PhD-FSTM-2021-083
The Faculty of Sciences, Technology and Medicine

DISSERTATION

Defence held on 22/11/2021 in Esch-sur-Alzette, Luxembourg
to obtain the degree of

DOCTEUR DE L'UNIVERSITÉ DU LUXEMBOURG

EN BIOLOGIE

by

Luana FRIAS GUERRA
Born on 22nd October 1992 in Loures, Portugal

THE ANTIOXIDANT GLUTATHIONE AS A REGULATOR OF NATURAL KILLER CELL IMMUNITY

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Amar la trama…

The work presented in this thesis was conducted at the:

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Affidavit

I hereby confirm that the PhD thesis entitled “The antioxidant glutathione as a regulator of natural killer cell immunity” has been written independently and without any other sources than cited. All necessary ethical approvals have been obtained in accordance with the law, following the EU Directive **2010/63/EU** of care and use of laboratory animals.

Luxembourg, 14th September 2021,

Luana Frias Guerra

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Table of Content

List of Figures

List of Abbreviations

2-DG – 2-deoxyglucose

4E-BP1 - eukaryotic initiation factor 4E binding protein 1

AAO – amino acid oxidation

ADCC – antibody dependent cellular cytotoxicity

ADP – Adenosine diphosphate

ALT – alanine aminotransferase

AML – acute myeloblastic leukemia

AMP – Adenosine monophosphate

AMPK – AMP-activated protein kinase

APC – antigen presenting cell

AST – aspartate aminotransferase

ATP – Adenosine triphosphate

BCR – B cell receptor

CCL – chemokine ligand

CCR – chemokine receptor

CD – cluster of differentiation

cDC – conventional dendritic cell

CLP – common lymphoid progenitor

CMP – common myeloid progenitors

CMS – citrate-malate shuttle

CNS – central nervous system

CXCR – C-X-C motif chemokine receptors

DC – Dendritic cell

DCFDA - dichlorofluorescein diacetate

DNA – Deoxyribonucleic acid

EAE – experimental autoimmune encephalitis

ECAR – extracellular acidification rate

ELISA – Enzyme-linked immunosorbent assay

ETC – electron transport chain

FADH – flavin adenine dinucleotide

FAO – fatty acid oxidation

FasL – Fas ligand

FBP1 - fructose biphosphatase 1

FLT3 – FMS-like tyrosine kinase 3

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GCL – glutamate cysteine ligase

GCLC – glutamate cysteine ligase, catalytic subunit

GCLM – glutamate cysteine ligase, modifier subunit

G-CSF – granulocyte colony stimulating factor

GLS – glutathione synthase

GLUT1 – glucose transporter 1

GM-CSF – granulocyte-macrophage colony stimulating factor

GPX – glutathione peroxidase

GR – glutathione reductase

GSH – glutathione reduced

GSSG – glutathione oxidized

GvHD – graft versus host disease

H_2O_2 – hydrogen peroxide

HCV – Hepatitis C virus

HIF1α - hypoxia-inducible-factor 1-alpha

HIV – human immunodeficiency virus

HLA – human leukocyte antigen

HSC – hematopoietic stem cell

ICAM – Intercellular Adhesion Molecule 1

ICB – immune checkpoint blockade

IDO – indoleamine 2,3-dioxygenase

IFN – interferon

Ig – Immunoglobulin

IL – interleukin

ILC – innate lymphoid cell

iNK – immature NK cell

iNKR – inhibitory NK cell receptors

ITAM – tyrosine-based activation motif

ITIM – tyrosine-based activation motif

JAK - Janus kinase

KEAP1 - Kelch-like ECH-associated protein 1

KIR – killer immunoglobulin receptors

LCMV – Lymphocytic choriomeningitis virus

LDH – lactate dehydrogenase

MCA – methylcholanthrene

MCMV – mouse cytomegalovirus

MDSCs – myeloid derived suppressor cell

MFI – mean fluorescence intensity

MHC – major histocompatibility complex

mNK – mature NK cell

moDC – monocyte-derived dendritic cell

MS – multiple sclerosis

MTDR – Mitotracker Deep Red

MTG – Mitotracker Green

mTOR – mechanistic target of rapamycin

NADH – nicotinamide adenine dinucleotide

NADPH – Nicotinamide adenine dinucleotide phosphate

NCR1 (NKp46) – NK cell receptor 1

NET – neutrophil extracellular trap

NFAT – Nuclear factor of activated T-cells

NK – Natural killer

NKP – NK cell precursor

NKT – Natural killer T cells

NO• - nitric oxide

NOX – NADPH oxidase

NRF2 – Nuclear factor erythroid 2-related factor 2

O_2• - superoxide

OCR – oxygen consumption rate

OH• - hydroxyl

OXPHOS – oxidative phosphorylation

pDC – plasmocytoid dendritic cell

PPAR – peroxisome proliferator-activated receptor

PPP – pentose phosphate pathway

PRDX – peroxiredoxin

RAG – Recombination-activating gene

RO• - peroxide

ROS – reactive oxygen species

Sca-1: stem cell antigen 1

SOD – superoxide dismutase

SREBP – sterol regulatory element-binding protein

STAT – signal transducer and activator of transcription

TCA – tricarboxylic acid

TCR – T cell receptor

T_{FH} – T follicular helper cells

TGF-β – Transforming growth factor beta

Th – T helper cell

TIL – tumor infiltrating lymphocyte

TLR – toll like receptor

TME – tumor microenvironment

TNF – tumor necrosis factor

TRAIL – Tumor necrosis factor-related apoptosis-inducing ligand

Treg – Regulatory T cell

TRX – thioredoxin

WT – wild type

Summary

Natural killer (NK) cells are cytotoxic lymphocytes that belong to the innate branch of the immune system. Regulation of NK cell activity relies on the expression and engagement of a wide range of inhibitory and activating receptors that detect signals arising from cells in distress. Besides their cytotoxic function, NK cells are effective producers of cytokines that participate in the regulation of other immune cells, such as dendritic cells and T cells. These innate lymphocytes control microbial infections and malignant cell growth, which are pathological conditions where reactive oxygen species (ROS) play a crucial role. ROS participate in cell signaling events and constitute important secondary messengers for immune cell proliferation and growth. However, when accumulated, their presence leads to oxidative stress due to their high reactivity against biomolecules. In order to ensure coordinated levels of ROS, cells are endowed of antioxidant systems that allow for ROS detoxification. One of the most important intracellular antioxidants is glutathione (GSH), which our group has shown to modulate effector and regulatory T function in a subset specific manner. Given the subset specificity of GSH regulation in immunity, we aimed to investigate the role of this antioxidant in NK cells.

Using a genetic-based approach, through a flox-Cre system, we specifically abrogated GSH production in NK cells. Mutant mice had a reduced abundance of NK cells, when compared to controls. Furthermore, *in vitro* stimulation of NK cells with IL-15, showed that ablation of GSH production renders NK cells unable to proliferate and these cells were less cytotoxic. NK cells lacking GSH accumulated mitochondrial ROS, resulting in reduced mitochondrial fitness. This was paralleled by a general metabolic shutdown, and reduced mTOR and STAT5 signaling. *In vivo*, GSH and redox regulation were demonstrated to be key for NK cell-mediated regulation of T cells, in a viral model of lymphocytic choriomeningitis virus (LCMV). Moreover, in an experimental tumor model, deletion of GSH resulted in an NK cell intrinsic impairment of tumor dissemination and increased exhaustion. Interestingly, we showed that depletion of glucose and glutamine, often seen in the tumor microenvironment, can negatively affect glutathione production by NK cells. These results give new insights on GSH-dependent NK cell regulation and link NK cell dysfunction to nutrient availability in pathologies. Taken together, our results indicate GSH as a key checkpoint for NK cell homeostasis and function.

I. Introduction

The immune system

Permanent exposure to potentially deleterious microorganisms or defective cellular processes results in tissue damage. Whether those can cause disease depends on the pathogenicity of the organism, or the severity of the damage, and the integrity of the host immune system. The immune system comprises three levels: (1) anatomical barriers, (2) the innate immunity and (3) the adaptive immunity. The epithelial barriers are the first line of defense against an infection, and they are constituted by the skin and mucosal organs, such as the gastrointestinal tract, lungs and oral cavity. Primary lymphoid organs, such as the bone marrow and thymus, are key structures for lymphocyte generation and maturation. In the bone marrow, hematopoietic stem cells give rise to virtually all the cells of the immune system, including B and T cells. The thymus, on the other hand, is highly specialized in the process of T cell maturation. Secondary lymphoid organs are responsible for the maintenance of naïve lymphocytes and initiation of the adaptive immune response. In the lymph nodes and spleen, lymphocytes are activated by antigens, which leads to their clonal expansion and affinity maturation. Lymphoid tissues are connected through blood and lymph vessels, where the immune cells circulate. The innate immune system exerts an immediate response to infection or sterile inflammation, which is followed by a coordinated adaptive response, specific to the pathogen. Adaptive immunity allows memory formation, enabling a more efficient response upon re-exposure. (Murphy et al., 2017).

- **The innate immune response**

The innate immune system relies on a limited repertoire of receptors for the detection of invading pathogens. Their cognate ligands are normally conserved microbial components, shared by a large variety of pathogens. The key characteristic of the innate system is the promptness of the response. A reaction is triggered within minutes after pathogen exposure. This is mediated by monocytes, macrophages, neutrophils, mast cells, basophils, dendritic cells and natural killer (NK) cells, among others. Besides providing an effector response, these cells signal the presence of the pathogen and coordinate efforts with the adaptive immune system to effectively eliminate it (Turvey and Broide, 2010).

Monocytes and macrophages are mononuclear phagocytes. Monocytes derive from the bone marrow and patrol the body in the blood circulation, while macrophages are tissue-resident innate immune cells. As phagocytes, they are capable of ingesting and destroying microbes. Through projections of the cytoplasmic membrane, phagocytes engulf the particle and form a phagosome. The latter is subsequently fused with cytoplasmic granules of the monocytes and macrophages, creating the phagolysosome. The elimination of the microbial organism, allergen or stressed cell in the phagolysosome occurs by a combination of two

mechanisms. First, the oxygen-dependent *respiratory burst* involves the activity of NADPH oxidase (NOX) and leads to the production of reactive oxygen species (ROS), which, based on their oxidative capacity, have bactericidal effects. Moreover, enzymes present in the phagolysosome, such as myeloperoxidase and lysozyme, participate in the processing and destruction of the threat. Immediately after recognition of the infection or tissue damage, macrophages start secreting cytokines. Namely, granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin 8 (IL-8) are released and stimulate proliferation of myeloid precursors and neutrophil differentiation (Ginhoux and Jung, 2014).

Neutrophils are highly mobile cells and, in homeostasis, travel throughout the body. The onset of inflammation stimulates neutrophils to migrate to the site of infection or tissue damage. Inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1), produced by macrophages, induce the expression of adhesion molecules. Namely, E-selectin is upregulated on the vascular endothelium, and L-selectin on the neutrophils. This increased adhesion enables an intermittent tethering motion, named *rolling*, allowing neutrophil migration through the vessel to site of infection. The local production of neutrophil attractants drives neutrophil recruitment. The high concentration of chemoattractants at the site of inflammation results in the downregulation of specific receptors on neutrophils and these cells remain at the inflammatory site. Besides being able to perform phagocytosis, neutrophils can also form neutrophil extracellular traps (NETs), providing a physical barrier that prevents pathogen spreading (Mayadas et al., 2014).

Eosinophils are particularly important for the defense against parasitic infections. Those infections induce the production and secretion of the immunoglobulin E (IgE). The opsonization of the parasite with IgE induces the binding of eosinophils by the low affinity receptor, FcεRII. Eosinophils are not phagocytic but are endowed to release large granules containing basic proteins, such as eosinophilic cationic protein, eosinophil peroxidase and eosinophil-derived neurotoxin, which are highly cytotoxic. Eosinophils can also induce pathological inflammation in allergic reactions (Klion et al., 2020). **Mast cells and basophils** bear high affinity antigen receptors for IgE, the FcεRI (CD23). Crosslinking of these receptors leads to the degranulation of these cells, releasing histamine, serotonin, leukotrienes and prostaglandins, which increase vascular permeability and induce the inflammatory responses (Varricchi et al., 2018).

Of note, **innate lymphoid cells (ILCs)** are the most recently discovered family of the innate immune system and they mirror the phenotypes and function of the adaptive T cells. **Natural killer cells**, belonging to this family, will be covered in a more detailed fashion and can be considered the innate counterparts of CD8+ T cells. On the other hand, ILC1, ILC2 and

ILC3 may represent the T helper cell subsets Th1, Th2 and Th17, respectively. ILCs do not express specific antigen receptors, nor undergo clonal selection. As innate immune cells, ILCs recognize inflammatory signals and produce cytokines according to the stimuli they sense, as discussed later for T cell subsets. Much remains to be uncovered on the development, activation and function of ILCs, especially due to the plasticity between subsets. Nevertheless, they seem to be key regulators of the immune response and essential keepers of mucosal tissue homeostasis (Vivier et al., 2018).

Finally, **dendritic cells (DCs)** are professional antigen presenting cells and important regulators of innate and adaptive immune response. Due to their phenotypical and functional heterogeneity, they can be subdivided in two groups: conventional (cDCs) and plasmacytoid (pDCs). pDCs morphologically resemble antibody-producing (plasma) cells and constitute important mediators of antiviral immunity, secreting type I interferons (IFN, IFN-α and IFN-β) (Reizis, 2019). On the other hand, cDCs can be further classified in cDC1 and cDC2. cDC1 participate in antitumor response and clearance of intracellular pathogens and are specialized on CD8+ and T helper 1 (Th1) immune responses. The function of cDC2 is closely related to CD4+ T cells, mainly inducing humoral immunity, as well as a Th2 or Th17 response (Anderson et al., 2021). Monocyte-derived DCs (moDCs) are present in inflammatory environments and have been associated with immunosuppresion (Wculek et al., 2020). Dendritic cells have the capacity to internalize and destroy invading microbes through phagocytosis and have a prominent role as professional antigen presenting cells (APCs). Professional APCs internalize the microbe or debris and generate peptide fragments by proteasomal degradation. Those fragments (antigens) are bound to the major histocompatibility complex (MHC) and shuttled to the surface, where they are presented. In lymphoid sites, professional APCs, such as DCs and macrophages, come in contact with T or B lymphocytes that recognize the antigen at the surface of the APC. The activation of naïve T cells is only possible by professional APCs, since, in addition to the MHC, they express co-stimulatory molecules, CD80, CD86 and CD40. These co-stimulatory ligands deliver a second signal for full activation. Noteworthy, there are two types of MHC receptors. MHC class I molecules are ubiquitously expressed and bind to proteins derived from intracellular pathogens, such as viruses and bacteria, and can present self-antigens. In contrast, MHC class II is expressed only at the surface of antigen presenting cells and presents extracellular proteins, derived from bacteria and parasites (Murphy et al., 2017). Antigen presentation bridges the innate immune response with an antigen-specific response mediated by the adaptive immune system. In secondary lymphoid organs, professional APCs “prime” T and B cells by presenting the captured antigens and providing key activation signals.

