of the *IFNG* locus[226]. NV and CE NK cells expressed equal *IFNG* transcript levels upon stimulation, suggesting regulation at the post-transcriptional or post-translational level. Increased IFN-γ expression among CE NK cells could be due to enhanced translation or protein stabilization, or the removal of a negative regulator[227]. Further, miRNAs have also been implicated in the control of IFN-γ expression[228,229].

Continuous transcription and translation were needed to support CD71 expression in NV and CE NK cells alike. Importantly, our experiments provided evidence that glycolysis was required for expression of CD71 in NV and CE NK cells, possibly regulated at the transcriptional level. Thus, both NV and CE NK cells symmetrically rely on glycolytic reprogramming upon stimulation for induction of CD71.

Pre-clinical studies have demonstrated that CE NK cells have substantial potential as anti-cancer cellular therapy. Specifically, in *in vivo* tumor models of lymphoma or melanoma CE NK cells had enhanced effector function (IFN-γ production and cytotoxicity); and they substantially reduced AML burden and improved overall survival in AML xenografted NSG mice[106,107,110]. In a first-in-human-phase 1 clinical trial with CE NK cells, five out of nine AML patients demonstrated clinical responses, including 4 complete remissions (NCT01898793)[110]. Several clinical trials are currently ongoing, further testing the efficacy of CE NK cells in the treatment of hematologic malignancies (NCT03068819 and NCT02782546). The striking feature of CE NK cells in our study was their higher proliferative rate upon encountering a second stimulus. The

fact that NK cell proliferation strictly depended on iron suggests that iron availability may influence NK cell-mediated anti-tumor responses *in vivo* during the course of immunotherapy.

Engineered therapeutic T cells, e.g. CAR T cells, provide an emerging platform for the treatment of cancer. One key aspect to improve sustained functionality of CAR T cells is to engineer stably proliferating cells, which is of particular importance for the treatment of solid tumors[230]. The here described concept of a pseudo iron deficiency – that facilitated increased proliferation – might be relevant when aiming to improve proliferation of engineered immune cells used to treat cancer.

In summary, our study identified CD71-mediated iron uptake as a key metabolic checkpoint for activated NK cells, acting as a go/no-go gatekeeper with regard to cell proliferation. In CE NK cells, surplus levels of IRPs created a pseudo iron deficient state, selectively enhancing translation of CD71 – hence enabling increased proliferation.

8 MATERIALS AND METHODS

Mice

Animal experiments performed at the University of Rijeka, Faculty of Medicine were approved by Ethical Committee of the Faculty of Medicine, University of Rijeka and Ethical Committee at the Croatian Ministry of Agriculture, Veterinary and Food Safety Directorate (UP/I-322-01/18-01/44). Mice were strictly age- and sex-matched within experiments and were held in SPF conditions. Animal handling was in accordance with the guidelines contained in the International Guiding Principles for Biomedical Research Involving Animals. Wild-type C57BL/6J (B6, strain 000664), B6 Ly5.1 (strain 002014), *Tfrc*$^{fl/fl}$ (strain 028363) and *Rag2*$^{-/-}$*γc*$^{-/-}$ (strain 014593) mice were purchased from the Jackson laboratory. *Ncr1*^{Cre} mice were kindly provided by V. Sexl (Vienna, Austria) and B6.*Ly49h*$^{-/-}$ were kindly provided by Silvia M. Vidal (Montreal, Canada). In some experiments mice were put on iron-deficient diet and corresponding control diet for 6 weeks (C1038 and C1000, Altromin).

Animal experiments at the University of Basel were performed in accordance with local rules for the care and use of laboratory animals. Mice were strictly age- and sex-matched within experiments and were held in SPF conditions. Wild-type C57BL/6J (B6, strain 000664) mice were purchased from Jackson Laboratories (USA) and *Tfrc*$^{Y20H/Y20H}$ mice were kindly provided by R. Geha (Boston, USA).

Hematologic analyses

The serum iron, ferritin, unsaturated iron binding capacity (UIBC) and total iron binding capacity (TIBC) were determined using AU5800 Analyzer (Beckman Coulter). Hematocrit was determined using hematology analyzer DxH500 (Beckman Coulter). Measurements were conducted at the Clinical Institute of Laboratory Diagnostics (Clinical Hospital Center, Rijeka, Croatia).

Viruses

The bacterial artificial chromosome-derived murine cytomegalovirus (BAC-MCMV) strain pSM3fr-MCK-2fl clones 3.3 has previously been shown to be biologically equivalent to MCMV Smith strain (VR-1399; ATCC) and is herein after referred to as wild-type (WT) MCMV[231]. pSM3fr-MCK-2fl clone3.3 and Δ*m157* were propagated on mouse embryonic fibroblasts

(MEFs)[232]. *Animals were infected* intravenously (i.v.) with $2x10^5$ plaque forming units (PFU). Viral titers were determined on MEFs by standard plaque assay[233].

Adoptive transfer experiments

Adoptive co-transfer studies were performed by transferring splenocytes from WT B6 (CD45.1) and *Tfrc*$^{fl/fl}$*Ncr1Cre* (CD45.2) mice in an equal ratio into B6.*Ly49h*$^{-/-}$ and, respectively, into *Rag2*$^{-/-}$*γc*$^{-/-}$ recipients 1 day prior to MCMV infection.

For cell proliferation assays *in vivo*, splenocytes were loaded, prior to transfer, with cell-proliferation dye carboxyfluorescein succinimidyl ester (5 µM CFSE, Molecular probes, USA).

Human NK cell isolation and cell culture

Blood samples were obtained from healthy donors after written informed consent. Peripheral blood mononuclear cells were isolated by standard density-gradient centrifugation protocols (Lymphoprep; Fresenius Kabi). NK cells were negatively selected using EasySep negative NK cell isolation kit (Stemcell). Human NK cells were maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% heat-inactivated human AB serum, 50 U/ml penicillin (Invitrogen) and 50 µg/ml streptomycin (Invitrogen) (R10AB). To generate CE NK cells, isolated NK cells were primed in R10AB containing IL-12 (10 ng/ml, R&D systems), IL-15 (1ng/ml, PeproTech) and IL-18 (50 ng/ml, R&D systems) over-night. The next day cells were washed twice with PBS and maintained in R10AB containing IL-15 (1ng/ml) until stimulation. Every 2-3 days 50% of the medium was replaced with fresh IL-15 (1ng/ml). After 7 days cells were stimulated in R10AB containing IL-12 (10ng/ml), IL-15 (1ng/ml) and IL-18 (50ng/ml) or with K562 leukemia targets (effector: target ratio, 5:1) for 6 hours. When indicated, cells were pre-incubated with 2-deoxy-D-glucose (10mM, Sigma-Aldrich), Actinomycin D (1 and 10 µM, Sigma-Aldrich), Cycloheximide (10 and 100 µg/ml, Sigma-Aldrich), 2,2′-Bipyridyl (1, 10, 50 and 100 µM, Sigma-Aldrich) or 6-aminonicotinamide (50 µM, Sigma-Aldrich) for 30 min and then stimulated in R10AB containing IL-12 (10ng/ml), IL-15 (1ng/ml) and IL-18 (50ng/ml) for 6 hours.

Flow cytometry analysis of human cells

For surface staining NK cells were stained for 30 min at 4°C with saturating concentrations of antibodies. Following antibodies were used: anti-human CD71 (clone CY1G4, Biolegend), anti-human CD69 (clone FN50, Immunotools), anti-human CD98 (clone MEM-108, Biolegend) Samples were acquired using a BD AccuriC6 or a CytoFLEX flow cytometer (Beckman Coulter). Data were analyzed with Flowjo®_V10.5 (Tree Star, USA).

For cell proliferation assays, NK cells were loaded prior to activation with the cell-proliferation dye CFSE (1 µM, Molecular probes, USA) and seeded in 96-well plates. Cells were washed twice and maintained in R10AB with IL-15 (1ng/ml), when indicated in the presence of inhibitors. A fixable live-dead cell stain (Fixable Viability Dye, eBioscience or Zombie Aqua, Biolegend) was used to exclude dead cells prior to sample acquisition. CFSE dilution was analyzed 65 hours post-stimulation by flow cytometry. Samples were acquired using a BD AccuriC6 or a CytoFLEX flow cytometer (Beckman Coulter). Data were analyzed with Flowjo®_V10.5 (Tree Star, USA).

Flow cytometry analysis of murine cells

Lymphocytes from spleens were isolated by meshing organs and filtering them through a 100-µm strainer. To isolate lymphocytes from liver, the tissue was meshed and filtered through a 100 µm strainer and purified using a discontinuous gradient of 40% over 80% Percoll. Red blood cells in spleen and liver were lysed using erythrocyte lysis buffer. Cells were pretreated with Fc block (clone 2.4G2) and a fixable live-dead cell stain (Fixable Viability Dye, eBioscience) was used to exclude dead cells. Cells were stained for 30 min at 4°C with saturating concentrations of antibodies. Following antibodies purchased from Thermo Fisher Scientific were used: anti-mouse CD8α (clone 53-6.7), anti-mouse CD45.2 (clone 104), anti-mouse CD4 (clone RM4-5), anti-mouse CD69 (clone H1.2F3), anti-mouse CD45.1 (clone A20), anti-mouse CD3ε (clone 145-2C11), anti-mouse CD19 (clone 1D3), anti-mouse NK1.1 (clone PK136), anti-mouse NKp46 (clone 29A1.4), anti-mouse CD62L (clone MEL-14), anti-mouse Ly6c (clone HK1.4), anti-mouse KLRG1 (clone 2F1), anti-mouse Ly49H (clone 3D10), anti-mouse CD11b (clone M1/70) and anti-mouse CD27 (clone O323). Samples were acquired using a BD FACSAria. Data were analyzed with Flowjo®_V10.5 (Tree Star, USA).

For intracellular cytokine staining upon MCMV infection, lymphocytes from spleen and liver of MCMV-infected mice were isolated as indicated above. Cells were resuspended in RPMI-1640 medium supplement with 10% fetal bovine serum (Thermo Fisher Scientific), 50 U/ml penicillin (Invitrogen), 50 µg/ml streptomycin (Invitrogen) and 50 µM 2-*mercaptoethanol* (Thermo Fisher Scientific) (R10FBS) in the presence of IL-2 (500 IU/ml). Cells were incubated at 37°C in the presence of brefeldin A (eBioscience) for 5 hours. Cells were surface-stained, followed by fixation and permeabilization according to the manufacturer's protocol (BD Biosciences). Intracellular cytokines were stained using mouse-anti IFN-γ (clones XMG1.2, Thermo Fisher Scientific). Samples were acquired using a BD FACSAria. Data were analyzed with Flowjo®_V10.5 (Tree Star, USA).

For cell proliferation assays, lymphocytes were loaded prior to activation with the cell-proliferation dye CFSE (1 µM, Molecular probes, USA) and seeded in U-bottom 96-well plates ($5x10^5$ cells/well). Cells were were stimulated in R10FBS containing IL-12 (10ng/ml, PeproTech), IL-15 (10 ng/ml, PeproTech) and IL-18 (50 ng/ml, R&D Systems) for 16 hours. Cells were washed twice and maintained in R10FBS containing IL-15 (10ng/ml). CFSE dilution was analyzed 65 hours post-stimulation by flow cytometry. A fixable live-dead cell stain (Fixable Viability Dye, eBioscience or Zombie Aqua, Biolegend) was used to exclude dead cells. Samples were acquired using a BD FACSAria or CytoFLEX flow cytometer (Beckman Coulter). Data were analyzed with Flowjo®_V10.5 (Tree Star, USA).

Seahorse metabolic flux analyzer

A Seahorse XF-96^e extracellular flux analyzer (Seahorse Bioscience, Agilent) was used to determine the metabolic profile of cells. NK cells were plated ($3x10^5$ cells/well) onto Celltak (Corning, USA) coated cell plates. Mitochondrial perturbation experiments were carried out by sequential addition of oligomycin (1 µM, Sigma), FCCP (2 µM, Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, Sigma), and rotenone (1 µM, Sigma). Oxygen consumption rates (OCR, pmol/min) and extracellular acidification rates (ECAR, mpH/min) were monitored in real time after injection of each compound.

2-NBDG uptake

NK cells were seeded in U-bottom 96-well plates ($2x10^5$ cells/well). When indicated cells were pre-incubated for 30 min with inhibitors and stimulated in R10AB containing IL-12 (10ng/ml), IL-15 (1 ng/ml) and IL-18 (50ng/ml) for 6 hours. Cells were then incubated in medium containing 20 µM 2-NBDG (Invitrogen) for 15 min and analyzed by flow cytometry. Samples were acquired using a BD AccuriC6 flow cytometer. Data were analyzed with Flowjo®_V10.5 (Tree Star, USA).

IFN-γ measurement in human NK cells

NK cells were seeded in U-bottom 96 well plates ($2x10^5$ cells/well) using R10AB. When indicated cells were pre-incubated for 30 min with inhibitors and stimulated in R10AB containing IL-12 (10ng/ml), IL-15 (1 ng/ml) and IL-18 (50ng/ml) for 6 hours. Cell supernatants were harvested after stimulation and IFN-γ was measured using a human Th1 cytokine bead-based immunoassay (Legendplex, Biolegend) according to manufacturer's protocol.

Transferrin uptake assay

NK cells were seeded in U-bottom 96-well plates ($2x10^5$ cells/well). When indicated cells were pre-incubated for 30 min with inhibitors and stimulated in R10AB containing IL-12 (10ng/ml), IL-15 (1 ng/ml) and IL-18 (50ng/ml) for 4 hours. Cells were then stimulated in RPMI-1640 medium containing 5% BSA and IL-12 (10ng/ml), IL-15 (1 ng/ml) and IL-18 (50ng/ml) for 2 hours. After stimulation cells were washed with RPMI-1640 containing 0.5% BSA before incubation with transferrin-alexa488 conjugate (Tf-488, 10 µg/ ml, Thermo Fisher Scientific) for 15 min. Transferrin uptake was stopped by washing cells in ice-cold acidic buffer (150 mM NaCl, 20 mM citric acid and pH: 5). Cells were resuspended in FACS buffer and analyzed by flow cytometry. Samples were acquired using a BD AccuriC6 flow cytometer. Data were analyzed with Flowjo®_V10.5 (Tree Star, USA).

HPG incorporation assay

NK cells were seeded in 96-well plates ($2x10^5$ cells/well). Cells were stimulated in R10AB containing IL-12 (10ng/ml), IL-15 (1 ng/ml) and IL-18 (50ng/ml) for 4.5h and afterwards incubated for 1.5h in methionine-free RPMI-1640 medium containing 10% dialyzed FBS and

IL-12 (10ng/ml), IL-15 (1 ng/ml) and IL-18 (50ng/ml). Click-IT®HPG (50 µM, Life Technologies) was added for the last 30 min of the incubation. HPG incorporation into NK cells was stained with Click-iT® reaction cocktail (Thermo Fisher Scientific) and detected by flow cytometry. Samples were acquired using a BD AccuriC6 flow. Data were analyzed with Flowjo®_V10.5 (Tree Star, USA).

Immunoblot analysis

Protein concentrations were determined by BCA protein assay kit (Thermo Fisher Scientific). Total cell lysates were separated using 4%–15% Mini Protean TGX Gel (Bio-Rad, Hercules CA, USA), and transferred to nitrocellulose membranes using Trans-Blot Turbo Transfer (Bio-Rad, Hercules CA, USA). Membranes were probed with the following antibodies: anti-human CD71 mAb (13113), anti-human IRP1 mAb (20272), anti-human IRP2 mAb (37135), anti-human FTH1 mAb (4393), anti-human eIF4E mAb (2067), anti-human c-Myc mAb (5605) and anti-human β-actin mAb (3700) (all from Cell Signaling, USA). Blots were stained with appropriate secondary antibodies and the odyssey imaging system (LICOR, Lincoln NE, USA) was used for visualization, and the ImageJ software (1.48v) for quantification.

RNA sequencing

RNA-seq was performed by Admera Health (USA). In brief, samples were isolated using ethanol precipitation. Quality check was performed using Tapestation RNA HS Assay (Agilent Technologies, USA) and quantified by Qubit RNA HS assay (Thermo Fisher Scientific). Ribosomal RNA depletion was performed with Ribo-zero Magnetic Gold Kit (MRZG12324, Illumina Inc., USA). Samples were randomly primed and fragmented based on manufacturer's recommendation (NEBNext® Ultra™ RNA Library Prep Kit for Illumina®). First strand was synthesized using Protoscript II Reverse Transcriptase with a longer extension period (40 min for 42°C). All remaining steps for library construction were used according to the NEBNext® Ultra™ RNA Library Prep Kit for Illumina®. Illumina 8-nt dual-indices were used. Samples were pooled and sequencing on a HiSeq with a read length configuration of 150 paired-end.

Quantitative real-time PCR

RNA was isolated from NK cells using Trizol (Thermo Fisher Scientific) and chloroform (Sigma-Aldrich) according to manufacturer's protocol, then purified with RNeasy RNA purification mini kit (QIAGEN, Germany). RNA concentration was determined using the NanoDrop 2000C (Thermo Fisher Scientific). From purified RNA, cDNA was synthesized using the reverse-transcriptase kit GoScript™ Reverse Transcriptase (Promega). Quantitative PCR for *IFNG, TFRC* and *18S* mRNA was done in triplicate using commercially designed primers from Life Technologies (Hs00989291_m1, Hs00951083_m1, Hs03003631_g1). PCR reactions were performed using Go Tag G2 DNA Polymerase (Promega) according to manufacturer's protocol.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was determined by either using unpaired two-tailed Student's t test or paired two-tailed Student's t test using GraphPad Prism 8.00 (GraphPad Software). For comparison of increases (before versus after) in paired samples, a simple linear-regression model was used. *P* values of less than 0.05 were considered statistically significant.