- **The adaptive immune response**

T cells derive from progenitor cells in the bone marrow and, at early stage, migrate to the thymus. The generation of antigen-specific receptors is the result of a process of random rearrangements of multiple DNA segments coding for antigen-binding areas of the T cell receptors (TCR). Combinatorial diversity arises from the number of possible rearrangements between the segments of the TCR. The resulting amino acid sequence dictates the binding specificity of the receptor. Productive rearrangement of two TCR genes (α and β or γ and δ) marks the transition from a pre-T cell to a double positive T cell, where they express both CD4 and CD8 molecules on their surface. In the thymus, double positive T cells go through a process of selection to achieve central tolerance. T cells clones that present low affinity to self-MHC molecules are eliminated (*positive selection*). At the same time, T cells that possess TCRs that respond with very high affinity to self MHC/self-peptide complexes are also eliminated (*negative selection*). Surviving double positive lymphocytes, mature to single positive CD4+ or CD8+ T cells by means of further interaction with MHC class II or MHC class I, respectively. Later on, T cells are activated upon TCR recognition of the cognate peptide/MHC complex on a professional APC. The engagement of the TCR by the MHC complex leads to a rapid clustering of TCR-associated molecules at the immunological synapse. Sequentially, a cascade of signaling events occurs to permit T cell activation and differentiation. Depending on the type of APC and cytokine milieu at the site of antigen encounter, activated T cells differentiate into distinct subsets that mediate context-dependent effector functions in an antigen-dependent manner. **CD8+ T cells** are cytotoxic T cells that recognize antigens presented by MHC class I. CD8+ T cells are able to trigger apoptotic cell death of the target, through the release of cytotoxic granules and expression of death receptors. Furthermore, **natural killer T (NKT) cells** represent an additional subset of T cells defined by the simultaneous expression of CD3, T cell antigen, and CD56 or NK1.1, a natural killer (NK) cell antigen. Activated NKT are able to produce large amounts of IL-4 and IFN-γ and have been implicated in allergic reactions. The largest group of T cells is the **CD4+ T cells**, which can be further subdivided. **Th1** cells differentiate under the influence of IL-12, IFN-γ and the transcription factor Tbet. Th1 cells produce IFN-γ and IL-2 and participate in antibacterial and antiviral response. **Th2** cell differentiation is driven by IL-4 and GATA-3. When activated, Th2 cells produce IL-4, IL-5 and IL-13 and are important in mounting a response against extracellular pathogens, namely parasites. Th2 cells also participate in humoral and hypersensitivity reactions. In addition, **Th17** cells differentiate in the presence of IL-6 and TGF-β, driven by the transcription factor retinoic acid receptor related orphan receptor γt (RORγt). Th17 cells produce IL-17 and IL-22 and, through their highly pro-inflammatory profile, have been described to mediate autoimmune

processes, as well as anti-bacterial and anti-fungal responses. CD4+ regulatory T (**Tregs**) cells express the transcription factor FOXP3 and have been shown to suppress immune response by secretion of TGF-β and IL-10. Follicular T helper (**TFH)**, localized in follicles of the spleen and lymph nodes, trigger B cell activation, leading to germinal center formation, crucial for humoral immunity. Overall, cytokine signals, mainly produced by APCs, orchestrate the lineage differentiation of CD4+ T cells and shape the adaptive immune response (Bonilla and Oettgen, 2010; Saravia et al., 2019).

B cells are the major contributors of humoral immunity, which is mediated by antibodies that neutralize toxins and prevent pathogen adhesion to mucosal surfaces. Immunoglobulins IgA, IgG and/or IgM can also induce complement activation and the opsonization of particles. Opsonization triggers phagocytosis of the pathogens, as well as sensitization of target cells for antibody-dependent cellular cytotoxicity (ADCC). B cells arise from hematopoietic stem cells in the bone marrow. There, pre-B cells acquire their antigen specificity, in a process similar to the TCR rearrangement in T cells. The second phase of B cell development occurs upon encounter with the cognate antigen. Depending on the stimuli received, a B cell can have several fates. Mature B cells recirculate through secondary lymphoid organs. In the lymph nodes, B cells are concentrated in the cortex, in primary follicles. There, B cells are activated by circulating antigens or by antigens presented on the surface of APCs (macrophages, DCs or B cells themselves). Concomitantly, B cells express co-stimulatory molecules, CD40, CD80, CD86, which engage with receptors of T cells, in the immunological synapse. Activated B cells can either become short-lived plasma cells or enter a follicle and establish a germinal center. Short-lived plasma cells have low affinity antibodies. B cells in the germinal center can switch from the production of IgM and IgD to higher affinity isotypes (IgG, IgA and IgE), a phenomenon named *class switching*. This occurs through a mechanism analogous to the TCR and BCR gene rearrangement. The process of class switching is partially mediated by cytokines. For example, IL-4 and IL-13 promote switching to IgE, while IL-10 and TGF-β promote switching to IgA. Concomitant to class switching, B cells in the germinal center undergo *somatic hypermutation*, in which the immunoglobulin heavy and light chain variable regions accumulate point mutations through a mechanism of nucleotide substitution. As selection mechanisms are in place in the germinal center, this leads to the production of antibodies with higher affinity for the antigen (Cyster and Allen, 2019). The adaptive immune system relies on the existence of a memory response, formed during the primary response to the pathogen. **Memory T and B cells**, upon subsequent exposure to the antigen, are activated more quickly and, particularly B cells, are equipped with higher affinity antibodies to respond more efficiently.

Natural killer cells

Natural killer (NK) cells are innate lymphocytes that lack specificity in antigen recognition and respond rapidly against bacterial and viral infections, as well as tumor cells. Contrarily to T and B cells, which need rearrangements of their respective T and B cell receptors for specific antigen recognition, NK cells are able to respond to a broad range of stress signals (Lanier, 2005). NK cells contribute to target cell death by the production of cytotoxic molecules, such as granzymes and perforin, and shape the inflammatory environment with the production of cytokines, namely INF-γ and TNF-α. NK cells are, therefore, crucial keepers of immune homeostasis (Mujal et al., 2021; Vivier et al., 2008). NK cells were first described in the late half of the 1970s as a new subset capable of eliminating tumor cells (Herberman et al., 1975a; Herberman et al., 1975b; Kiessling et al., 1975a; Kiessling et al., 1975b).

NK cell development

NK cells develop from hematopoietic stem cells (HSCs) in the bone marrow (Figure 1). A 3-stage model for NK development has been suggested, where mature NK cells derive from HSCs transitioning through an immature state (Kumar et al., 1979; Seaman and Gindhart, 1979). Human HSCs can be classified as lineage negative (Lin-) $CD34^{+}CD38^{dim/-}$ $CD45RA^{-}CD10^{-}$ and their murine homologues are defined as Lin^{-}stem cell antigen 1 (Sca-1)$^{+}$ c-Kit^{+} (Galy et al., 1995). The thymus and extramedullary tissues, namely spleen and liver, can represent sites of NK cell development outside of the bone marrow (Douagi et al., 2002). However, they do not seem to be essential for NK generation. Normal numbers of functional NK cells are found in congenitally athymic nude mice and patients with Di George syndrome, which present thymic aplasia or hypoplasia (Sihvola and Hurme, 1984). In the same line, thymectomy or splenectomy does not seem to affect NK cell development, neither in mice nor in humans (Passlick et al., 1991; Ramos et al., 1996; Schwarz and Hiserodt, 1990). NK cell progenitors are found in the fetal liver. However, it remains to be determined if this organ is a site for NK development in adults. It is important to note that thymus, spleen and liver might have a role in diversification and heterogeneity of peripheral NK cells. For example, in the liver, where NK cells constitute more than 50% of intrahepatic lymphocytes, this population was shown to be further subdivided in three subsets with distinct transcriptional and phenotypical profiles: liver-resident NK cells, memory NK cells and recirculating NK cells (Mikulak et al., 2019). The importance of the microenvironment to shape the NK cell compartment has also become evident in the lung or the gut (Hervier

et al., 2019; Poggi et al., 2019). More studies arise to explain how differentiation and homeostasis of distinct NK cell subsets can contribute to pathological conditions.

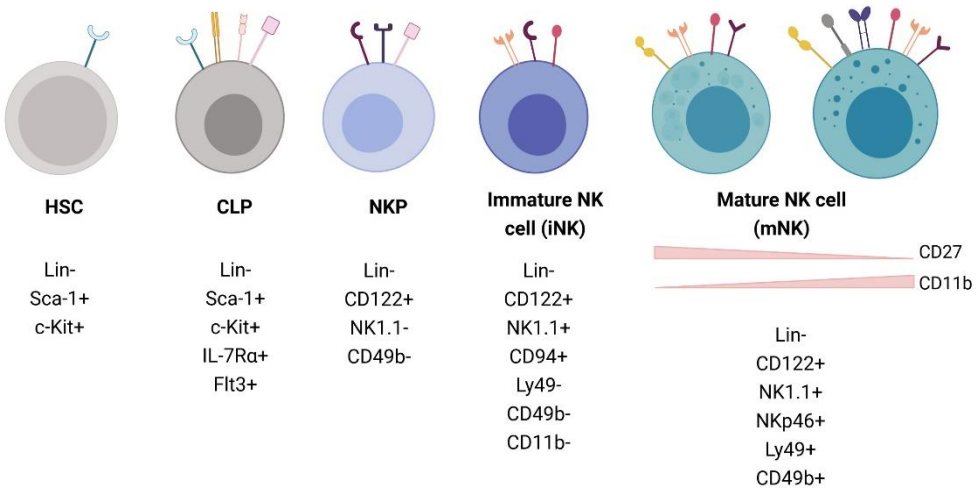

Figure 1 – Murine NK cell development
Mouse NK cells arise in the bone marrow from HSC, defined by the absence of lineage markers (Lin-) and the expression of the transcription factors Sca-1 and c-Kit (Lin$^-$ Sca-1+ c-Kit+). The acquisition of IL-7Rα and Flt3 drives the differentiation to common lymphoid progenitors (CLP), which can also give rise to T and B lymphocytes. The first step for pre-NK cell differentiation, into NK cell precursors (NKP) is the acquisition of CD122, the common IL-2/IL-15R β chain. Further in the development process, immature NK cells (iNK) start acquiring the expression of NK cells markers, such as NK1.1, Ncr1/NKp46 and CD94. The activation markers CD49b and CD11b are expressed in the final steps of NK cell maturation (mature NK cells, mNK). Murine mNK can be subdivided into further subsets, based on the expression of CD27 and CD11b. CD27 is expressed in highly proliferative, cytotoxic, less mature NK cells, whereas CD11b expression is higher in in terminally mature, more quiescent NK cells. Adapted from EMJ Allergy & Immunol. . 2019;4[1]:108-116. Created with Biorender.com.
HSC: hematopoietic stem cell; CLP: common lymphoid progenitor; Lin: lineage markers: CD2, CD3, CD4, CD8, CD5, NK1.1, B220, TER-119, Sca-1: stem cell antigen 1; CLP: common lymphoid progenitor

- **Pre-NK cells**

In the classical model of hematopoiesis, HSCs can differentiate into common myeloid progenitors (CMPs), which give rise to myeloid cells, erythrocytes and megakaryocytes, or common lymphoid progenitors (CLPs), that mature into lymphocytic lineages. CLPs acquire the expression of CD45RA and CD10 in humans, being described as $CD34^+CD38^+CD45RA^+CD10^+$. Mouse CLPs start expressing IL-17Rα, and are classified as Lin$^-$ Sca-1^{lo} c-Kit^{lo} IL-7Rα$^+$ FLT3$^+$ (Figure 1). CLPs are able to originate T cells, B cells or NK cells and pDCs (Kondo et al., 1997). A crucial step for the commitment to NK cell differentiation is the acquisition of the beta chain of the interleukin 15 (IL-15) receptor (IL-

15Rβ), downstream of the CLPs (Rosmaraki et al., 2001). On one hand, mice deficient in IL-15 signaling elements, such as Janus kinase 3 (Jak3) or signal transducer and activator of transcription 5 (STAT5), exhibit a defective NK cell development (Eckelhart et al., 2011; Nosaka et al., 1995). On the other hand, overexpression of IL-15 in transgenic mice, results in increased NK cell numbers, which can be followed by the development of NK cell leukemia (Fehniger et al., 2001).

The acquisition of IL-15R establishes the differentiation of CLPs to NK precursors (NKPs) in the bone marrow, expressing the common γ chain (CD132) and the shared IL-2/IL-15R β chain (CD122) (Carotta et al., 2011; Rosmaraki et al., 2001). NKPs are, in addition, described as lacking lineage-specific markers, such as CD3, CD19 and CD14, as well as immunophenotypic markers of mature NK cells, such as NKp46, NKG2D, NKp30, killer immunoglobulin receptors (KIRs). Murine NKPs are described as Lin^- $CD122^+$ $NK1.1^-$ $DX5^-$ cells and are not able to induce cell-targeted cytotoxicity nor producing IFN-γ (Rosmaraki et al., 2001). Furthermore, it was shown that c-Kit and FLT3 ligands are important, but not essential, for NKP commitment to the NK lineage, probably by directly inducing the expression of CD122 in HSCs or CLPs (Yu et al., 1998). Transcription factors regulate the developmental process of committed NKPs from HSCs. Mice deficient in Ikaros, a transcription factor involved in lymphopoiesis, showed reduced numbers of NK cells (Wang et al., 1996). The same was true for mice carrying defects on various transcription factors involved in the differentiation of lymphoid cells, ETS1, PU.1 and members of the family of inhibitors of DNA binding (ID) proteins, ID2 and ID3 (Barton et al., 1998; Colucci et al., 2001; Heemskerk et al., 1997; Yokota et al., 1999). The transcription factors described for T and B cell lineage commitment partially overlap with the transcription program for NK differentiation, denoting that the latter is established prematurely in the progression from HSCs to NKPs (Colucci et al., 2003). It is important to note that the details of differentiation of human NKPs are not fully understood. However, following *in vitro* culture of HSCs with FLT3 and c-KIT ligands, the first $CD122^+$ cells to arise are negative for CD7, CD16, CD56 and NKG2A, markers of NK cell maturation (Bennett et al., 1996; Miller et al., 1994). Thus, murine and human NKPs show great similarity in development and commitment of NK cells (Williams et al., 1997).

- **Immature NK (iNK) cells**

The maintenance of IL-15 signaling and cell-to-cell interactions between NK cells and bone marrow stromal cells are essential for NK cell survival and differentiation (Kennedy et al., 2000; Lodolce et al., 1998). NKPs develop to immature NK cells by expressing a family of

C-type lectin of NK-cell receptors (NKRP1C, CD161), recognized by NK1.1 antibodies in C57BL/6 mice (Glimcher et al., 1977; Huntington et al., 2007b) (Figure 1). Murine iNK cells do not express CD49b (α integrin, DX5) and their human homologues lack CD56 expression. Moreover, in both species, iNK cells do not express MHC-class I specific receptors, Ly49 in mice and the killer immunoglobulin-like receptors (KIR) family in humans (Bennett et al., 1996; Rosmaraki et al., 2001). Similar to NKPs, iNK cells are not able to lyse sensitive targets in a perforin-dependent manner nor produce IFN-γ (Zamai et al., 1998). During murine NK development, $NK1.1^+$ iNK cells progress in the lineage commitment by the gradual and orderly expression of functional receptors: NK1.1 is followed by the expression of CD94/NKG2A, NCR1 (also known as NKp46), Ly49 receptors, CD49b and, finally, CD11b (or macrophage 1 antigen, Mac-1) (Kim et al., 2002). Despite the considerable heterogeneity of bone-marrow iNK cells, mouse iNK cells are described as: Lin^- $CD122^+$ $NK1.1^+$ $CD94^+$ $Ly49^-$ $DX5^-$, $CD11b^-$ (Rosmaraki et al., 2001). In humans, more mature $CD161^+$ NK cells, upon sensing differentiation signals, enhance the sequential expression of CD56, CD94/NKG2A and KIRs (Grzywacz et al., 2006; Perussia et al., 2005).

- **NK cell maturation and subsets**

NK cell maturation is accompanied by the expression of receptors recognizing specific MHC class I molecules, as well as other cell surface ligands (see Table 1). Mature NK cells can produce IFN-γ and mediate perforin-dependent cytotoxicity (Grzywacz et al., 2006; Kim et al., 2002). Mature NK cells leave the bone marrow and populate peripheral lymphoid and non-lymphoid organs. Peripheral NK cells are activated by sensing missing or altered self MHC class I molecules (*missing-self* hypothesis) or by the presence of pro-inflammatory stimuli (Colucci et al., 2003; Ljunggren and Kärre, 1990). In the mouse, NK cells represent 2% of the lymphocytes in the spleen and 10% in the lung. In humans, NK cells can account from 2 to 18% of peripheral blood lymphocytes, with an average life span of 2 weeks (Grégoire et al., 2007). Interestingly, in wild type mice, transferred mature NK cells can persist in circulation for 5 weeks (Ranson et al., 2003). However, when the host is IL-15-incompetent, NK cells are not detectable in the blood, spleen or liver after 5 days, evidencing once more the importance of this cytokine for NK homeostasis (Cooper et al., 2002).

Anatomical, phenotypical and functional differences in NK cells led to the definition of distinct subsets in mice and humans. In humans, mature NK cells can be subdivided in two subsets based on the expression of CD56. $CD56^{dim}$ $CD16^+$ NK cells are mostly located in the peripheral blood and spleen and display a more cytotoxic profile, producing IFN-γ upon

interaction with target cells *in vitro*. In contrast, $CD56^{bright}$ $CD16^{dim}$ cells are primarily located in secondary lymphoid tissues, namely tonsils and lymph nodes and are less prone to perform perforin-mediated cell-targeted cytotoxicity. Upon stimulation, the $CD56^{bright}$ subset promptly upregulates cytokine production, namely IFN-γ, TNF-α, GM-CSF, IL-3 and IL-10 (Cooper et al., 2001).

In mice, the receptors CD27 and CD11b allow the differentiation of NK cell terminally mature NK cell subsets (Figure 1). Increased expression of CD11b and concomitant loss of CD27 mark murine NK cell maturation and acquisition of effector functions in a 4-stage program: (i) $CD11b^{lo}$ $CD27^{lo}$ (ii) $CD11b^{lo}$ $CD27^{hi}$ (iii) $CD11b^{hi}$ $CD27^{hi}$ (iv) $CD11b^{hi}$ $CD27^{lo}$ (Chiossone et al., 2009; Hayakawa and Smyth, 2006). $CD11b^{lo}$ $CD27^{lo}$ (double negative) and $CD11b^{lo}$ $CD27^{hi}$ NK cells show a higher proliferation rate than the other subsets and act as precursors of more mature, effector NK cells. $CD11b^{lo}$ $CD27^{hi}$ NK cells are more prevalent in the lymph nodes and bone marrow. Double positive and $CD11b^{hi}$ $CD27^{lo}$ mature NK cells show comparable cytotoxic capacity and secrete IFN-γ when stimulated in vitro (Chiossone et al., 2009; Hayakawa and Smyth, 2006). In the final maturation stage, $CD11b^{hi}$ $CD27^{lo}$, NK cells appear to be in a senescent state, lack the CXC motif chemokine receptor 3 (CXCR3) and, therefore, their migration capacity is reduced. This subset is less cytotoxic and produces a reduced amount of cytokines (Hayakawa and Smyth, 2006). This is paralleled by an increased expression of the inhibitory receptor killer-cell lectin-like receptor subfamily G, member 1 (KLRG1), induced upon chronic stimulation and proliferation (Robbins et al., 2002). Due to their lower cytotoxic capacity, this highly mature NK cells were coined “regulatory NK cells” and are enriched in the blood, spleen and lungs. (Hayakawa and Smyth, 2006). Crinier et al. recently used single-cell RNA sequencing (scRNAseq) to analyze different subsets of mouse and human NK cells. Interestingly, the high throughput analysis highlighted similar transcriptional profiles between the two major NK cell subsets across species. Human $CD56^{dim}$ overlap with the mouse $CD11b^{hi}$ $CD27^{lo}$, subset, whereas $CD56^{bright}$ NK cells transcriptionally resemble the murine $CD11b^{lo}CD27^{hi}$ subgroup (Crinier et al., 2018). This study indicates that, despite the differences between the two species and the subset heterogeneity, mouse NK cells studies can be translated to human diseases.

Table 1 – Activating and inhibitory NK cell receptors and their ligands

Receptors	Species	Ligands
Activating Receptors		
CD16	Human (mouse)	IgG
NKp46	Human (mouse)	Influenza hemagglutinin
CD94/NKG2C/E	Human (mouse)	HLA-E (Qa-1)
NKG2D	Human (mouse)	MIC, ULBP (RAE-1, H60)
2B4 (CD244)	Human (mouse)	CD48
Ly49D	Mouse	H-2D
Ly49H	Mouse	MCMV-induced?
KLRB1 (CD161)	Mouse	N/A
NKp44	Human	Influenza hemagglutinin
NKp30	Human	B7-H6
KIR2DS	Human	HLA-C
Inhibitory receptors		
CD94/NKG2A	Human (mouse)	HLA-E (Qa-1^{b})
Ly49	Mouse	H-2K, H-2D
KIR2DL	Human	HLA-C
KIR3DL	Human	HLA-A, HLA-Bw4

HLA-human leukocyte antigen; KIR-killer cell immunoglobulin receptor; MCMV-mouse cytamegalovirus; MIC-MHC class I chain-related molecules; ULBP – UL16-binding protein;

NK cell education

NK cells cytotoxic response is induced upon recognition of missing or altered expression of a self MHC class I molecule (Ljunggren and Kärre, 1990). One of the main questions of NK cell biology is: how do they become self-tolerant? Each individual encodes a set of 10 or more inhibitory receptors for self MHC class I molecules (inhibitory NK cell receptors, iNKRs) and each iNKR is specific for polymorphic variants of the respective MHC molecules (Boudreau and Hsu, 2018). The diversity of the NK cell repertoire arises from the fact that each NK cell expresses a selection of 3 to 5 iNKRs (Kubota et al., 1999). Besides those inhibitory receptors, NK cells have, on their surface, receptors directed to ligands expressed by cells with DNA damage or in stress, molecules encoded by virus or other constitutively expressed ligands (Raulet et al., 2001) (Table 1).

Interestingly, MHC molecules are inherited in an independent fashion of iNKR genes that recognize them (namely Ly49 for mice, and KIRs for humans) and present great allelic variation (Yokoyama and Plougastel, 2003). NK cell *education* is the process of acquiring self-tolerance and selecting NK cells with appropriate specificity to self-MHC molecules.

Uneducated and educated NK cells co-exist and the individual NK cell repertoire exhibits graded levels of responsiveness towards recognition of low or absent self MHC class I expression (Raulet and Vance, 2006). The underlying molecular mechanisms of NK cell education have not yet been established and, thus, several models have been proposed to explain the process of NK cell education.

The *licensing* and *arming* models propose that the functional maturation of NK cells arises from the expression of "at least one" iNKR that recognizes self MHC class I molecules. NK cells that lack the expression of such receptors are either deleted or rendered hyporesponsive (Kim et al., 2005). These models may appear paradoxical, since signaling through inhibitory receptors would enable NK cell function. However, there is evidence supporting such mechanisms. Inhibitory receptors on NK cells can signal through SRC homology 2 (SH2)-domain containing protein tyrosine phosphatases (SHP). Interfering with signaling of key pathways mediators, namely through the deletion of SHP-1 or other protein phosphatases, results in the accumulation of immature NK cells (Banh et al., 2012; Wu et al., 2016). A similar scenario is described upon deletion of the cytoplasmic region of Ly49A receptor or through mutation of immune receptor tyrosine-based inhibition motif (ITIM), crucial for inhibition signaling (Bern et al., 2017).

In the *disarming model*, however, NK cells are proposed to be initially autoreactive towards self-MHC class I molecules. Thus, education would consist in the sequential expression and engagement of the cognate inhibitory receptors. With the signals delivered by these receptors, NK cells become tolerant to self by losing their reactivity. Alternatively, those NK cells that do not express receptors recognizing self MHC class I molecules, suffer activation-induced anergy and become unresponsive to subsequent stimulation. In this model, NK cells expressing iNKRs mismatched with self MHC are not clonally deleted but rather unlicensed (Coudert et al., 2008). Several experimental evidence supports this model. Persistent stimulation of the activating receptors CD94/NKG2A or Ly49H, render NK cells hyporesponsive to their respective triggering ligands. Moreover, individual homozygous for HLA-C2, present anergic KIR2DS1+ NK cells due to chronic stimulation by the overexpression of its cognate ligand (Kim et al., 2005; Pittari et al., 2013).

The models aforementioned are not mutually exclusive and the convergent *rheostat model* has been suggested. As previously stated, it is allelic variation that determines Ly49 or KIR expression on NK cells, independently of the expression of cognate ligands (Frazier et al., 2013). Moreover, other NK cell receptors can interfere in the dynamics of the interaction of each cell with self MHC class I molecules (Boudreau et al., 2016; Narni-Mancinelli et al., 2012). Therefore, the rheostat model hypothesizes that potential of NK cell effector function is calibrated by the dose of inhibition sensed by the cell. In other words, the status of

tolerance or reactivity, and the magnitude of the response of a given NK cell is determined by the density and diversity of inhibitory receptors expressed on its surface and the presence of cognate ligands (Brodin et al., 2009; Johansson et al., 2005; Patel et al., 2010; Yu et al., 2007). This model can apply to both arming and disarming models, where the process of NK education can be tuned depending on changes of MHC expression occurring over short periods of time (Boudreau et al., 2016; Elliott et al., 2010). The adjustment of NK cell effector function ensures that tolerance is maintained, while circumventing auto-immunity and enabling successful pregnancies, for example. Supporting evidence of this model arises from experiments using antibodies that interfere with inhibitory signaling or modulation of the expression of self MHC molecules. The NK cell effector response is sensitive to the environmental changes of iNKR and MHC expression over time (Joncker et al., 2010; Wagner et al., 2016). Educated NK cells are effector cells capable of lysing target cells and participate in the immune response to viral infections or tumor development, via two mechanisms. NK cells can release cytotoxic granules, containing granzyme and perforin, directly to target cells (Blakely et al., 1987). Alternatively, these cytolytic cells can induce cell death through the expression of TRAIL or Fas ligand (FasL) (Prager and Watzl, 2019).