9 REFERENCES

1 Kiessling, R., Klein, E., Pross, H. & Wigzell, H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *European journal of immunology* **5**, 117-121, doi:10.1002/eji.1830050209 (1975).

2 Herberman, R. B., Nunn, M. E. & Lavrin, D. H. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *International journal of cancer* **16**, 216-229 (1975).

3 Sun, J. C., Beilke, J. N. & Lanier, L. L. Adaptive immune features of natural killer cells. *Nature* **457**, 557-561, doi:10.1038/nature07665 (2009).

4 O'Sullivan, T. E., Johnson, L. R., Kang, H. H. & Sun, J. C. BNIP3- and BNIP3L-Mediated Mitophagy Promotes the Generation of Natural Killer Cell Memory. *Immunity* **43**, 331-342, doi:10.1016/j.immuni.2015.07.012 (2015).

5 Hammer, Q. *et al.* Peptide-specific recognition of human cytomegalovirus strains controls adaptive natural killer cells. *Nat Immunol* **19**, 453-463, doi:10.1038/s41590-018-0082-6 (2018).

6 Schlums, H. *et al.* Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function. *Immunity* **42**, 443-456, doi:10.1016/j.immuni.2015.02.008 (2015).

7 Beziat, V. *et al.* CMV drives clonal expansion of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. *European journal of immunology* **42**, 447-457, doi:10.1002/eji.201141826 (2012).

8 Guma, M. *et al.* Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood* **104**, 3664-3671, doi:10.1182/blood-2004-05-2058 (2004).

9 Spits, H., Bernink, J. H. & Lanier, L. NK cells and type 1 innate lymphoid cells: partners in host defense. *Nat Immunol* **17**, 758-764, doi:10.1038/ni.3482 (2016).

10 Seillet, C. & Belz, G. T. Differentiation and diversity of subsets in group 1 innate lymphoid cells. *International immunology* **28**, 3-11, doi:10.1093/intimm/dxv051 (2016).

11 Seillet, C. *et al.* Deciphering the Innate Lymphoid Cell Transcriptional Program. *Cell Reports* **17**, 436-447, doi:https://doi.org/10.1016/j.celrep.2016.09.025 (2016).

12 Vivier, E. *et al.* Innate Lymphoid Cells: 10 Years On. *Cell* **174**, 1054-1066, doi:10.1016/j.cell.2018.07.017 (2018).

13 Freud, A. G., Mundy-Bosse, B. L., Yu, J. & Caligiuri, M. A. The Broad Spectrum of Human Natural Killer Cell Diversity. *Immunity* **47**, 820-833, doi:10.1016/j.immuni.2017.10.008 (2017).

14 Yu, J., Freud, A. G. & Caligiuri, M. A. Location and cellular stages of natural killer cell development. *Trends in immunology* **34**, 573-582, doi:10.1016/j.it.2013.07.005 (2013).

15 Caligiuri, M. A. Human natural killer cells. *Blood* **112**, 461-469, doi:10.1182/blood-2007-09-077438 (2008).

16 Scoville, S. D., Freud, A. G. & Caligiuri, M. A. Modeling Human Natural Killer Cell Development in the Era of Innate Lymphoid Cells. *Frontiers in immunology* **8**, 360-360, doi:10.3389/fimmu.2017.00360 (2017).

17 Sun, J. C. & Lanier, L. L. NK cell development, homeostasis and function: parallels with $CD8^+$ T cells. *Nature reviews. Immunology* **11**, 645-657, doi:10.1038/nri3044 (2011).

18 Intlekofer, A. M. *et al.* Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat Immunol* **6**, 1236-1244, doi:10.1038/ni1268 (2005).
19 Cooper, M. A. *et al.* In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. *Blood* **100**, 3633-3638, doi:10.1182/blood-2001-12-0293 (2002).
20 Marçais, A. *et al.* Regulation of mouse NK cell development and function by cytokines. *Frontiers in immunology* **4**, 450-450, doi:10.3389/fimmu.2013.00450 (2013).
21 Meazza, R., Azzarone, B., Orengo, A. M. & Ferrini, S. Role of common-gamma chain cytokines in NK cell development and function: perspectives for immunotherapy. *Journal of biomedicine & biotechnology* **2011**, 861920-861920, doi:10.1155/2011/861920 (2011).
22 Horowitz, A. *et al.* Genetic and Environmental Determinants of Human NK Cell Diversity Revealed by Mass Cytometry. *Science translational medicine* **5**, 208ra145-208ra145, doi:10.1126/scitranslmed.3006702 (2013).
23 Strauss-Albee, D. M., Horowitz, A., Parham, P. & Blish, C. A. Coordinated Regulation of NK Receptor Expression in the Maturing Human Immune System. *The Journal of Immunology* **193**, 4871-4879, doi:10.4049/jimmunol.1401821 (2014).
24 Strauss-Albee, D. M. *et al.* Human NK cell repertoire diversity reflects immune experience and correlates with viral susceptibility. *Science translational medicine* **7**, 297ra115, doi:10.1126/scitranslmed.aac5722 (2015).
25 Moretta, L. Dissecting CD56^{dim} human NK cells. *Blood* **116**, 3689-3691, doi:10.1182/blood-2010-09-303057 (2010).
26 De Maria, A., Bozzano, F., Cantoni, C. & Moretta, L. Revisiting human natural killer cell subset function revealed cytolytic CD56^{dim}CD16⁺ NK cells as rapid producers of abundant IFN-γ on activation. *Proceedings of the National Academy of Sciences* **108**, 728-732, doi:10.1073/pnas.1012356108 (2011).
27 Goh, W. & Huntington, N. D. Regulation of Murine Natural Killer Cell Development. *Front Immunol* **8**, 130, doi:10.3389/fimmu.2017.00130 (2017).
28 Long, E. O., Kim, H. S., Liu, D., Peterson, M. E. & Rajagopalan, S. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annual review of immunology* **31**, 227-258, doi:10.1146/annurev-immunol-020711-075005 (2013).
29 Lanier, L. L. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* **9**, 495-502, doi:10.1038/ni1581 (2008).
30 Hewitt, E. W. The MHC class I antigen presentation pathway: strategies for viral immune evasion. *Immunology* **110**, 163-169 (2003).
31 Raulet, D. H. & Vance, R. E. Self-tolerance of natural killer cells. *Nature Reviews Immunology* **6**, 520, doi:10.1038/nri1863 (2006).
32 Koch, J., Steinle, A., Watzl, C. & Mandelboim, O. Activating natural cytotoxicity receptors of natural killer cells in cancer and infection. *Trends in Immunology* **34**, 182-191, doi:https://doi.org/10.1016/j.it.2013.01.003 (2013).
33 Bryceson, Y. T., March, M. E., Ljunggren, H. G. & Long, E. O. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* **107**, 159-166, doi:10.1182/blood-2005-04-1351 (2006).
34 Fehniger, T. A. *et al.* Potential mechanisms of human natural killer cell expansion in vivo during low-dose IL-2 therapy. *The Journal of clinical investigation* **106**, 117-124, doi:10.1172/jci6218 (2000).
35 Biron, C. A., Young, H. A. & Kasaian, M. T. Interleukin 2-induced proliferation of murine natural killer cells in vivo. *J Exp Med* **171**, 173-188 (1990).