Mechanisms of NK cell cytotoxicity

- **Production and release of lytic molecules**

Due to the broad toxicity, granzyme and perforin production and storage are tightly regulated in NK cells (Blakely et al., 1987) (Figure 2). **Granzymes** belong to a family of serine proteases and distinct members have been described: granzymes A, B, H, K, M. Although all sustain lymphocytic cytotoxic function, each granzyme signals distinct pathways, in addition to complementing roles (Ewen et al., 2012). Granzymes are expressed as a pre-proteins (zymogens) and contain an inhibitory dipeptide, which renders them pro-enzymes (Ewen et al., 2012). This inhibitory peptide is removed by cysteine proteases cathepsin C or H, activating the granzyme (Griffiths and Isaaz, 1993; Kummer et al., 1996; Smyth et al., 1995). Granzymes are then stored in the lytic granules of the effector cells, at low pH and in association with serglycin, thereby preventing the cleavage of host cell proteins at homeostatic conditions (Metkar et al., 2002). Granzymes A and B are the best described. The main substrate of granzyme B, after being released in target cells, is caspase-3. Cleavage of caspase-3 and caspase-7 induces cell death by cleavage of several cellular components, including structural and regulatory proteins in the nucleus and cytosol (Fischer et al., 2003). Granzyme B is also able to induce cell death by cleaving Bid, which,

when truncated, relocates to the mitochondria and signals apoptotic proteins Bax and/or Bak (Sutton et al., 2000). The disruption of the mitochondrial membrane integrity results in release of pro-apoptotic factors and target cell death. Granzyme B-induced apoptosis can be inhibited by overexpression of the anti-apoptotic protein Bcl-2 (Pinkoski et al., 2001; Sutton et al., 1997). On the other hand, Granzyme A has a trypsin-like activity and induces a fast form of cell death. Its mechanism of action passes through the formation of large DNA fragments that activate reactive oxygen species (ROS) production from the mitochondria and disrupt mitochondrial membrane potential (Beresford et al., 1999; Martinvalet et al., 2008; Martinvalet et al., 2005). This form of cell death is independent of caspases and apoptotic factors. Granzymes work in conjunction with **perforin**, which is capable of forming pores in the target cells to mediate the release of the lytic granules (Praper et al., 2010; Voskoboinik et al., 2005). All these strictly regulated processes allow a controlled production, release and activity of pro-apoptotic enzymes in the cytosol of the effector cells.

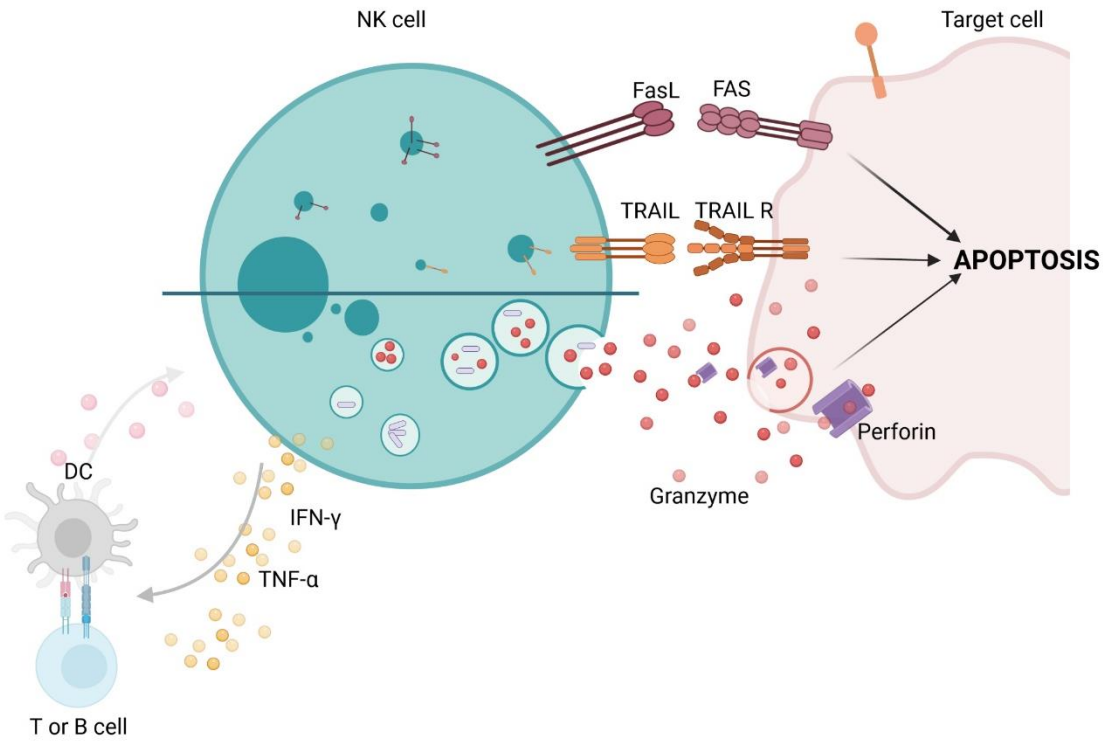

Figure 2 –NK cell cytotoxic mechanisms
NK cells recognize attached target cells and can eliminate them through two distinct mechanisms: release of cytotoxic granules (bottom) or through engagement of death receptors (top). Bottom: granzymes and perforin are produced and transported in the cytotoxic granules of NK cells. Granzymes can reach the target cell by endocytosis or through formation of perforin pores in the plasma membrane. Top: NK cells can also express death receptor ligands, FasL and TRAIL, which engage FAS and TRAIL receptor in the target cells, respectively. (continues in the next page).

(Figure 2) Distinct cytotoxic mechanisms can induce target cell death by caspase activation, mitochondrial dysfunction or caspase-independent apoptosis. NK cells can also induce T or B cell activation through the production of pro-inflmmatory cytokines, such as IFN-γ and TNF-α, boosting the adaptive immune response. Adapted from (Prager and Watzl, 2019). Created with Biorender.com

NK cell-mediated cytotoxic response is initiated by integrin-dependent adhesion to form the immunologic synapse with the target cell (Hoffmann et al., 2011; Mace et al., 2014). Lytic granules are transported along the microtubules in the direction of the microtubule organizing center (MTOC) and are polarized to the synapse, where the secretory vesicles are fused with the plasma membrane (James et al., 2013; Mentlik et al., 2010). In the immunological synapse, the increased pH and the presence of Ca^{2+} allows perforin monomers to aggregate. Hence, perforin forms pores in the membrane of the target cell, through which granzyme B is able to pass (Lopez et al., 2013). To protect against self cytotoxicity, NK cells express serpin B9 (SB9), which protects from granzyme B escaping the exocytic pathway (Bird et al., 1998). Additionally, some phospholipids are more compactly connected in the effector cell, which this can reduce the capacity of perforin to bind or to intercalate in the lipid layer (Antia et al., 1992).

- **Death Receptor Mediated Killing**

NK cells are also able to induce cytotoxicity through the engagement of cell-death receptors of the TNF superfamily: FasL, engaging the CD95/Fas receptor, and TRAIL, which can bind different TRAIL receptors (Figure 2). **FasL** is expressed on activated NK cells (Furuke et al., 1999). After synthesis, FasL is transported to secretory lysosomes, whose surface expression is dependent on degranulation events (Bossi and Griffiths, 1999). Of note, in T cells the secretory granules that store FasL seem to be distinct from the ones containing granzyme and perforin and, hence, the required signals for externalization may be distinct also in NK cells (Schmidt et al., 2011; Schmidt et al., 2008). Upon surface expression, FasL binds to Fas, expressed by a target cell, and stimulates an intracellular program leading to caspase 8- and 10-dependent apoptosis (Mariani et al., 1995). Homologous to FasL, **TRAIL**, is a transmembrane protein expressed in effector T and NK cells (Fanger et al., 1999; Ishikawa et al., 2005). TRAIL surface expression by NK cells is induced by cytokines, such as IL-2, IL-15 or IL-12 (Kayagaki et al., 1999; Smyth et al., 2001a). TRAIL co-localizes with CD107a/LAMP1-expressing vesicles (Monleón et al., 2001). FasL and TRAIL activity can be shed by the activity of a protease, at the membrane. However, in contrast to soluble FasL that loses its activity, soluble TRAIL retains apoptotic activity (Schneider et al., 1998; Wiley et al., 1995).

Adaptive NK cells

Immunological memory, characterized by a quantitatively and qualitatively higher response upon a secondary stimulation, has traditionally been associated with adaptive immune cells. However, “trained immunity” was already described in the innate compartment, in myeloid cells. Upon stimulation, monocytes sustain epigenetic changes, which confer engagement of the AKT, mechanistic target of rapamycin and hypoxia-inducible-factor 1-alpha (AKT-mTOR-Hif1α) pathway and induce engagement of glycolysis. This metabolic switch enables them to sustain enhanced response to a similar restimulation (Cheng et al., 2014).

Several studies report that NK cells display immunological memory in chronic hypersensitivity, infection and cancer (Cerwenka and Lanier, 2016; Mujal et al., 2021). Rag2-deficient mice, that lack functional T and B cells, are able to develop hapten-induced chronic hypersensitivity. However, the same mice treated with a blocking antibody directed against NK1.1 or Rag-2/IL2rγ double knock-out mice, that lack functional NK cells, do not present chronic hypersensitivity reaction. Interestingly, the memory response was described as being hapten-specific and persistent upon rechallenge. Furthermore, adoptive transfer of NK cells derived from previously challenged mice maintained the immune response to the hapten (O'Leary et al., 2006). Anti-viral memory response was also described in a subset of NK cells that could mount an enhanced response against murine cytomegalovirus (MCMV). MCMV-specific memory NK cells could be found in the liver, spleen, blood, lung, kidney and other lymphoid tissues after infection (Sun et al., 2009). The same was shown for distinct pathogen sources, namely human immunodeficiency virus 1 (HIV-1), influenza virus or vesicular stomatitis virus (VSV). Interestingly, in line with the concept of immunological memory, the protective response driven by adoptively transferred NK cells was specific to the virus that the NK cells had encountered in the first host (Paust et al., 2010). Cooper et al. showed that NK cells briefly exposed to a cytokine cocktail of IL-12, IL-15 and IL-18 would develop immunological memory. *In vitro* stimulated memory NK cells would sustain high levels of IFN-γ upon restimulation with IL-12 or IL-15 (Cooper et al., 2009). Furthermore, memory response was maintained following homeostatic proliferation in host mice (Keppel et al., 2013). Cytokine-induced memory NK cells relevance was already described in the human setting (Romee et al., 2012).

The mechanisms leading to memory NK cell formation remain to be elucidated. Despite the differences between the hapten-, viral- or cytokine-induced memory NK cells, there are certain similarities. The fine-tuning of NK effector potential by the expression of different polarizing receptors, led to the hypothesis that signaling through activating or inhibitory receptor could be the basis for memory NK formation (Cerwenka and Lanier, 2016). For

instance, during MCMV infection, the activating NK receptor Ly49H (KLRA8) and its cognate ligand, m157, were described to have a primary role in memory formation. When the expression of the NK receptor Ly49H/m157 is inhibited, the subset of long-lived, self-renewed NK cells is absent upon MCMV challenge (Sun et al., 2009). In humans that persistently encountered the human cytomegalovirus (HCMV), a subset of CD94/NKG2C^{+} NK cells can persist as memory NK cells and constitute up to 70% of the total NK cell population (Gumá et al., 2004). In HCMV-seropositive donors, this subset expands upon acute HCMV infection, creating a significantly better response to the virus, when compared to seronegative donors (Foley et al., 2012). Memory NK cell formation upon HCMV infection requires the cell-to-cell contact and soluble factors produced by CD14^{+} monocytes *in vitro* (Rölle et al., 2014). Uncovering the adaptive features of NK cells, will create new avenues to redefine NK cell function, tolerance and the usage of these cells in therapy.

NK in Health and Disease

Cancer

The first evidence for a NK cell-dependent tumor control was given by a methylcholanthrene (MCA) murine model of fibrosarcoma (O'Sullivan et al., 2012). Upon antibody depletion of NK cells, tumors grew significantly faster (Smyth et al., 2001b; Smyth et al., 2000). Thenceforth, underlying mechanisms of the protective role of NK cells in cancer were studied in both preclinical models and in the clinic. High expression levels of NK activating receptors or enhanced NK cell cytotoxicity are correlated with a better prognosis in different types of cancer (Imai et al., 2000; Schantz and Ordonez, 1991; Tartter et al., 1987). For example, in clear cell renal cell carcinoma, NK cell infiltration was considered a good prognosis (Eckl et al., 2012). In contrast, in non-small-cell lung cancer, NK cell presence is not associated with clinical outcome. Nevertheless, a downregulation of activating receptors in tumor infiltrating NK cells is associated with poor prognosis (Platonova et al., 2011). Thus, depending on the type of tumor, disease outcomes can be more dependent on the abundance or the phenotype of NK cells, namely the repertoire of receptors expressed on the surface, cytotoxic potential or array of produced cytokines (Carrega et al., 2008). Interestingly, NK and CD8+ T cells, innate and adaptive cytotoxic effectors, respectively, share the expression of receptors for activating and inhibitory ligands, namely DNAM1, NKG2D, CD2 or 2-B4 , as well as TIGIT and NKG2A. Both cell types require regulation by transcription factors Tbet and Eomes, for their functions and exert cytotoxicity through IFN-γ, TNF, granzymes, perforin, engagement of the death receptor pathways FasL or TRAIL. However, NK cells are not restricted to a specific antigen and exert their function by

the recognition of MHC-class I downregulation, which can hinder CD8+ T cell function. Therefore, NK cells are now regarded as main targets for cancer immunotherapy. In order to enhance their infiltration in the tumor microenvironment (TME), cancer cell recognition, effector and regulatory function, it is key to understand the molecular mechanisms governing those steps in NK cells (Huntington et al., 2020).

- **NK cell recruitment to the TME**

The two main subsets of NK cells, in human and in mice, express different repertoires of chemokine receptors, although similar across species. $CD56^{bright}/CD11b^{lo}$ $CD27^{hi}$ NK cells normally express and respond to ligands for CCR2, CCR5, CCR7, CXCR3, CXCR4 and CD62L, whilst $CD56^{dim}/CD11b^{hi}$ $CD27^{lo}$ subsets express CXCR1, CXCR2, CXCR4 and CX3CR1 (Bald et al., 2020; Bernardini et al., 2016; Hayakawa and Smyth, 2006; Lima et al., 2015; Morandi et al., 2011). It was shown that $CD56^{bright}$ NK cells are the dominant subset of NK cells in several tumors, including NSCLC and breast cancer (Carrega et al., 2014; Carrega et al., 2008). In the TME, there is an upregulation of CXCL9, CXCL10 and CCL19, which signal through the chemokine receptors CCR7 and CXCR3 on NK cells (Wendel et al., 2008). CCL5 specifically recruits $CD56^{bright}/CD11b^{lo}$ $CD27^{hi}$ subsets, where the majority of NK cells express CCR5 (Bald et al., 2020). In a mouse model of melanoma lung metastasis, IL-33 was responsible for the induction of CCL5 expression by CD8+ T cells and eosinophils. This led to an increased recruitment of NK cells to the TME and a decreased metastatic burden (Qi et al., 2020). The expression of other NK cell chemotactic molecules have been associated with a better disease prognosis, as it is the case for CX3CL1 and CCL27, signaling through CX3CR1 and CCR10, respectively (Park et al., 2012; Simonetti et al., 2006).