36 Lotzova, E., Savary, C. A. & Herberman, R. B. Induction of NK cell activity against fresh human leukemia in culture with interleukin 2. *J Immunol* **138**, 2718-2727 (1987).
37 Wang, K. S., Frank, D. A. & Ritz, J. Interleukin-2 enhances the response of natural killer cells to interleukin-12 through up-regulation of the interleukin-12 receptor and STAT4. *Blood* **95**, 3183-3190 (2000).
38 Becknell, B. & Caligiuri, M. A. Interleukin-2, interleukin-15, and their roles in human natural killer cells. *Advances in immunology* **86**, 209-239, doi:10.1016/s0065-2776(04)86006-1 (2005).
39 Hashimoto, W. *et al.* Differential antitumor effects of administration of recombinant IL-18 or recombinant IL-12 are mediated primarily by Fas-Fas ligand- and perforin-induced tumor apoptosis, respectively. *J Immunol* **163**, 583-589 (1999).
40 Kodama, T. *et al.* Perforin-dependent NK cell cytotoxicity is sufficient for anti-metastatic effect of IL-12. *European journal of immunology* **29**, 1390-1396, doi:10.1002/(sici)1521-4141(199904)29:04<1390::Aid-immu1390>3.0.Co;2-c (1999).
41 Orange, J. S., Wang, B., Terhorst, C. & Biron, C. A. Requirement for natural killer cell-produced interferon gamma in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. *J Exp Med* **182**, 1045-1056 (1995).
42 Kobayashi, M. *et al.* Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *The Journal of experimental medicine* **170**, 827-845 (1989).
43 Lehmann, D. *et al.* IL-12 directs further maturation of ex vivo differentiated NK cells with improved therapeutic potential. *PLoS One* **9**, e87131, doi:10.1371/journal.pone.0087131 (2014).
44 Sun, J. C. *et al.* Proinflammatory cytokine signaling required for the generation of natural killer cell memory. *J Exp Med* **209**, 947-954, doi:10.1084/jem.20111760 (2012).
45 Hyodo, Y. *et al.* IL-18 up-regulates perforin-mediated NK activity without increasing perforin messenger RNA expression by binding to constitutively expressed IL-18 receptor. *J Immunol* **162**, 1662-1668 (1999).
46 Kawakami, K. *et al.* IL-18 contributes to host resistance against infection with Cryptococcus neoformans in mice with defective IL-12 synthesis through induction of IFN-gamma production by NK cells. *J Immunol* **165**, 941-947 (2000).
47 Pien, G. C., Satoskar, A. R., Takeda, K., Akira, S. & Biron, C. A. Cutting edge: selective IL-18 requirements for induction of compartmental IFN-gamma responses during viral infection. *J Immunol* **165**, 4787-4791 (2000).
48 Rowland, C. A. *et al.* Critical role of type 1 cytokines in controlling initial infection with Burkholderia mallei. *Infection and immunity* **74**, 5333-5340, doi:10.1128/iai.02046-05 (2006).
49 Takeda, K. *et al.* Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity* **8**, 383-390 (1998).
50 Hoshino, K. *et al.* Cutting edge: generation of IL-18 receptor-deficient mice: evidence for IL-1 receptor-related protein as an essential IL-18 binding receptor. *J Immunol* **162**, 5041-5044 (1999).
51 Freeman, B. E., Raué, H.-P., Hill, A. B. & Slifka, M. K. Cytokine-Mediated Activation of NK Cells during Viral Infection. *Journal of virology* **89**, 7922-7931, doi:10.1128/JVI.00199-15 (2015).

52 Fehniger, T. A., Carson, W. E. & Caligiuri, M. A. Costimulation of human natural killer cells is required for interferon gamma production. *Transplantation proceedings* **31**, 1476-1478 (1999).
53 Strowig, T., Brilot, F. & Münz, C. Noncytotoxic functions of NK cells: direct pathogen restriction and assistance to adaptive immunity. *Journal of immunology (Baltimore, Md. : 1950)* **180**, 7785-7791 (2008).
54 Lodoen, M. B. & Lanier, L. L. Natural killer cells as an initial defense against pathogens. *Curr Opin Immunol* **18**, 391-398, doi:10.1016/j.coi.2006.05.002 (2006).
55 Lee, S. H., Miyagi, T. & Biron, C. A. Keeping NK cells in highly regulated antiviral warfare. *Trends Immunol* **28**, 252-259, doi:10.1016/j.it.2007.04.001 (2007).
56 Vivier, E., Tomasello, E., Baratin, M., Walzer, T. & Ugolini, S. Functions of natural killer cells. *Nat Immunol* **9**, 503-510, doi:10.1038/ni1582 (2008).
57 Baran, K. *et al.* The molecular basis for perforin oligomerization and transmembrane pore assembly. *Immunity* **30**, 684-695, doi:10.1016/j.immuni.2009.03.016 (2009).
58 Thiery, J. *et al.* Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nat Immunol* **12**, 770-777, doi:10.1038/ni.2050 (2011).
59 Alter, G., Malenfant, J. M. & Altfeld, M. CD107a as a functional marker for the identification of natural killer cell activity. *J Immunol Methods* **294**, 15-22, doi:10.1016/j.jim.2004.08.008 (2004).
60 Krzewski, K., Gil-Krzewska, A., Nguyen, V., Peruzzi, G. & Coligan, J. E. LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. *Blood* **121**, 4672-4683, doi:10.1182/blood-2012-08-453738 (2013).
61 Law, R. H. *et al.* The structural basis for membrane binding and pore formation by lymphocyte perforin. *Nature* **468**, 447-451, doi:10.1038/nature09518 (2010).
62 Kurschus, F. C., Fellows, E., Stegmann, E. & Jenne, D. E. Granzyme B delivery via perforin is restricted by size, but not by heparan sulfate-dependent endocytosis. *Proc Natl Acad Sci U S A* **105**, 13799-13804, doi:10.1073/pnas.0801724105 (2008).
63 Kaiserman, D. *et al.* The major human and mouse granzymes are structurally and functionally divergent. *The Journal of cell biology* **175**, 619-630, doi:10.1083/jcb.200606073 (2006).
64 Ross, M. E. & Caligiuri, M. A. Cytokine-induced apoptosis of human natural killer cells identifies a novel mechanism to regulate the innate immune response. *Blood* **89**, 910-918 (1997).
65 Roda, J. M. *et al.* Natural killer cells produce T cell-recruiting chemokines in response to antibody-coated tumor cells. *Cancer Res* **66**, 517-526, doi:10.1158/0008-5472.Can-05-2429 (2006).
66 Fehniger, T. A. *et al.* Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J Immunol* **162**, 4511-4520 (1999).
67 Fauriat, C., Long, E. O., Ljunggren, H.-G. & Bryceson, Y. T. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* **115**, 2167-2176, doi:10.1182/blood-2009-08-238469 (2010).
68 Perona-Wright, G. *et al.* Systemic but not local infections elicit immunosuppressive IL-10 production by natural killer cells. *Cell host & microbe* **6**, 503-512, doi:10.1016/j.chom.2009.11.003 (2009).

69 Brandstadter, J. D. & Yang, Y. Natural killer cell responses to viral infection. *Journal of innate immunity* **3**, 274-279, doi:10.1159/000324176 (2011).
70 Bukowski, J. F. & Welsh, R. M. Inability of interferon to protect virus-infected cells against lysis by natural killer (NK) cells correlates with NK cell-mediated antiviral effects in vivo. *J Immunol* **135**, 3537-3541 (1985).
71 Bukowski, J. F., Woda, B. A. & Welsh, R. M. Pathogenesis of murine cytomegalovirus infection in natural killer cell-depleted mice. *J Virol* **52**, 119-128 (1984).
72 Scalzo, A. A., Corbett, A. J., Rawlinson, W. D., Scott, G. M. & Degli-Esposti, M. A. The interplay between host and viral factors in shaping the outcome of cytomegalovirus infection. *Immunology and cell biology* **85**, 46-54, doi:10.1038/sj.icb.7100013 (2007).
73 Biron, C. A., Byron, K. S. & Sullivan, J. L. Severe Herpesvirus Infections in an Adolescent without Natural Killer Cells. *New England Journal of Medicine* **320**, 1731-1735, doi:10.1056/nejm198906293202605 (1989).
74 Etzioni, A. *et al.* Fatal varicella associated with selective natural killer cell deficiency. *The Journal of pediatrics* **146**, 423-425, doi:10.1016/j.jpeds.2004.11.022 (2005).
75 Eidenschenk, C. *et al.* A Novel Primary Immunodeficiency with Specific Natural-Killer Cell Deficiency Maps to the Centromeric Region of Chromosome 8. *The American Journal of Human Genetics* **78**, 721-727, doi:10.1086/503269 (2006).
76 Robbins, S. H. *et al.* Natural killer cells promote early CD8 T cell responses against cytomegalovirus. *PLoS Pathog* **3**, e123, doi:10.1371/journal.ppat.0030123 (2007).
77 Fodil-Cornu, N. *et al.* Ly49h-Deficient C57BL/6 Mice: A New Mouse Cytomegalovirus-Susceptible Model Remains Resistant to Unrelated Pathogens Controlled by the NK Gene Complex. *The Journal of Immunology* **181**, 6394-6405, doi:10.4049/jimmunol.181.9.6394 (2008).
78 Lee, S. H. *et al.* Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. *Nat Genet* **28**, 42-45, doi:10.1038/88247 (2001).
79 Arase, H., Mocarski, E. S., Campbell, A. E., Hill, A. B. & Lanier, L. L. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* **296**, 1323-1326, doi:10.1126/science.1070884 (2002).
80 Brodbeck, T., Nehmann, N., Bethge, A., Wedemann, G. & Schumacher, U. Perforin-dependent direct cytotoxicity in natural killer cells induces considerable knockdown of spontaneous lung metastases and computer modelling-proven tumor cell dormancy in a HT29 human colon cancer xenograft mouse model. *Molecular cancer* **13**, 244-244, doi:10.1186/1476-4598-13-244 (2014).
81 van den Broek, M. F., Kagi, D., Zinkernagel, R. M. & Hengartner, H. Perforin dependence of natural killer cell-mediated tumor control in vivo. *European journal of immunology* **25**, 3514-3516, doi:10.1002/eji.1830251246 (1995).
82 van den Broek, M. E. *et al.* Decreased tumor surveillance in perforin-deficient mice. *J Exp Med* **184**, 1781-1790 (1996).
83 Smyth, M. J. *et al.* Perforin Is a Major Contributor to NK Cell Control of Tumor Metastasis. *The Journal of Immunology* **162**, 6658-6662 (1999).
84 Kim, S., Iizuka, K., Aguila, H. L., Weissman, I. L. & Yokoyama, W. M. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 2731-2736, doi:10.1073/pnas.050588297 (2000).