- **NK cell recognition of malignant transformation**

The process of oncogenic transformation in a cell is signaled through the expression of “danger signals” to prime its destruction by the immune system. In many cases, cancer cells downregulate the expression of MHC class I and this was thought to be an immediate trigger for NK cell cytotoxic activity (“*missing self*”) (Figure 3) (Ljunggren and Kärre, 1990). NK cell activity towards a transformed cell also depends on the repertoire of activating and inhibitory receptors for self MHC class I molecules, as well as additional signals through co-receptors and adhesion receptors (“*altered-self recognition*”) (Morvan and Lanier, 2016; Raulet and Guerra, 2009) (Figure 3).

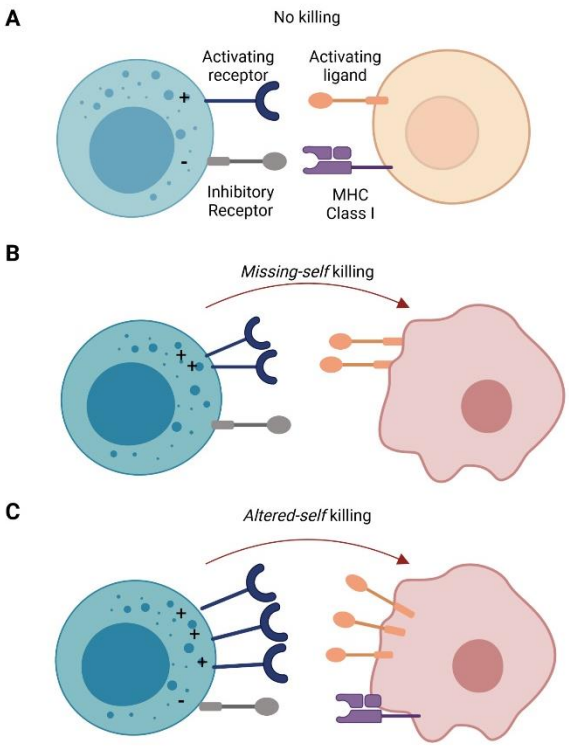

Figure 3 – NK cell activation and cytotoxicity induction.
NK cells recognize target cells and mediate cytotoxic mechanisms through the integration of activating and inhibitory receptors. Inhibitory signals are provided by different molecules, being the recognition of *self* MHC Class I molecules the key signal for NK cell inhibition. On the contrary, activating ligands for NK cell receptors are expressed by cells in *stress*, such as virally infected or malignant cells. **(A)** Resting NK cells recognized a balanced number of activating and inhibitory ligands expressed by a healthy cell and do not activate cytotoxic pathways. **(B)** Loss of MHC class I expression by infected or transformed cells induces NK cell activation through "missing self" recognition and leads to targeted cytotoxicity. **(C)** *Stressed* cells overexpress activating ligands and, when those predominate, NK cell cytoxic mechanisms are induced by "altered-self killing". Adapted from EMJ Allergy & Immunol. 2019; 4[1]:108-116. Created with Biorender.com
+: activating signal; -: inhibitory signal; MHC: major histocompatibility complex.

Human NK cells can recognize cancer cells through the activating receptor NKG2D, MHC class I polypeptide-related sequence A (MICA) and MICB, glycoproteins expressed on the surface of cells suffering genotoxic stress or DNA damage (Diefenbach et al., 2002). In preclinical models, deficiency of the receptor NKG2D increased the burden of spontaneous and transplantable tumors (Cerwenka et al., 2000; Gasser et al., 2005; Guerra et al., 2008). In patients with acute myeloblastic leukemia (AML), NKG2D was shown to play a role in tumor immunosurveillance (Paczulla et al., 2019). Furthermore, it was shown that the engagement of the leukocyte function-associated molecule 1 (LFA-1) and the intercellular adhesion molecule 1 (ICAM1), that contribute to the immune synaptic formation, improved NK cell recognition and lysis of tumor cells (Saga et al., 2019; Schmits et al., 1996). The activating receptors NCR1 (NKp46), NKp44 and NKp30 are associated with several tyrosine-based activation motif (ITAM)-containing adaptor proteins, in order to recruit

downstream kinases, such as Lck, Fyn, Syk and ZAP-70, and activate NK cells (Barrow et al., 2019). B7-H6 expression is upregulated in several tumor cells lines, where it can be recognized by NKp30 (Fiegler et al., 2013). *Bona fide* tumor ligands for NKp44 and NKp46 have not yet been identified. However, it was shown that mice with NCR1 deletion have an increased metastatic burden and NK cells show a reduced cytotoxic activity, marked by low degranulation, as well as reduced IFN-γ and TNF production (Glasner et al., 2012). In cancer patients, increased NCR1 expression is related to an improved prognosis of the disease (Cursons et al., 2019). Furthermore, engagement of CD2 on NK cells with its cognate ligand CD58, as well as 2B4 and CD48, seem to increase tumor cell cytotoxicity, likely by synergizing with other activating receptors (Messmer et al., 2006; Schmidt et al., 1988). Interestingly, CD155, expressed on the surface of malignant cells, can be recognized by NK activating receptors DNAM1 and CD96, or by an inhibitory receptor, TIGIT. Those receptors compete for the binding to the cognate ligand and, hence, influence NK cell signaling and the cytotoxic response (Chan et al., 2014; Sanchez-Correa et al., 2019). HLA-E, that can be expressed on tumor cells, can also engage the heterodimer CD94/NKG2C or CD94/NKG2A, enhancing or hindering NK cell function, respectively (Lanier et al., 1998; Le Dréan et al., 1998). Finally, KIR2DL1, KIR2DL2 and KIR2DL3 are NK inhibitory receptors with an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic tail that recognize HLA-C and seem to be involved in NK cell inhibition *in vivo* (Romagné et al., 2009).

- **NK cell killing of transformed cells and immune escape**

Reduced cytotoxicity of peripheral blood NK cells was correlated with higher incidence of tumor (Imai et al., 2000), suggesting that NK cell-mediated killing is important for tumor control. The selective pressure imposed by NK cells leads to the elimination of malignant cells expressing a high activating-to-inhibitory ligand ratio. The shaping of the repertoire of ligands expressed by residual tumor cells ("*immune editing*") renders the tumor cells insensitive to NK cell-mediated killing ("*immune escape*") (Huntington et al., 2020). Experimental evidence of such occurrence comes from MCA-induced fibrosarcoma, where NK cell-deficient mice present a higher tumor incidence (Smyth et al., 2000). Interestingly, when transferred to another host, these tumors are more immunogenic and result in a lower tumor burden, when compared to those derived from NK cell-competent hosts (O'Sullivan et al., 2012). Cancer cells can acquire intrinsic features that reduce the likelihood of being recognized, namely by downregulating the expression of NK cell-activating ligands and increasing the inhibitory counterparts. Moreover, they are able to downregulate the expression of FAS on their surface or generate soluble variants of NKG2D ligands that act

as decoys for the cognate activating receptors (Benson et al., 2010; Deng et al., 2015a; Elboim et al., 2010; Maecker et al., 2002; Reiners et al., 2013). In addition, TME-derived factors contribute to dampen NK cell-dependent anti-tumor response. The immunosuppressive factors tumor growth factor β (TGF-β), activin-A and adenosine have direct inhibitory effects on NK cell function (Bruno et al., 2013; Robson et al., 2009; Tang et al., 2017; Young et al., 2018). Namely, TGF-β and activin-A bind to specific receptors on NK cells and induce the phosphorylation of the transcription factors SMAD2 and SMAD3, with consequent downregulation of EOMES, whose expression has been involved in maintenance of NK cell survival, proliferation, cytotoxicity and IFN-γ production (Robson et al., 2009; Tang et al., 2017). Moreover, IL-10 and TGF-β participate in the recruitment of immunosuppressive cells, such as Treg cells, myeloid derived suppressor cells (MDSCs), neutrophils and indoleamine 2,3-dioxygenase (IDO1)-expressing DCs, all of which inhibit NK cell effector functions (Della Chiesa et al., 2006; Li et al., 2009; Pedroza-Pacheco et al., 2013; Sceneay et al., 2012). When the tumor cells escape from the immune response, immune infiltration is subverted, dampened or inexistent. Interestingly, chemo- and radiotherapy or targeted therapy with antibodies or small molecules can induce activating ligands in tumor cells, increasing their sensitivity to NK-mediated cell lysis. Enhanced immune activation leads to an increased immune infiltration and better tumor control (Huntington et al., 2020).

Autoimmunity

An autoimmune disease arises from the loss of self-tolerance and it is characterized by an exacerbated immune reaction that causes tissue damage. Depending whether or autoantibody production and T cell hyperactivation are localized, an autoimmune disease can be classified as organ-specific or systemic. Besides the *bona fide* role of the adaptive immune system, the innate immunity, particularly NK cells, have recently been implicated in the onset of autoimmune diseases (Gianchecchi et al., 2021; Liu et al., 2021).

Namely, in rheumatoid arthritis, one of the most prevalent chronic autoimmune diseases (Smolen et al., 2016), NKG2D and MICA genes were associated with disease susceptibility, as well as severity (Mariaselvam et al., 2017). In the peripheral blood of active RA patients, the total numbers and percentages of NK cells are elevated, and high levels of IL-2 and IL-15 are present in the serum (Chalan et al., 2016; Li et al., 2020). In the inflamed joints, the $CD56^{bright}$ NK cell subset accumulates in response to IL-12, IL-15 and IL-18 produced by monocytes and macrophages. These NK cells rapidly secrete IFN-γ and GM-CSF, which contribute to macrophage activation, creating a pro-inflammatory feedback loop (Dalbeth

and Callan, 2002). Synovial NK cells express multiple activating receptors, namely NKG2D, DNAM-1, NKp44 and NKp46, which engage to ligands in the synovial fibroblasts (Nielsen et al., 2014).

In multiple sclerosis (MS), both clinical studies and experimental autoimmune encephalitis (EAE) animal model of the disease, imply a role of NK cells in the disease development (Van Kaer et al., 2019). It was shown that blocking the inhibitory signaling driven by NKG2A on NK cells promoted cytolytic effects of T cells and of microglia, ameliorating the CNS inflammation (Lu et al., 2007). It has also been demonstrated that IL-2-driven activation of CNS-resident NK cells suppressed Th17 responses against myelin and decreased disease severity. In this case, NK cells had a regulatory role, by promoting the secretion of IL-1β, TGF-β and TNF (Langeneckert et al., 2019). In patients, the accumulation of NK cells in the CNS, driven by CX3CR1/CX3CL1 signaling, negatively correlated with disease severity (Huang et al., 2006). On the other hand, studies have shown that NK cells may exacerbate MS. In a EAE model, NK cells promote the expansion of macrophages and T cells in the CNS, through the secretion of IFN-γ and can exert cytotoxic effects against oligodendrocytes (Zhao et al., 2020). Notably, following treatment of patients with MS, the most prevalent NK cells belong are $CD56^{bright}$, which exert an immune regulatory function in MS, suppressing Th17 activity. Therefore, regulating the $CD56^{bright}$ NK cell subset might become a therapeutic strategy for preventing MS onset and/or relapse (Gilmore et al., 2020; Rodríguez-Martín et al., 2015).

Infections

- **NK cells in viral infection**

The importance of NK cells in the control of viral infections has long been recognized. Evidence has mainly originated from patients with severe combined immunodeficiencies. Impairment of NK cell function was concomitant with an increased susceptibility to viral infections, specifically with herpes simplex virus (HSV), Varicella Zoster virus (VZV), CMV and human papilloma virus (HPV) (Orange, 2013). Many viruses are able to modify the surface membranes of their hosts, resulting in increased expression of stress ligands or downregulation of MHC class I molecules. These characteristics lead to enhanced NK cell recognition and subsequent killing of the target cell (Hammer et al., 2018).

NK cells can be directly activated by viral ligands expressed on the surface of infected cells. It is well known that the activating receptor NKp46 in NK cells recognizes and binds influenza hemagglutinin on human NK cells, inducing target-cell cytotoxicity (Mandelboim et al., 2001). In mice, MCMV is recognized by activating receptors belonging to the Ly49

family, which bind the viral glycoprotein m157. Ly49H-expressing mouse strains are highly resistant to MCMV infection. In fact, Ly49H binding to m157 is sufficient for NK cell activation and MCMV elimination (Arase et al., 2002; Daniels et al., 2001; Smith et al., 2002). Likewise, viral proteins of West Nile virus and Dengue virus are known to bind NKp44. However, as this receptor is only induced upon activation, NK-mediated cytotoxicity requires accessory signals for virus elimination (Hershkovitz et al., 2009). Other specific viral proteins-NK receptor pairs have been described to be important for the anti-viral response (Chisholm and Reyburn, 2006; Fang et al., 2008). However, inhibitory receptors, such as KIRs, were also shown to participate in NK-mediated viral control. In acute hepatitis C virus (HCV) infections, expression of KIR2DL3 in NK cells, combined with a downregulation of its cognate ligand, HLA-C1, has been associated with virus clearance and positive outcomes in the treatment of HCV patients (Khakoo et al., 2004). In HIV-1, some polymorphisms have been reported to select for increased recognition and binding to KIR2DL2, in chronically infected patients (Alter et al., 2011). On the other hand, viral infections are able to induce NK cell activation by increasing the secretion of type I IFNs and IL-12 by plasmacytoid DCs, as described for MCMV and HSV (Dalod et al., 2003; Krug et al., 2004). During MCMV infection, IL-12 production sustains IFN-γ production by NK cells, while NK cell cytotoxicity seems to be dependent on the presence of IFN-α or IFN-β. In the late phase of the infection, decreased IL-12 availability leads to a reduction in IFN-γ secretion by NK cells (Delale et al., 2005) (Figure 2). One of the most recent key studies in the importance of NK cells for viral control was the report of enhanced NK cell function during secondary antigen exposure, as previously elaborated. The adaptive feature of NK cells would provide a number of therapeutic and prophylactic avenues for viral infections (Sun et al., 2009).

- **NK cells in bacterial infections**

Regarding bacterial infections, NK cell response seems to be induced in an indirect way. The direct recognition of bacterial cell-wall-derived proteins by NK cell receptors is, so far, only described in *Mycobacterium bovis* (BCG), *Nocardia farcinica* and *Pseudomonas aeruginosa,* by engagement of NKp44. This direct signaling does not seem essential for cytotoxic NK cell activity but contributes to increased cytokine production (Esin et al., 2008; Marcenaro et al., 2008) Nevertheless, during the most part of bacterial infections, namely with *Listeria monocytogenes, Staphylococcus aureus, Mycobacterium tuberculosis* and BCG, an indirect activation of NK cells is described. Generally, dendritic cells and monocytes are activated via TLR and secrete IL-12, IL-18 and type I IFN, inducing NK cell function (Newman and Riley, 2007). *M. tuberculosis* infections are aggravated by the depletion of NK cells, due to an uncontrolled replication of the pathogen in macrophages (Denis et al., 2007; Feng et al., 2006). Some evidence point towards a regulatory role of IL-

18, as well as IL-12 and IL-2, also produced by CD4+ T cells (Evans et al., 2011). In case of acute infections with *L. monocytogenes* or *Yersinia pestis*, NK cells are able to produce IL-10. This prevents immune pathology during systemic infection, by suppressing IL-12 secretion by DCs, leading to a reduced NK cell activation (Perona-Wright et al., 2009).

NK cell metabolism

Metabolic modulation is at the core of homeostatic maintenance of any cell type in the organism. Immune cells are not an exception and the field of “immunometabolism” has markedly grown over the past years. The rewiring of energetic sources and its utilization has unraveled many details of the regulation of immune function. This additional layer of regulation is particularly important in disease environments, where energy supply and demands are in constant change, and influence the functional outcome of the immune cells (Kobayashi and Mattarollo, 2019; O'Brien and Finlay, 2019). The importance of metabolic regulation can be exemplified with effector CD8+ T cells, which are in many aspects comparable to NK cells. Upon activation, CD8+ T cells, switch from basal oxidative phosphorylation to glycolysis and glutaminolysis. This rewiring of energetic sources, even in the presence of normal levels of oxygen, enables CD8+ T cells to fulfill the energetic demands of clonal proliferation. Upon antigen clearance, surviving CD8+ T form memory T cells, restructure the mitochondria and increase their mitochondrial mass. These structural changes enable a more efficient fatty acid oxidation process and improve their maintenance and survival (Geltink et al., 2018). Likewise, NK cell activation has been associated with major metabolic changes, which influence their functional capacity and role in diseases.

Distinct **metabolic pathways** are used to generate energy levels adequate to the needs of the cells: survival, growth and proliferation. Despite their diversity on the fuel source, those pathways are closely interrelated through the need of by-products from an alternative pathway to ensure the completion of a distinct metabolic process. The glycolytic pathway is initiated with the uptake of glucose from the extracellular environment and, the catabolism of this product yields pyruvate. In the mitochondria, pyruvate is converted to acetyl-CoA, which feeds into the tricarboxylic acid (TCA) cycle. The TCA cycle-generated reducing agents, NADH and $FADH_2$ transfer electrons to complex I and II of the electron transport chain (ETC). This creates a proton gradient driving the activity of ATP synthase, generating ATP. Alternatively, β oxidation of fatty acids (also known as fatty acid oxidation, FAO) in the mitochondria can form acetyl-CoA, through the TCA cycle, which is used to fuel oxidative phosphorylation (OXPHOS) (Figure 4). Furthermore, the catabolism of glutamine (glutaminolysis) to α-ketoglutarate, can additionally feed the TCA cycle. Glucose and

glutamine can be used to generate products involved in the biosynthesis of lipids, nucleotides and proteins. Cells with high energetic demands, metabolize glucose to lactate, while also feeding pyruvate into the mitochondria for the TCA cycle. This metabolic configuration diverts glycolytic intermediates towards biosynthesis, whilst sustaining ATP production (O'Neill et al., 2016).

- **Metabolic modulation of NK cell activation**

Resting NK cells have low basal levels of OXPHOS and glycolysis, which are essential to sustain acute NK cell responses. Short cytokine- or receptor-mediated NK cell stimulation does not result in increased metabolic rates. Nevertheless, inhibition of OXPHOS or glycolysis at these time points results in impaired IFN-γ production. Interestingly, receptor stimulation of NK cells was demonstrated to be more sensitive to metabolic inhibition than cytokine stimulation (Keppel et al., 2015; Marçais et al., 2014). A more prolonged stimulation of NK cells, however, drives more robust metabolic changes, which are needed for the effector function of these cells. Overnight cytokine-stimulation with IL-15, IL-2 and IL-12, alone or in different combinations, increases rates of glycolysis and OXPHOS (Donnelly et al., 2014; Marçais et al., 2014). *In vivo*, MCMV infection results in NK cell activation and increased mitochondrial mass for optimal respiratory capacity, depicting the necessary metabolic configuration for NK cell function (O'Sullivan et al., 2015). Activated NK cells treated with 2-deoxyglucose (2-DG) and oligomycin, which respectively inhibit glycolysis and OXPHOS, show significantly reduced expression of IFN-γ and granzyme B (Donnelly et al., 2014; Keating et al., 2016). Human NK cells cultured with IL-15 and 2-DG have an impaired cytolytic activity directed against K-562, a cell line sensitive to NK cell activity (Felices et al., 2018). Mouse models of *in vivo* NK cell activation, through injection of polyinosinic:polycytidylic (Poly I:C) or MCMV, show that NK cell IFN-γ production is drastically inhibited when animals are concomitantly injected with 2-DG. In the case of MCMV, viral clearance was also impaired (Donnelly et al., 2014; Mah et al., 2017). In fact, activated NK cells increase their glucose uptake by upregulating the glucose transporter GLUT1 and the transcription of glycolytic enzymes. The expression of CD71, a transferrin receptor, as well as amino acid transporters, SLC1A5, SCL7A5 and CD98 is increased upon activation (Jensen et al., 2017; Keating et al., 2016). The inhibition of the amino acid transporters SLC1A5 and SCL7A5, in human NK cells activated through NKG2D stimulation, impaired their cytotoxic potential, impacting IFN-γ secretion and degranulation (Jensen et al., 2017).

The energetic demands of activated NK cells are primarily answered through glucose metabolism, resulting in the production of pyruvate and lactate, through aerobic glycolysis. Notably, in NK cells, the pyruvate that enters the mitochondria is not used to fuel the TCA

cycle. Instead, it is converted to mitochondrial citrate and metabolized by citrate-malate shuttle (CMS). The CMS generates NADH to, alternatively, fuel OXPHOS and generate ATP. The CMS can also generate NAD+, a co-factor for the glycolytic enzyme GAPDH (glyceraldehyde-3-phosphate dehydrogenase), enabling higher glycolytic flux (Assmann et al., 2017). As the CMS is only fueled by glucose, NK cells do not use glutamine as an energetic source. In fact, inhibition of glutaminolysis, does not impact OXPHOS in NK cells, nor their effector functions (Keppel et al., 2015; Loftus et al., 2018). On the other hand, the utilization of fatty acids by these cells is still a matter of debate. It seems that, despite its recently reported off-target effects, the inhibitor of fatty acid oxidation, etomoxir, had no effect on activated NK cells (Keppel et al., 2015; Raud et al., 2018).

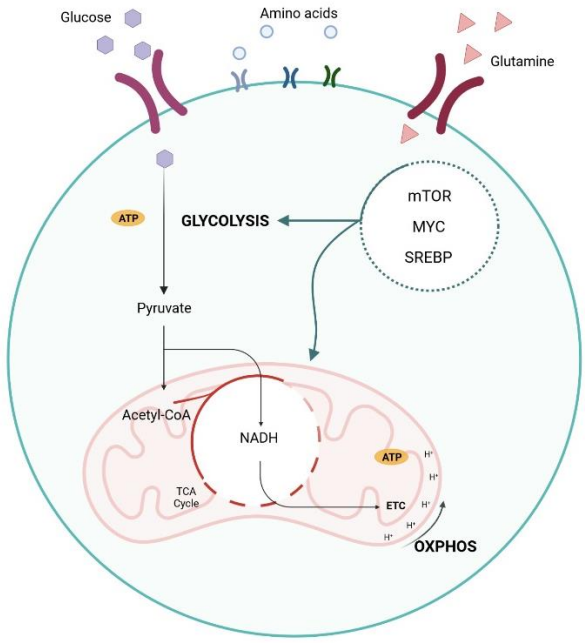

Figure 4 – Metabolic modulation of NK cells.
Glucose is metabolized to pyruvate, through glycolysis, and further to acetyl-CoA, yielding NADH. Acetyl-coA feeds into the TCA cycle, which fuels OXPHOS through the generation of reducing agent NADH (and $FADH_2$, not shown). NADH and $FADH_2$ transfer electrons to the ETC, leading to the creation of a proton gradient, resulting in the production of ATP. Alternative energetic sources, such as fatty acids and amino acids, are used to fuel OXPHOS and ATP production. Cytokine stimulation and sustained glucose and glutamine levels induce mTOR activity and MYC expression, which contribute to enhanced OXPHOS and glycolysis, sustaining NK cell function. mTOR signaling induces the accumulation of MYC protein, which is later independently sustained by amino acid uptake. Likewise, mTOR activity contributes to SREBP signaling, which controls the expression of the citrate-malate shuttle and preserves NK cell metabolic activity and function. Adapted from (O'Brien and Finlay, 2019). Created with Biorender.com.
mTOR: mechanistic target of rapamycin; SREBP: sterol regulatory element-binding protein; OXPHOS: oxidative phosphorylation; TCA cycle: tricarboxylic acid cycle; ETC: electron transport chain

- **Metabolic modulation of NK cell development and maturation**

To this date, studies on metabolic modulation of NK cells focus on mature NK cells and there has not been direct investigation of the role of metabolism during NK development, from precursors to immature NK cells (O'Brien and Finlay, 2019). In mice, $CD11b^{lo}$ $CD27^{hi}$ immature NK cell have a high proliferation rate, which correlates with the expression of the amino acid transporter SLC3A2 and the transferrin receptor CD71 (Marçais et al., 2014). Single cell RNA sequencing data from this NK cell subset, localized in the bone marrow, shows an increased transcription of genes related with nutrient transporters and metabolic enzymes, namely hexokinases and phosphofructose kinase (Pfkl), from the glycolytic pathway, as well as enzymes involved in lipid synthesis, such as fatty acid synthase (Fasn) and ATP citrate lyase (Acly) (Consortium, 2018). Throughout maturation, as NK cells terminally differentiate to $CD11b^{hi}$ $CD27^{hi}$ and $CD11b^{hi}$ $CD27^{lo}$, they present a more quiescent state and reduce the expression of nutrient transporters. Interestingly, there is mounting evidence for a role of metabolic regulation in the education or licensing of NK cells (Marçais et al., 2014). In humans, when compared to unlicensed NK cells, educated NK cells show an increased expression of glucose transporters and higher glycolysis rates, even in a resting state. In contrast, respiratory capacity of immature NK cells remained unchanged (Pfeifer et al., 2018; Schafer et al., 2019).

- **Metabolic regulators of NK cells**

Cell metabolism is regulated by a number of enzymes and proteins. In immune cells, the coordinated activity of these regulators ensures the engagement of different metabolic pathways according to changes in the environment, enabling an effective immune response. In NK cells, the mTOR complex 1 (**mTORC1**) constitutes one of the major regulators of metabolism and activity. mTORC1 is a serine/threonine kinase whose downstream targets are the ribosomal protein S6 kinase (S6) and eukaryotic initiation factor 4E (4E-BP1), which can lead to the initiation of protein translation. mTOR activity is regulated by external amino acid levels or growth factors (Figure 4) (Kim and Guan, 2019). mTORC1 is crucial for NK cell development. The more immature $CD11b^{lo}$ $CD27^{hi}$ NK cell subset has a higher mTORC1 activity, which correlates with the higher expression of nutrient transporters and metabolic enzymes (Consortium, 2018; Marçais et al., 2014). Mice with a NK-specific deletion of the regulatory associated protein of mTOR (Raptor), essential for mTORC1 activity, present an abnormal accumulation of $CD11b^{lo}$ NK cells in the bone marrow. There, differentiation is blocked at the $CD11b^{lo}$ to $CD11b^{hi}$ stage, due to a defect in proliferation (Yang et al., 2018). Moreover, the more mature NK cells ($CD11b^{hi}$ $CD27^{lo}$) show lower expression of mTORC1, which is related to their resting status. This is associated with lower expression of CD98, CD71 and decreased glucose uptake

(Consortium, 2018; Marçais et al., 2014). Nevertheless, upon NK cell activation, mTORC1 activity increases, which is essential for the expression of nutrient transporters, glycolytic enzymes, as well as mitochondrial remodeling and expression of effector molecules, such as IFN-γ and granzyme B (Donnelly et al., 2014; Keating et al., 2016; Marçais et al., 2014). Activated NK cells treated with rapamycin, an inhibitor of mTORC1, show a decreased glycolytic rate (Donnelly et al., 2014; Viel et al., 2016). In human NK cells, mTORC1-driven metabolic changes depend on the specificity of the cytokine stimulation. For example, overnight stimulation of NK cells with IL-2 enhances glycolysis in an mTORC1-dependent fashion. However, a combination of IL-12 and IL-15 increased glucose metabolism in the absence mTORC1 activity (Keating et al., 2016). Nevertheless, rapamycin-treated mice, infected with MCMV show a reduced mTORC1 activity in NK cells, with reduced proliferation and cytotoxic capacity, resulting in high viral loads (Nandagopal et al., 2014). These data highlight the fine regulation of NK cell metabolism, activity and signaling in a stimulation-specific manner.