85 Imai, K., Matsuyama, S., Miyake, S., Suga, K. & Nakachi, K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet (London, England)* **356**, 1795-1799, doi:10.1016/s0140-6736(00)03231-1 (2000).
86 Souza-Fonseca-Guimaraes, F., Cursons, J. & Huntington, N. D. The Emergence of Natural Killer Cells as a Major Target in Cancer Immunotherapy. *Trends Immunol* **40**, 142-158, doi:10.1016/j.it.2018.12.003 (2019).
87 Whiteside, T. L. & Herberman, R. B. Role of human natural killer cells in health and disease. *Clinical and diagnostic laboratory immunology* **1**, 125-133 (1994).
88 Sung, P. S. & Jang, J. W. Natural Killer Cell Dysfunction in Hepatocellular Carcinoma: Pathogenesis and Clinical Implications. *International journal of molecular sciences* **19**, 3648, doi:10.3390/ijms19113648 (2018).
89 Chiu, J., Ernst, D. M. & Keating, A. Acquired Natural Killer Cell Dysfunction in the Tumor Microenvironment of Classic Hodgkin Lymphoma. *Frontiers in immunology* **9**, 267-267, doi:10.3389/fimmu.2018.00267 (2018).
90 Burns, L. J. *et al.* IL-2-based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: a phase I/II trial. *Bone marrow transplantation* **32**, 177-186, doi:10.1038/sj.bmt.1704086 (2003).
91 Cichocki, F. *et al.* The Past, Present, and Future of NK Cells in Hematopoietic Cell Transplantation and Adoptive Transfer. *Current topics in microbiology and immunology* **395**, 225-243, doi:10.1007/82_2015_445 (2016).
92 Ruggeri, L. *et al.* Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood* **94**, 333-339 (1999).
93 Ruggeri, L. *et al.* Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* **295**, 2097-2100, doi:10.1126/science.1068440 (2002).
94 Miller, J. S. *et al.* Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood* **105**, 3051-3057, doi:10.1182/blood-2004-07-2974 (2005).
95 Porrata, L. F. *et al.* Interleukin-15 affects patient survival through natural killer cell recovery after autologous hematopoietic stem cell transplantation for non-Hodgkin lymphomas. *Clinical & developmental immunology* **2010**, 914945, doi:10.1155/2010/914945 (2010).
96 Björklund, A. T. *et al.* Complete Remission with Reduction of High-risk Clones following Haploidentical NK Cell Therapy against MDS and AML. *Clinical Cancer Research*, clincanres.3196.2017, doi:10.1158/1078-0432.Ccr-17-3196 (2018).
97 Vitale, M., Cantoni, C., Pietra, G., Mingari, M. C. & Moretta, L. Effect of tumor cells and tumor microenvironment on NK-cell function. *European journal of immunology* **44**, 1582-1592, doi:10.1002/eji.201344272 (2014).
98 Hasmim, M. *et al.* Critical Role of Tumor Microenvironment in Shaping NK Cell Functions: Implication of Hypoxic Stress. *Frontiers in Immunology* **6**, doi:10.3389/fimmu.2015.00482 (2015).
99 Berrien-Elliott, M. M., Romee, R. & Fehniger, T. A. Improving natural killer cell cancer immunotherapy. *Current opinion in organ transplantation* **20**, 671-680, doi:10.1097/mot.0000000000000243 (2015).

100 Boerman, G. H. *et al.* Role of NKG2D, DNAM-1 and natural cytotoxicity receptors in cytotoxicity toward rhabdomyosarcoma cell lines mediated by resting and IL-15-activated human natural killer cells. *Cancer immunology, immunotherapy : CII* **64**, 573-583, doi:10.1007/s00262-015-1657-9 (2015).
101 van Ostaijen-ten Dam, M. M. *et al.* Preparation of Cytokine-activated NK Cells for Use in Adoptive Cell Therapy in Cancer Patients: Protocol Optimization and Therapeutic Potential. *Journal of immunotherapy (Hagerstown, Md. : 1997)* **39**, 90-100, doi:10.1097/cji.0000000000000110 (2016).
102 Wu, Y., Tian, Z. & Wei, H. Developmental and Functional Control of Natural Killer Cells by Cytokines. *Front Immunol* **8**, 930, doi:10.3389/fimmu.2017.00930 (2017).
103 Iliopoulou, E. G. *et al.* A phase I trial of adoptive transfer of allogeneic natural killer cells in patients with advanced non-small cell lung cancer. *Cancer immunology, immunotherapy : CII* **59**, 1781-1789, doi:10.1007/s00262-010-0904-3 (2010).
104 Romee, R. *et al.* Cytokine activation induces human memory-like NK cells. *Blood* **120**, 4751-4760, doi:10.1182/blood-2012-04-419283 (2012).
105 Cooper, M. A. *et al.* Cytokine-induced memory-like natural killer cells. *Proc Natl Acad Sci U S A* **106**, 1915-1919, doi:10.1073/pnas.0813192106 (2009).
106 Ni, J., Miller, M., Stojanovic, A., Garbi, N. & Cerwenka, A. Sustained effector function of IL-12/15/18-preactivated NK cells against established tumors. *J Exp Med* **209**, 2351-2365, doi:10.1084/jem.20120944 (2012).
107 Boieri, M. *et al.* IL-12, IL-15, and IL-18 pre-activated NK cells target resistant T cell acute lymphoblastic leukemia and delay leukemia development in vivo. *Oncoimmunology* **6**, e1274478, doi:10.1080/2162402x.2016.1274478 (2017).
108 Ni, J. *et al.* Adoptively transferred natural killer cells maintain long-term antitumor activity by epigenetic imprinting and CD4(+) T cell help. *Oncoimmunology* **5**, e1219009, doi:10.1080/2162402x.2016.1219009 (2016).
109 Leong, J. W. *et al.* Preactivation with IL-12, IL-15, and IL-18 induces CD25 and a functional high-affinity IL-2 receptor on human cytokine-induced memory-like natural killer cells. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* **20**, 463-473, doi:10.1016/j.bbmt.2014.01.006 (2014).
110 Romee, R. *et al.* Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. *Science translational medicine* **8**, 357ra123, doi:10.1126/scitranslmed.aaf2341 (2016).
111 Bantug, G. R. *et al.* Mitochondria-Endoplasmic Reticulum Contact Sites Function as Immunometabolic Hubs that Orchestrate the Rapid Recall Response of Memory CD8(+) T Cells. *Immunity* **48**, 542-555 e546, doi:10.1016/j.immuni.2018.02.012 (2018).
112 Gubser, P. M. *et al.* Rapid effector function of memory CD8+ T cells requires an immediate-early glycolytic switch. *Nat Immunol* **14**, 1064-1072, doi:10.1038/ni.2687 (2013).
113 van der Windt, G. J. *et al.* CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability. *Proc Natl Acad Sci U S A* **110**, 14336-14341, doi:10.1073/pnas.1221740110 (2013).
114 Buck, M. D., Sowell, R. T., Kaech, S. M. & Pearce, E. L. Metabolic Instruction of Immunity. *Cell* **169**, 570-586, doi:10.1016/j.cell.2017.04.004 (2017).

115 Zheng, J. Energy metabolism of cancer: Glycolysis versus oxidative phosphorylation (Review). *Oncology letters* **4**, 1151-1157, doi:10.3892/ol.2012.928 (2012).
116 Gardiner, C. M. & Finlay, D. K. What Fuels Natural Killers? Metabolism and NK Cell Responses. *Frontiers in immunology* **8**, 367-367, doi:10.3389/fimmu.2017.00367 (2017).
117 MacIver, N. J., Michalek, R. D. & Rathmell, J. C. Metabolic regulation of T lymphocytes. *Annual review of immunology* **31**, 259-283, doi:10.1146/annurev-immunol-032712-095956 (2013).
118 Frauwirth, K. A. *et al.* The CD28 signaling pathway regulates glucose metabolism. *Immunity* **16**, 769-777 (2002).
119 Jacobs, S. R. *et al.* Glucose uptake is limiting in T cell activation and requires CD28-mediated Akt-dependent and independent pathways. *J Immunol* **180**, 4476-4486 (2008).
120 Wang, R. *et al.* The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. *Immunity* **35**, 871-882, doi:10.1016/j.immuni.2011.09.021 (2011).
121 Liberti, M. V. & Locasale, J. W. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends in biochemical sciences* **41**, 211-218, doi:10.1016/j.tibs.2015.12.001 (2016).
122 Chang, C. H. *et al.* Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* **153**, 1239-1251, doi:10.1016/j.cell.2013.05.016 (2013).
123 Donnelly, R. P. *et al.* mTORC1-Dependent Metabolic Reprogramming Is a Prerequisite for NK Cell Effector Function. *The Journal of Immunology* **193**, 4477-4484, doi:10.4049/jimmunol.1401558 (2014).
124 Keppel, M. P., Saucier, N., Mah, A. Y., Vogel, T. P. & Cooper, M. A. Activation-specific metabolic requirements for NK Cell IFN-gamma production. *J Immunol* **194**, 1954-1962, doi:10.4049/jimmunol.1402099 (2015).
125 Marçais, A. *et al.* The metabolic checkpoint kinase mTOR is essential for IL-15 signaling during the development and activation of NK cells. *Nature Immunology* **15**, 749-757, doi:10.1038/ni.2936 (2014).
126 Keating, S. E. *et al.* Metabolic Reprogramming Supports IFN-gamma Production by CD56bright NK Cells. *J Immunol* **196**, 2552-2560, doi:10.4049/jimmunol.1501783 (2016).
127 Viel, S. *et al.* TGF-beta inhibits the activation and functions of NK cells by repressing the mTOR pathway. *Sci Signal* **9**, ra19, doi:10.1126/scisignal.aad1884 (2016).
128 Assmann, N. *et al.* Srebp-controlled glucose metabolism is essential for NK cell functional responses. *Nat Immunol* **18**, 1197-1206, doi:10.1038/ni.3838 (2017).
129 O'Brien, K. L. & Finlay, D. K. Immunometabolism and natural killer cell responses. *Nat Rev Immunol*, doi:10.1038/s41577-019-0139-2 (2019).
130 Jensen, H., Potempa, M., Gotthardt, D. & Lanier, L. L. Cutting Edge: IL-2-Induced Expression of the Amino Acid Transporters SLC1A5 and CD98 Is a Prerequisite for NKG2D-Mediated Activation of Human NK Cells. *J Immunol* **199**, 1967-1972, doi:10.4049/jimmunol.1700497 (2017).
131 Mah, A. Y. *et al.* Glycolytic requirement for NK cell cytotoxicity and cytomegalovirus control. *JCI Insight* **2**, doi:10.1172/jci.insight.95128 (2017).
132 Loftus, R. M. *et al.* Amino acid-dependent cMyc expression is essential for NK cell metabolic and functional responses in mice. *Nat Commun* **9**, 2341, doi:10.1038/s41467-018-04719-2 (2018).

133 Horton, J. D., Goldstein, J. L. & Brown, M. S. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *The Journal of clinical investigation* **109**, 1125-1131, doi:10.1172/jci15593 (2002).
134 O'Neill, L. A. J., Kishton, R. J. & Rathmell, J. A guide to immunometabolism for immunologists. *Nature reviews. Immunology* **16**, 553-565, doi:10.1038/nri.2016.70 (2016).
135 Jabara, H. H. *et al.* A missense mutation in TFRC, encoding transferrin receptor 1, causes combined immunodeficiency. *Nat Genet* **48**, 74-78, doi:10.1038/ng.3465 (2016).
136 Almutairi, S. M. *et al.* Interleukin-18 up-regulates amino acid transporters and facilitates amino acid-induced mTORC1 activation in natural killer cells. *J Biol Chem* **294**, 4644-4655, doi:10.1074/jbc.RA118.005892 (2019).
137 Bernardino de la Serna, J. *et al.* Tissue-resident NK cells differ in their expression profile of the nutrient transporters Glut1, CD98 and CD71. *Plos One* **13**, doi:10.1371/journal.pone.0201170 (2018).
138 Caro-Maldonado, A., Gerriets, V. A. & Rathmell, J. C. Matched and mismatched metabolic fuels in lymphocyte function. *Seminars in immunology* **24**, 405-413, doi:10.1016/j.smim.2012.12.002 (2012).
139 Maciver, N. J. *et al.* Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival. *Journal of leukocyte biology* **84**, 949-957, doi:10.1189/jlb.0108024 (2008).
140 Horecker, B. L. The pentose phosphate pathway. *J Biol Chem* **277**, 47965-47971, doi:10.1074/jbc.X200007200 (2002).
141 Poffenberger, Maya C. & Jones, Russell G. Amino Acids Fuel T Cell-Mediated Inflammation. *Immunity* **40**, 635-637, doi:https://doi.org/10.1016/j.immuni.2014.04.017 (2014).
142 Napolitano, L. *et al.* LAT1 is the transport competent unit of the LAT1/CD98 heterodimeric amino acid transporter. *The international journal of biochemistry & cell biology* **67**, 25-33, doi:10.1016/j.biocel.2015.08.004 (2015).
143 Cantor, J., Slepak, M., Ege, N., Chang, J. T. & Ginsberg, M. H. Loss of T cell CD98 H chain specifically ablates T cell clonal expansion and protects from autoimmunity. *J Immunol* **187**, 851-860, doi:10.4049/jimmunol.1100002 (2011).
144 Cantor, J. M. & Ginsberg, M. H. CD98 at the crossroads of adaptive immunity and cancer. *J Cell Sci* **125**, 1373-1382, doi:10.1242/jcs.096040 (2012).
145 Lederman, H. M., Cohen, A., Lee, J. W., Freedman, M. H. & Gelfand, E. W. Deferoxamine: a reversible S-phase inhibitor of human lymphocyte proliferation. *Blood* **64**, 748-753 (1984).
146 Thelander, L., Gräslund, A. & Thelander, M. Continual presence of oxygen and iron required for mammalian ribonucleotide reduction: Possible regulation mechanism. *Biochemical and Biophysical Research Communications* **110**, 859-865, doi:10.1016/0006-291X(83)91040-9 (1983).
147 Oexle, H., Gnaiger, E. & Weiss, G. Iron-dependent changes in cellular energy metabolism: influence on citric acid cycle and oxidative phosphorylation. *Biochim Biophys Acta* **1413**, 99-107 (1999).
148 Dlouhy, A. C. & Outten, C. E. The iron metallome in eukaryotic organisms. *Metal ions in life sciences* **12**, 241-278, doi:10.1007/978-94-007-5561-1_8 (2013).