Paralleled by mTOR-dependent nutrient sensing, NK cells are regulated by AMP-activated protein kinase (**AMPK**). When subjected to nutrient deprivation, cells lack the substrates to produce ATP. This results in the accumulation of adenosine diphosphate (ADP) and monophosphate (AMP), which leads to the activation of the kinase AMPK. This results in induced lipid oxidation through the inhibition of de novo lipogenesis and activation of limiting step enzymes of FAO. Indeed, AMPK activity inhibits NK cell activation (Wang et al., 2016). In humans, the terminally differentiated $CD56^{dim}$ NK cell subset shows higher AMPK activity. Pharmacological activation of the AMPK pathway results in suppressed NK cell cytotoxicity, IFN-γ and granzyme B production (Müller-Durovic et al., 2016).

Another factor involved in fatty acid and cholesterol metabolism is the sterol regulatory element-binding protein (**SREBP**). In NK cells, SREBP activity is induced by cytokine stimulation and mTORC1 signaling. In NK cells, SREBP executes a very specific role, inducing the expression of two enzymes involved in the CMS, the citrate-malate antiporter, SLC25A1 and the ACLY. SREBP activity in NK cells drives the maintenance of the high rates of glycolysis and OXPHOS and is essential for NK cell effector functions, such as IFN-γ production and cytotoxicity. *In vivo*, SREBP plays a prominent role in tumor control. These findings were recapitulated in human NK cells, where SREBP regulates metabolic and functional changes induced by cytokine stimulation, by enhancing glucose-dependent CMS activity (Assmann et al., 2017). Recently, O'Brien et al. described an additional layer in SREBP-dependent NK metabolic regulation. SREBP activity induces polyamine synthesis through facilitating high expression of Myc (O'Brien et al., 2021). The transcription factor **MYC** has also been shown to be a major metabolic regulator of NK cells. MYC activity

increases the expression of glucose transporters and glycolytic enzymes to support glycolysis and mitogenesis in order to maintain high rates of OXPHOS. At long term, MYC expression is completely independent of mTORC1 activity, but it is instead regulated by the availability of amino acids. As previously mentioned, glutamine is not an important source of energy for NK cells. Nevertheless, glutamine induces MYC signaling, as it facilitates the transport of amino acids through SLC7A5. Glutamine withdrawal or SLC7A5 inhibition lead to decreased MYC expression (Loftus et al., 2018). Interestingly, $CD56^{bright}$ human NK cells have a higher expression of MYC, which is consistent with their higher metabolic activity (Collins et al., 2019). Other regulators, such as **HIF-1α** or the DNA-binding protein **Rfx7** have been shown to participate in the control of NK metabolism and activity (Castro et al., 2018; Ni et al., 2020).

- **Metabolic constraints of NK cell function**

Accumulating evidence points towards NK cell metabolic dysfunction in pathologic conditions, such as cancer and obesity. Recently, Michelet et al. demonstrated that NK cells from **obese** mice and humans, are not able to increase their glycolytic or respiratory rates after activation. The activation of the peroxisome proliferator-activated receptor (PPAR) leads to lipid accumulation in the NK cells, downregulating the expression of mTORC1 and MYC and generally altering gene expression. NK cells from obese mice and humans fail to correctly form an immune synapse to lyse the target cell (Michelet et al., 2018). This study was in line with previous data showing that, both in mice and humans, obesity leads to a decreased NK cell number and impaired cytotoxic activity (Smith et al., 2007; Tobin et al., 2017). Likewise, several metabolic perturbations for NK cells have been described in **cancer**. Primarily, the TME is known to be nutrient-deprived, with low levels of glucose and glutamine, which restrict NK cell metabolism. It was also shown that high concentrations of lactate and the low pH of the TME can inhibit NK cell antitumor function (Brand et al., 2016; Pötzl et al., 2017). In colorectal liver metastasis, the decreased intracellular pH of NK cells led to mitochondrial dysfunction and apoptosis (Harmon et al., 2019). Other molecules, such as TGF-β can inhibit mTORC1 activity and impair NK cell metabolism (Viel et al., 2016). In an experimental model of lung cancer, TGF-β was suggested to induce the expression of fructose biphosphatase 1 (FBP1) in NK cells, counteracting the glycolytic flux and impairing cytotoxic activity (Cong et al., 2018). In a similar way to what was described for obesity, some tumors are rich in cholesterol, which could potentially impair NK cell activity (Michelet et al., 2018). Interestingly, in aggressive B cell lymphoma, NK cells have increased lipid metabolism, which suppresses their effector response and cellular metabolism (Kobayashi et al., 2020). Moreover, cholesterol can be hydrolyzed by macrophages in the TME and

converted to hydroxycholesterol. The latter was shown to inhibit SREBP activity, a master regulator of NK cell metabolism. Other factors of the TME have been shown to alter the function of effector immune cells, namely tryptophan metabolites, adenosine, hypoxia or reactive oxygen species. The dissection of the specific contribution for NK dysfunction in disease settings is of great importance to unravel the fine regulation of metabolism and homeostasis. This would allow the generation of new therapeutic avenues and will shed light on the molecular mechanisms of NK cell function (Guerra et al., 2020).

Reactive oxygen species (ROS)

One specific constraint of highly inflammatory environments, such as the TME, adipose tissue or microbial infections, is the presence of reactive oxygen species (ROS).

Oxidation-reduction (redox) reactions are intrinsically coupled to different metabolic programs occurring in the cell. ROS are chemically active oxygen-containing molecules and include free radicals such as hydroxyl (OH•), superoxide (O_2•), peroxides (RO•), nitric oxide (NO•) and the non-radical hydrogen, peroxide (H_2O_2). **Mitochondria** are a primary source of ROS generation. During aerobic metabolism, in the electron transport chain, electrons pass through mitochondrial complexes to react with O_2. However, leakage of electrons occurring during this process provokes a reaction with molecular oxygen, leading to the formation of O_2•. The latter is rapidly converted to H_2O_2 by superoxide dismutase (SOD) present in the mitochondria. Other significant ROS generators are NADPH oxidases (**NOXs**). They are mainly localized in the plasma membrane, although they can be expressed in other cellular membranous organelles, such as the endoplasmic reticulum (ER), nucleus or mitochondria. NOXs generate O_2• from O_2 and NADPH, and O_2• is rapidly converted to H_2O_2 by SODs. In the ER, besides NOX activity, ROS can be generated during protein post-translational modifications. Namely, the flavoenzyme ERO1 initiates the formation of disulfide bonds using O_2 as an electron acceptor (Apel and Hirt, 2004).

The effects of ROS on cellular homeostasis are strictly dependent on their levels in the cell and, therefore, their rate of production, detoxification and their spatial and temporal regulation. At low levels, given their defined subcellular compartmentalization, ROS can inhibit tyrosine phosphatases, leading to subsequent phosphorylation and activation of growth factors. ROS can also target transcription factors, metabolic enzymes or DNA. This promotes cell proliferation, metabolic adaptation and survival. However, their high reactivity and specific targeting of proteins and DNA can be detrimental to several cellular components. High rates of ROS production coupled to a deficiency in their detoxification is the basis for **oxidative stress** and may cause cell senescence or death. Therefore, a

structured adjustment of redox signaling enables cells to maintain antioxidant activity. As previously mentioned, O_2• is readily converted by SODs into H_2O_2, which can be converted into water (H_2O) by distinct antioxidants (Gorrini et al., 2013). There is a complex organization of cellular ROS detoxification.

- **Counterbalancing ROS: glutathione**

The cellular antioxidant system is composed, among others, by glutathione (GSH), thioredoxin (TRX) and catalase (Figure 5). Catalase is mainly located in peroxisomes and directly converts hydrogen peroxide to water. On the other hand, GSH and TRX may directly be oxidized by ROS or participate in peroxidase-catalyzed reactions, which require NADPH as co-factor (Perillo et al., 2020).

GSH is the most abundant intracellular antioxidant. GSH is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) and its redox activity is based on the presence of an active thiol group in the cysteine residue. GSH production occurs in the cytosol in a two-step, energy-consuming process (Figure 5). The first step, catalyzed by L-glutamate cysteine ligase (GCL), ensures the formation of a peptide bond between glutamate and cysteine: γ-glutamyl-cysteine. Subsequently, glutathione synthase (GLS) participates in the combination of γ-glutamyl-cysteine with glycine, forming GSH. The first part of the process constitutes the rate-limiting step of GSH production and the presence of GSH can feedback into γGLCL activity and control it (Lu, 2013).

GSH can act as an antioxidant in two distinct ways. The cysteine residue present in GSH can be directly oxidized by ROS, leading to the formation of GSSG. Alternatively, GSH can be used as co-substrate by glutathione peroxidase (GPX), reducing H_2O_2 and, thereby, forming GSSG, water or alcohols. GSSG can be restored by glutathione reductase (GR) to GSH or be excreted from the cell. GSH is synthesized in the cytosol but its function is not limited to this sub-cellular localization. As mitochondria produce 90% of all ROS, GSH presence in this organelle is crucial for the maintenance of cellular redox status (Ribas et al., 2014). In the inner mitochondrial membrane, cytochrome c is normally bound to the phospholipid cardiolipin. Cardiolipin is sensitive to oxidative damage and, therefore, the action of mitochondrial GSH prevents the release of cytochrome c and the activation of the apoptotic cascade. In the same line, when mitochondrial ROS levels rise, Ca^{2+} influx can be triggered, leading to the activation of cell death machinery. The presence of GSH in the mitochondria enables an efficient scavenging of the oxidants and keeps calcium levels compatible with cell survival (Marí et al., 2009). The importance of GSH for cell homeostasis is also depicted in its role in the ER, where GSSG can support the efficient conformation of newly formed polypeptides. GSH constitutes the main source of oxidative power for the

formation of disulfide bonds between residues. Moreover, in the nucleus, GSH-driven maintenance of redox status, allows an efficient function of proteins involved in nucleic acid biosynthesis and DNA repair. GSH participates in the reduction of ribonucleotides to deoxyribonucleotides, essential for DNA formation. The role of this tripeptide also comprises protein glutathionylation, synthesis of leukotrienes, prostaglandins and constitutes an important intracellular cysteine reserve. Given its remarkable spatial and functional extent, the importance of GSH for the maintenance of cellular homeostasis cannot be overestimated (Desideri et al., 2019).

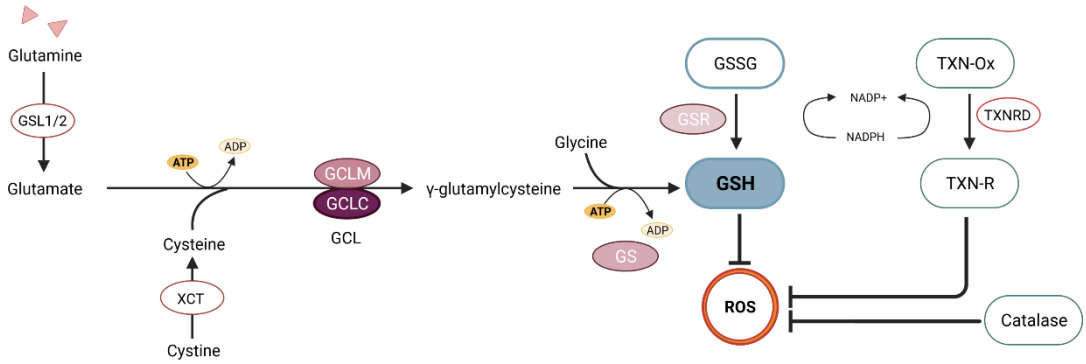

Figure 5 – Glutathione synthesis and alternative antioxidant pathways for ROS detoxification.
The three main pathways for ROS scavenging involve the activity of catalase, TXN and GSH. Catalase is mainly located in peroxisomes and acts directly on ROS, independently of NADPH. TXN and GSH detoxify ROS at the expense of NADPH consumption, for their regeneration to the reduced form, by TXNRD and GSR, respectively. *De novo* GSH synthesis is a two step ATP-consuming process. The first step is catalyzed by GLC (composed by GCLC and GCLM) and involves the ligation of glutamate and cysteine to form γ-glutamylcysteine. The latter is further conjugated with glycine, forming GSH. GSL1/2 produce glutamate, from glutamine, and XCT provides cysteine for the first reaction of GSH synthesis. Adapted from (Gorrini et al., 2013) and (Lu, 2013). Created with Biorender.com
GSL1/2: Glutaminase 1 or Glutaminase 2; XCT: cysteine/glutamate transporter; GCL: glutamate cysteine-ligase; GCLM: glutamate cysteine-ligase modifier subunit; GCLC: glutamate cysteine-ligase catalytic subunit; GS: glutathione synthase; GSR: glutathione reductase; TXN: thioredoxin (Ox, oxidized, R-reduced); TXNRD: thioredoxin reductase; ROS: reactive oxygen species

- **ROS regulation in the immune response**

Oxidative stress contributes to the onset of several pathological conditions. Increased protein oxidation was associated with metabolic diseases, such as diabetes type 2, arteriosclerosis, respiratory diseases or neurological disorders, namely Alzheimer disease or multiple sclerosis. Moreover, ROS have been strongly implicated in the onset and progression of infections and cancer. Thus, it is key to recognize the significance of

oxidative stress in perpetuating chronic inflammation and subsequent tissue damage in distinct disease settings (Garcia-Garcia et al., 2012).

In pathological conditions, ROS can be produced and released by damaged cells, as well as immune cells. At the initiation phase of the immune response, ROS can act as a chemoattractant, promoting infiltration of innate immune cells, namely neutrophils. Moreover, it is well established that monocytes, macrophages and neutrophils use ROS to eliminate pathogens or *stressed* cells (Dupré-Crochet et al., 2013). The **adaptive immune system** can also benefit from low levels of ROS during the initiation of the immune response. B and T cell activation through the BCR and TCR, respectively, triggers ROS production. Herein, ROS act as second messengers, amplify the BCR/TCR signal and promote cell proliferation and clonal expansion. In T cells, redox regulation is also involved in the differentiation in distinct T helper cell subsets. In particular, while low ROS levels promote a Th1 and Th17 response, increased ROS lead to a Th2 skewed response (Frossi et al., 2008). However, upon ROS accumulation, the immune response is altered. A more oxidative environment, such as the tumor microenvironment (TME), was shown to induce effector T cell hyporesponsiveness and to promote the accumulation or differentiation of Tregs. These cells seem to be more resistant to ROS and can use these reactive species as means of suppression. Therefore, in the TME, the heightened ROS production and release promotes Treg infiltration and enhances immune suppression (Maj et al., 2017). Likewise, MDSCs, immature myeloid cells present in the tumor microenvironment (TME), exert their suppressive capacity also by producing ROS. This can lead to T cell inhibition, by loss of the TCR ζ chain or, in extreme, to T cell apoptosis (Schmielau and Finn, 2001). Increased levels of ROS also promote the recruitment of pro-inflammatory macrophages and neutrophils. The accumulation of these inflammatory cells results in the inhibition of effector cells, such as, conventional effector T cells, NK cells and IL-17^{+} γδ T cells (Kraaij et al., 2010). At the same time, T and B cell activation is diminished due to a deficient cross-presentation by antigen presenting cells. In particular, increased ROS levels in DCs promote chronic ER stress, which leads to a deficient antigen presentation and poor B and T cell stimulation in the TME (Chen et al., 2016; Cubillos-Ruiz et al., 2015).