149 White, M. F. & Dillingham, M. S. Iron-sulphur clusters in nucleic acid processing enzymes. *Current opinion in structural biology* **22**, 94-100, doi:10.1016/j.sbi.2011.11.004 (2012).
150 Le, N. T. & Richardson, D. R. The role of iron in cell cycle progression and the proliferation of neoplastic cells. *Biochim Biophys Acta* **1603**, 31-46 (2002).
151 Muckenthaler, M. U., Rivella, S., Hentze, M. W. & Galy, B. A Red Carpet for Iron Metabolism. *Cell* **168**, 344-361, doi:10.1016/j.cell.2016.12.034 (2017).
152 Heinzel, S., Marchingo, J. M., Horton, M. B. & Hodgkin, P. D. The regulation of lymphocyte activation and proliferation. *Curr Opin Immunol* **51**, 32-38, doi:10.1016/j.coi.2018.01.002 (2018).
153 O'Donnell, K. A. *et al.* Activation of transferrin receptor 1 by c-Myc enhances cellular proliferation and tumorigenesis. *Mol Cell Biol* **26**, 2373-2386, doi:10.1128/MCB.26.6.2373-2386.2006 (2006).
154 Salzberger, W. *et al.* Tissue-resident NK cells differ in their expression profile of the nutrient transporters Glut1, CD98 and CD71. *PLoS One* **13**, e0201170, doi:10.1371/journal.pone.0201170 (2018).
155 Spear, A. T. & Sherman, A. R. Iron deficiency alters DMBA-induced tumor burden and natural killer cell cytotoxicity in rats. *The Journal of nutrition* **122**, 46-55, doi:10.1093/jn/122.1.46 (1992).
156 Sherman, A. R. & Lockwood, J. F. Impaired natural killer cell activity in iron-deficient rat pups. *The Journal of nutrition* **117**, 567-571, doi:10.1093/jn/117.3.567 (1987).
157 Chandra, R. K. 1990 McCollum Award lecture. Nutrition and immunity: lessons from the past and new insights into the future. *The American journal of clinical nutrition* **53**, 1087-1101, doi:10.1093/ajcn/53.5.1087 (1991).
158 Aisen, P., Wessling-Resnick, M. & Leibold, E. A. Iron metabolism. *Current opinion in chemical biology* **3**, 200-206, doi:10.1016/s1367-5931(99)80033-7 (1999).
159 Eisenstein, R. S. Iron regulatory proteins and the molecular control of mammalian iron metabolism. *Annu Rev Nutr* **20**, 627-662, doi:10.1146/annurev.nutr.20.1.627 (2000).
160 Chen, O. S., Schalinske, K. L. & Eisenstein, R. S. Dietary iron intake modulates the activity of iron regulatory proteins and the abundance of ferritin and mitochondrial aconitase in rat liver. *The Journal of nutrition* **127**, 238-248, doi:10.1093/jn/127.2.238 (1997).
161 Beinert, H., Kennedy, M. C. & Stout, C. D. Aconitase as Ironminus signSulfur Protein, Enzyme, and Iron-Regulatory Protein. *Chemical reviews* **96**, 2335-2374 (1996).
162 Haile, D. J. Regulation of genes of iron metabolism by the iron-response proteins. *The American journal of the medical sciences* **318**, 230-240 (1999).
163 Iwai, K. *et al.* Iron-dependent oxidation, ubiquitination, and degradation of iron regulatory protein 2: implications for degradation of oxidized proteins. *Proc Natl Acad Sci U S A* **95**, 4924-4928 (1998).
164 Vashisht, A. A. *et al.* Control of Iron Homeostasis by an Iron-Regulated Ubiquitin Ligase. *Science* **326**, 718-721, doi:10.1126/science.1176333 (2009).
165 Salahudeen, A. A. *et al.* An E3 Ligase Possessing an Iron-Responsive Hemerythrin Domain Is a Regulator of Iron Homeostasis. *Science* **326**, 722-726, doi:10.1126/science.1176326 (2009).
166 Theil, E. C. & Eisenstein, R. S. Combinatorial mRNA regulation: iron regulatory proteins and iso-iron-responsive elements (Iso-IREs). *J Biol Chem* **275**, 40659-40662, doi:10.1074/jbc.R000019200 (2000).

167 Rouault, T. & Klausner, R. Regulation of iron metabolism in eukaryotes. *Current topics in cellular regulation* **35**, 1-19 (1997).
168 Hentze, M. W., Muckenthaler, M. U., Galy, B. & Camaschella, C. Two to tango: regulation of Mammalian iron metabolism. *Cell* **142**, 24-38, doi:10.1016/j.cell.2010.06.028 (2010).
169 Costain, G. *et al.* Absence of iron-responsive element-binding protein 2 causes a novel neurodegenerative syndrome. *Brain*, doi:10.1093/brain/awz072 (2019).
170 Chitambar, C. R., Massey, E. J. & Seligman, P. A. Regulation of transferrin receptor expression on human leukemic cells during proliferation and induction of differentiation. Effects of gallium and dimethylsulfoxide. *The Journal of clinical investigation* **72**, 1314-1325, doi:10.1172/jci111087 (1983).
171 Kuhn, L. C. The transferrin receptor: a key function in iron metabolism. *Schweizerische medizinische Wochenschrift* **119**, 1319-1326 (1989).
172 Kwok, J. C. & Richardson, D. R. The iron metabolism of neoplastic cells: alterations that facilitate proliferation? *Critical reviews in oncology/hematology* **42**, 65-78 (2002).
173 Romeo, A. M., Christen, L., Niles, E. G. & Kosman, D. J. Intracellular chelation of iron by bipyridyl inhibits DNA virus replication: ribonucleotide reductase maturation as a probe of intracellular iron pools. *J Biol Chem* **276**, 24301-24308, doi:10.1074/jbc.M010806200 (2001).
174 Koutcher, J. A. *et al.* Effect of 6-aminonicotinamide on the pentose phosphate pathway: 31P NMR and tumor growth delay studies. *Magnetic resonance in medicine* **36**, 887-892 (1996).
175 Tsouko, E. *et al.* Regulation of the pentose phosphate pathway by an androgen receptor-mTOR-mediated mechanism and its role in prostate cancer cell growth. *Oncogenesis* **3**, e103, doi:10.1038/oncsis.2014.18 (2014).
176 Bubic, I. *et al.* Gain of Virulence Caused by Loss of a Gene in Murine Cytomegalovirus. *Journal of Virology* **78**, 7536-7544, doi:10.1128/jvi.78.14.7536-7544.2004 (2004).
177 Eckelhart, E. *et al.* A novel Ncr1-Cre mouse reveals the essential role of STAT5 for NK-cell survival and development. *Blood* **117**, 1565-1573, doi:10.1182/blood-2010-06-291633 (2011).
178 Marcais, A. *et al.* Regulation of mouse NK cell development and function by cytokines. *Front Immunol* **4**, 450, doi:10.3389/fimmu.2013.00450 (2013).
179 Prlic, M., Blazar, B. R., Farrar, M. A. & Jameson, S. C. In vivo survival and homeostatic proliferation of natural killer cells. *J Exp Med* **197**, 967-976, doi:10.1084/jem.20021847 (2003).
180 Sun, J. C., Beilke, J. N., Bezman, N. A. & Lanier, L. L. Homeostatic proliferation generates long-lived natural killer cells that respond against viral infection. *J Exp Med* **208**, 357-368, doi:10.1084/jem.20100479 (2011).
181 Preston, G. C. *et al.* Single cell tuning of Myc expression by antigen receptor signal strength and interleukin-2 in T lymphocytes. *EMBO J* **34**, 2008-2024, doi:10.15252/embj.201490252 (2015).
182 Hentze, M. W. & Kuhn, L. C. Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. *Proc Natl Acad Sci U S A* **93**, 8175-8182 (1996).
183 Hentze, M. W., Muckenthaler, M. U. & Andrews, N. C. Balancing acts: molecular control of mammalian iron metabolism. *Cell* **117**, 285-297 (2004).

184 Campillos, M., Cases, I., Hentze, M. W. & Sanchez, M. SIREs: searching for iron-responsive elements. *Nucleic Acids Res* **38**, W360-367, doi:10.1093/nar/gkq371 (2010).
185 Anderson, E. R. & Shah, Y. M. Iron homeostasis in the liver. *Comprehensive Physiology* **3**, 315-330, doi:10.1002/cphy.c120016 (2013).
186 Ned, R. M., Swat, W. & Andrews, N. C. Transferrin receptor 1 is differentially required in lymphocyte development. *Blood* **102**, 3711-3718, doi:10.1182/blood-2003-04-1086 (2003).
187 Chan, R. Y., Ponka, P. & Schulman, H. M. Transferrin-receptor-independent but iron-dependent proliferation of variant Chinese hamster ovary cells. *Experimental cell research* **202**, 326-336 (1992).
188 Konijn, A. M., Meyron-Holtz, E. G., Fibach, E. & Gelvan, D. Cellular ferritin uptake: a highly regulated pathway for iron assimilation in human erythroid precursor cells. *Advances in experimental medicine and biology* **356**, 189-197 (1994).
189 Gelvan, D., Fibach, E., Meyron-Holtz, E. & Konijn, A. Ferritin uptake by human erythroid precursors is a regulated iron uptake pathway. *Blood* **88**, 3200-3207 (1996).
190 Meyron-Holtz, E. G. *et al.* Regulation of intracellular iron metabolism in human erythroid precursors by internalized extracellular ferritin. *Blood* **94**, 3205-3211 (1999).
191 Goto, Y., Paterson, M. & Listowsky, I. Iron uptake and regulation of ferritin synthesis by hepatoma cells in hormone-supplemented serum-free media. *J Biol Chem* **258**, 5248-5255 (1983).
192 Kaplan, J., Jordan, I. & Sturrock, A. Regulation of the transferrin-independent iron transport system in cultured cells. *J Biol Chem* **266**, 2997-3004 (1991).
193 Sturrock, A., Alexander, J., Lamb, J., Craven, C. M. & Kaplan, J. Characterization of a transferrin-independent uptake system for iron in HeLa cells. *J Biol Chem* **265**, 3139-3145 (1990).
194 Hodgson, L. L., Quail, E. A. & Morgan, E. H. Iron transport mechanisms in reticulocytes and mature erythrocytes. *Journal of cellular physiology* **162**, 181-190, doi:10.1002/jcp.1041620204 (1995).
195 Dixon, S. J. & Stockwell, B. R. The role of iron and reactive oxygen species in cell death. *Nature chemical biology* **10**, 9-17, doi:10.1038/nchembio.1416 (2014).
196 Bogdan, A. R., Miyazawa, M., Hashimoto, K. & Tsuji, Y. Regulators of Iron Homeostasis: New Players in Metabolism, Cell Death, and Disease. *Trends in Biochemical Sciences* **41**, 274-286, doi:https://doi.org/10.1016/j.tibs.2015.11.012 (2016).
197 Rouault, T. A. The role of iron regulatory proteins in mammalian iron homeostasis and disease. *Nature chemical biology* **2**, 406-414, doi:10.1038/nchembio807 (2006).
198 Wu, K. J., Polack, A. & Dalla-Favera, R. Coordinated regulation of iron-controlling genes, H-ferritin and IRP2, by c-MYC. *Science* **283**, 676-679 (1999).
199 Sanchez, M. *et al.* Iron regulatory protein-1 and -2: transcriptome-wide definition of binding mRNAs and shaping of the cellular proteome by iron regulatory proteins. *Blood* **118**, e168-e179, doi:10.1182/blood-2011-04-343541 (2011).
200 Galy, B., Ferring, D., Benesova, M., Benes, V. & Hentze, M. W. Targeted mutagenesis of the murine IRP1 and IRP2 genes reveals context-dependent RNA processing differences in vivo. *Rna* **10**, 1019-1025, doi:10.1261/rna.7220704 (2004).

201 Meyron-Holtz, E. G. *et al.* Genetic ablations of iron regulatory proteins 1 and 2 reveal why iron regulatory protein 2 dominates iron homeostasis. *The EMBO journal* **23**, 386-395, doi:10.1038/sj.emboj.7600041 (2004).
202 LaVaute, T. *et al.* Targeted deletion of the gene encoding iron regulatory protein-2 causes misregulation of iron metabolism and neurodegenerative disease in mice. *Nat Genet* **27**, 209-214, doi:10.1038/84859 (2001).
203 Wang, W. *et al.* IRP2 regulates breast tumor growth. *Cancer Res* **74**, 497-507, doi:10.1158/0008-5472.CAN-13-1224 (2014).
204 Miyazawa, M., Bogdan, A. R. & Tsuji, Y. Perturbation of Iron Metabolism by Cisplatin through Inhibition of Iron Regulatory Protein 2. *Cell Chem Biol* **26**, 85-97 e84, doi:10.1016/j.chembiol.2018.10.009 (2019).
205 Deng, Z., Manz, D. H., Torti, S. V. & Torti, F. M. Iron-responsive element-binding protein 2 plays an essential role in regulating prostate cancer cell growth. *Oncotarget* **8**, 82231-82243, doi:10.18632/oncotarget.19288 (2017).
206 Sonenberg, N. & Hinnebusch, A. G. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* **136**, 731-745, doi:10.1016/j.cell.2009.01.042 (2009).
207 Culjkovic, B., Topisirovic, I., Skrabanek, L., Ruiz-Gutierrez, M. & Borden, K. L. eIF4E promotes nuclear export of cyclin D1 mRNAs via an element in the 3'UTR. *The Journal of cell biology* **169**, 245-256, doi:10.1083/jcb.200501019 (2005).
208 Culjkovic, B., Topisirovic, I., Skrabanek, L., Ruiz-Gutierrez, M. & Borden, K. L. eIF4E is a central node of an RNA regulon that governs cellular proliferation. *The Journal of cell biology* **175**, 415-426, doi:10.1083/jcb.200607020 (2006).
209 Lazaris-Karatzas, A., Montine, K. S. & Sonenberg, N. Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. *Nature* **345**, 544-547, doi:10.1038/345544a0 (1990).
210 De Benedetti, A. & Graff, J. R. eIF-4E expression and its role in malignancies and metastases. *Oncogene* **23**, 3189-3199, doi:10.1038/sj.onc.1207545 (2004).
211 Joshi, B., Cameron, A. & Jagus, R. Characterization of mammalian eIF4E-family members. *European journal of biochemistry* **271**, 2189-2203, doi:10.1111/j.1432-1033.2004.04149.x (2004).
212 Truitt, M. L. *et al.* Differential Requirements for eIF4E Dose in Normal Development and Cancer. *Cell* **162**, 59-71, doi:10.1016/j.cell.2015.05.049 (2015).
213 Hsieh, A. C. *et al.* The translational landscape of mTOR signalling steers cancer initiation and metastasis. *Nature* **485**, 55-61, doi:10.1038/nature10912 (2012).
214 Mamane, Y. *et al.* Epigenetic activation of a subset of mRNAs by eIF4E explains its effects on cell proliferation. *PloS one* **2**, e242-e242, doi:10.1371/journal.pone.0000242 (2007).
215 Wang, Z. *et al.* Iron Drives T Helper Cell Pathogenicity by Promoting RNA-Binding Protein PCBP1-Mediated Proinflammatory Cytokine Production. *Immunity* **49**, 80-92 e87, doi:10.1016/j.immuni.2018.05.008 (2018).
216 Salmeron, A. *et al.* Transferrin receptor induces tyrosine phosphorylation in T cells and is physically associated with the TCR zeta-chain. *J Immunol* **154**, 1675-1683 (1995).
217 Schweier, O., Hofmann, M. & Pircher, H. KLRG1 activity is regulated by association with the transferrin receptor. *European journal of immunology* **44**, 1851-1856, doi:10.1002/eji.201344234 (2014).

218 Wellen, K. E. *et al.* ATP-citrate lyase links cellular metabolism to histone acetylation. *Science* **324**, 1076-1080, doi:10.1126/science.1164097 (2009).
219 Moussaieff, A. *et al.* Glycolysis-mediated changes in acetyl-CoA and histone acetylation control the early differentiation of embryonic stem cells. *Cell Metab* **21**, 392-402, doi:10.1016/j.cmet.2015.02.002 (2015).
220 Ducker, G. S. & Rabinowitz, J. D. One-Carbon Metabolism in Health and Disease. *Cell Metab* **25**, 27-42, doi:10.1016/j.cmet.2016.08.009 (2017).
221 Klose, R. J., Kallin, E. M. & Zhang, Y. JmjC-domain-containing proteins and histone demethylation. *Nature reviews. Genetics* **7**, 715-727, doi:10.1038/nrg1945 (2006).
222 Viel, S., Besson, L., Marotel, M., Walzer, T. & Marcais, A. Regulation of mTOR, Metabolic Fitness, and Effector Functions by Cytokines in Natural Killer Cells. *Cancers (Basel)* **9**, doi:10.3390/cancers9100132 (2017).
223 Heijmans, B. T. *et al.* Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A* **105**, 17046-17049, doi:10.1073/pnas.0806560105 (2008).
224 Ost, A. *et al.* Paternal diet defines offspring chromatin state and intergenerational obesity. *Cell* **159**, 1352-1364, doi:10.1016/j.cell.2014.11.005 (2014).
225 Pujadas, E. & Feinberg, A. P. Regulated noise in the epigenetic landscape of development and disease. *Cell* **148**, 1123-1131, doi:10.1016/j.cell.2012.02.045 (2012).
226 Luetke-Eversloh, M. *et al.* Human cytomegalovirus drives epigenetic imprinting of the IFNG locus in NKG2Chi natural killer cells. *PLoS Pathog* **10**, e1004441, doi:10.1371/journal.ppat.1004441 (2014).
227 Young, H. A. Regulation of interferon-gamma gene expression. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* **16**, 563-568, doi:10.1089/jir.1996.16.563 (1996).
228 Sullivan, R. P. *et al.* MicroRNA-deficient NK cells exhibit decreased survival but enhanced function. *J Immunol* **188**, 3019-3030, doi:10.4049/jimmunol.1102294 (2012).
229 Ma, F. *et al.* The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-gamma. *Nat Immunol* **12**, 861-869, doi:10.1038/ni.2073 (2011).
230 Lim, W. A. & June, C. H. The Principles of Engineering Immune Cells to Treat Cancer. *Cell* **168**, 724-740, doi:10.1016/j.cell.2017.01.016 (2017).
231 Jordan, S. *et al.* Virus progeny of murine cytomegalovirus bacterial artificial chromosome pSM3fr show reduced growth in salivary Glands due to a fixed mutation of MCK-2. *J Virol* **85**, 10346-10353, doi:10.1128/jvi.00545-11 (2011).
232 Hirche, C. *et al.* Systemic Virus Infections Differentially Modulate Cell Cycle State and Functionality of Long-Term Hematopoietic Stem Cells In Vivo. *Cell Rep* **19**, 2345-2356, doi:10.1016/j.celrep.2017.05.063 (2017).
233 Brizic, I., Lisnic, B., Brune, W., Hengel, H. & Jonjic, S. Cytomegalovirus Infection: Mouse Model. *Current protocols in immunology*, e51, doi:10.1002/cpim.51 (2018).