The modulation of ROS levels can constitute the answer between the resolution or perpetuation of immune suppression and chronic inflammation. In that line, our group has shown the importance of GSH for T cell responses. Genetic ablation of GSH, and consequent ROS accumulation, impaired the metabolic reprogramming of CD4+ T cells. In the absence of GSH, T cells showed reduced activation of NFAT, mTOR and Myc. The regulation of these signaling pathways by GSH seems to be of great importance for T cells to shift to glycolysis and glutaminolysis, in order to meet the heightened metabolic needs

upon activation. GSH-ablated T cells were unable to clear a viral infection. Additionally, in a model of multiple sclerosis, mice lacking GSH in CD4+ T cells showed reduced disease burden, due to lack of T cell activation (Mak et al., 2017). As previously mentioned, Tregs are more resistant to oxidative stress. This may rely on an increased intracellular GSH pool, when compared to conventional T cells. Mice lacking GSH in Tregs developed severe spontaneous autoimmunity and showed enhanced anti-tumor responses. GSH-deficient Tregs, contrarily to T cells, show increased mTOR pathway activation and skewing towards serine metabolism, with concomitant loss of Foxp3 expression and suppression capacity. Briefly, the antioxidant GSH in Tregs seems crucial not only to control ROS levels, but also to preserve Treg functionality by restricting serine metabolism (Kurniawan et al., 2020).

- **ROS regulation in NK cells**

ROS are implicated in the development of tumors and the progress of infections. NK cells play a prominent role in the resolution of these pathologies and, it is, therefore, of great importance to study the impact of oxidative metabolites on NK cell function and homeostasis.

Seaman and Hellstrand provided the initial evidence of the impact of reactive oxygen intermediates in NK cell homeostasis. ROS derived from monocytes and polymorphonuclear leukocytes suppressed human NK cell cytotoxicity, evaluated by the ability of K-562 target cell killing. NK ihibition was abrogated when monocytes derived from patients with chronic granulomatous disease were used. Those monocytes, due to a mutation causing the inactivity of NOX, do not have the ability to produce ROS and, therefore, were not able to inhibit NK cell cytotoxicity. ROS-dependent NK cell dysfunction was reversed by the addition of catalase (Hellstrand et al., 1994). In the same line, in chronic myelogenous leukemia (CML), malignant cells and stimulated granulocytes were shown to inhibit NK cell function through ROS production This inhibition was triggered by NOX-dependent and independent mechanisms, and could be reverted by the addition of catalase and histamine (Mellqvist et al., 2000). The importance of NOX-derived ROS for NK cell function was further demonstrated in the context of a mouse model of melanoma dissemination. When NOX-2 deficient mice were subjected to intravenous injection of B16-F10 melanoma cells, the number of colonizing metastasis was significantly decreased, when compared to wild-type mice. The reduced number of tumor nodules in the lung is concomitant with a heightened presence of IFN-γ+ NK cells. The authors noted that myeloid derived suppressor cells ($CD11b^+$ $Gr1^+$) were the main source of extracellular ROS in the blood and tumor tissue and would, therefore, be implicated in oxidant-mediated NK dysfunction (Aydin et al., 2017). Later, Kono et al. showed that monocyte- and macrophage-

derived ROS could directly repress T- and NK-mediated cytotoxic activity. *In vitro* activation of monocytes and macrophages by lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA) induced ROS formation and consequent downregulation of expression of CD3- and CD16-associated ζ chain in T and NK cells, respectively. Interestingly, this phenomenon was also observed in T and NK cells from advanced stage melanoma. The inhibition of signal-transducing ζ molecules on T and NK-cells and their reduced cytotoxicity was reversed by the addition of catalase. Additionally, it was demonstrated that co-culture of monocytes with NK cells and respective target cells, results in decreased NK cell cytotoxicity mediated by a reduced production of TNF-α and IFN-γ. This inhibition of cytokine production in human NK cells was reverted by the addition of GSH (Kono et al., 1996). Notably, when comparing the apoptotic rate of human T and NK cells, it was shown that human NK cells are much more susceptible to ROS-mediated induced cell death. Hansson et al. showed that a two to five times higher concentration of hydrogen peroxide is necessary to induce T cell apoptosis, when compared to NK cells (Hansson et al., 1996). Oxidation-induced NK cell death is driven by activation of poly (ADP-ribose) polymerase. This culminates in the nuclear translocation of the mitochondrial apoptosis-inducing factor (AIF), consequent DNA fragmentation and cell death (Thorén et al., 2006). Nakamura et al. hypothesize that detrimental effects of ROS on NK cell function may also arise from these ROS creating an anionic-charged surface on the effector cells. As target cells are anionic, the presence of ROS induces the same polarity in effector cells and inhibits cell-to-cell adhesion in vitro and in vivo (Nakamura and Matsunaga, 1998).

It was demonstrated that the redox balance could affect NK cell function through nuclear factor kB (NF-kB) activity. Co-culture of sensitive target cells, such as K-562, with human NK cells, leads to the engagement of NF-kB signaling pathway, translocation of NF-kB to the nucleus and DNA binding. Pretreatment of NK cells with an antioxidant results in decreased NF-kB activity and inhibition of NK spontaneous cytotoxicity. Importantly, these data show that a balanced redox status is crucial for human NK cell function, since ROS mediate transcriptional and post-transcriptional events related to NK cell cytotoxicity (Valle Blázquez et al., 1997). On the contrary, culture of the human NK cell line NK3.3 in medium deprived of thiol-related compounds, such as L-cystine or GSH, results in reduced proliferation, measured by ^{3}H-thymidine incorporation. This was associated with cell cycle arrest at the transition of G1 to S phases. The addition of reducing mediators, like N-acetyl-cysteine (NAC), GSH or β-ME, restored the expression and phosphorylation of retinoblastoma (RB), a cyclin-dependent kinase substrate, and DNA synthesis, preventing cell cycle arrest (Yamauchi and Bloom, 1997).

Interestingly, NK cell subsets seem to distinctly tolerate oxidative stress. Two groups have shown that human regulatory $CD56^{bright}$ NK cells seem to be more resistant to oxidative agents. This subset, in contrast to the $CD56^{dim}$ counterpart, remains functional and viable after exposure to oxygen radicals, either produced by phagocytes or exogenously added to the culture. $CD56^{bright}$ NK cells bear an increased capacity of neutralizing exogenous H_2O_2, partially due to an increased expression of thiols on their surface (Harlin et al., 2007; Thorén et al., 2007). Harlin et al. hypothesize that the increased resistance to oxidative stress may be the underlying factor for the accumulation of $CD56^{bright}$ NK cells in sites of chronic inflammation and tumors. In fact, in gastric and esophageal cancer, there is an inverse correlation between $CD56^{dim}$ NK cell infiltration and H_2O_2 levels. Moreover, this subset decreases with the progression of the disease, probably due to a heightened susceptibility to H_2O_2-mediated apoptosis (Harlin et al., 2007). Recently, it was shown that activated human NK cells were more resistant to H_2O_2-induced cell death than their resting counterparts. This is due to an upregulation of antioxidant pathways, such as thioredoxin, peroxiredoxin and SOD (Mimura et al., 2017). Furthermore, a different study has demonstrated that cytokine stimulation of NK cells results in increased expression of PRDX1 and PRDX1-related enzymes, namely thioredoxin, thioredoxin reductase, glutathione peroxidase, glutathione reductase and catalase. The use of adenanthin, a PRDX1 inhibitor, reduces NK cytotoxic activity, degranulation, cytokine production and impairs ADCC, while changing the expression of co-activating and inhibitory receptors. NAC treatment rescued the adenanthin-mediated inhibitory effects (Siernicka et al., 2015). IL-15-activated NK cells upregulate the expression of thioredoxin. Upon exposure to H_2O_2, or other reactive oxygen metabolites, these cells seem to sustain a higher degranulation and IFN-γ production, concomitant with a higher mTOR activation. Inhibition of the thioredoxin pathway prevented the accumulation of thiols on the NK cell surface, and reversed IL-15-mediated ROS resistance (Yang et al., 2020). In the same line, peroxiredoxin 1 (Prdx1)-deficient mice showed decreased NK cell presence and functionality, coincident with an increased susceptibility for tumor development (Neumann et al., 2003).

The maintenance of a balanced NK redox status also seems to be important in the context of viral or bacterial infections. It was shown that, in case of viral infection, depletion of MDSCs resulted in enhanced NK proliferation and activation. For both poxvirus and adenovirus infections, catalase was the only compound able to abrogate MDSC-mediated NK cell suppression (Fortin et al., 2012; Zhu et al., 2012). On the other hand, myeloid cells can also enhance NK cell function upon viral challenge. IL-27 derived from myeloid cells, in the initial stages of the infection, promotes NK cell effector response, mediated by Maf-Nrf2 signaling pathway. Interestingly, IL-27-deficient mice are not able to induce the expression

of GCLC in NK cells. These cells also show an impaired effector function, in the absence of IL-27 or its cognate receptor. Hence, a sustained redox balance, mediated by IL27 and Nrf2, seems to be essential for maintaining NK anti-viral capacity upon influenza infection (Kumar et al., 2019). Remarkably, in the context of HIV infections, a depleted pool of GSH correlates with an increased susceptibility for *M. tuberculosis* infections in patients (Venketaraman et al., 2006). Treatment of human NK cells with NAC results in upregulation of the activating receptors NKp30 and NKp44, potentiating cell-targeted cytotoxicity against K-562. Furthermore, when NK cells are co-cultured with monocytes and *M. tuberculosis*, the inhibition of bacterial growth is increased by the presence of NAC, (Millman et al., 2008). HIV-seropositive patients have an altered triglyceride metabolism, with increased ox-LDL levels, which could contribute to NK dysfunction (Viora et al., 2001). Notably, other studies of oxidative stress in chronic inflammation, such as in uremia or atherosclerosis, reported NK cell dysfunction or death (Peraldi et al., 2009; Peraldi et al., 2013).

Given the cumulative evidence for the importance of redox balance in NK cell homeostasis, it is crucial to dissect the mechanisms of antioxidant regulation that contribute to NK cell function. This is particularly important in viral infections or tumors, where both NK cells and ROS have a significant impact on immunity.

II. Scope and Aims

Reactive oxygen species (ROS) are chemically reactive compounds formed due to an incomplete reduction of oxygen. ROS include the superoxide anion (O_2•), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•). The main source of intracellular ROS are the mitochondria. During oxidative phosphorylation, leakage of electrons from the electron transport chain can result in ROS formation. The compartmentalization and chemical properties of the distinct reactive species ensure an optimal balance of reduction/oxidation (redox) reactions, contributing to cellular homeostasis. ROS can act as critical secondary messengers in cell signaling, promoting proliferation, differentiation and cell survival. However, the accumulation of ROS can be detrimental for cell fate by directly damaging nucleic acids, proteins and lipids and induce cell death. This paradox is the basis for the tight regulation between ROS-producing pathways and detoxifying agents, antioxidants (Gorrini et al., 2013). The accumulation of ROS leads to oxidative stress and it has been implicated in tumorigenesis, infections, neurodegeneration and chronic inflammatory diseases, such as atherosclerosis and obesity (Andersen, 2004; Manna and Jain, 2015; Reczek and Chandel, 2017; Schwarz, 1996).

Natural killer (NK) cells are innate lymphocytes, crucial for the early control of pathogen infections and malignancies. NK cells directly kill infected or transformed cells, through the release of cytotoxic granules or the engagement of death-inducing receptors. Furthermore, they have the ability to produce a wide range of cytokines, namely interferon-γ (IFN- γ), tumor necrosis factor (TNF) or CC-chemokine ligands, such as CCL4 and CCL5. Therefore, NK cells can shape the adaptive immune response, contributing to the resolution or maintenance of the inflammatory process (Martinet and Smyth, 2015). The role of ROS in diseases where NK cells are involved provides insights for the importance of redox balance in these cells. In the context of different malignancies, such as leukemia and melanoma, it was shown that extrinsic ROS are able to interfere with NK cell cytotoxic capacity (Aydin et al., 2017; Mellqvist et al., 2000). Importantly, NK cell function was restored by the addition of the antioxidant catalase, a H_2O_2 scavenger (Hellstrand et al., 1994). In viral infections, the oxidative environment and the depletion of the antioxidant glutathione (GSH) seem to interfere with NK cell function. In HIV patients, the low levels of GSH correlate with opportunistic bacterial infections with *Mycobacterium tuberculosis*. Notably, the presence of the antioxidant N-acetyl cysteine (NAC) restores NK cells cytotoxic function, cytokine production and proliferative capacity (Millman et al., 2008; Viora et al., 2001). The importance of a robust antioxidant capacity in NK cells was further elucidated by the dysfunctional state in mouse models of thioredoxin or peroxiredoxin depletion (Neumann et al., 2003; Yang et al., 2020). However, the role of glutathione has never been deciphered in NK cells, despite its importance in regulating NK cell function in an oxidative environment.

Our group has recently shown that GSH is a crucial gatekeeper of T cell function. In conventional CD4+ T cells, the absence of GSH and subsequent increased levels of ROS, inhibited the signaling pathways regulated by NFAT, mTOR and cMyc. This resulted in an impaired metabolic switch, from oxidative phosphorylation to glycolysis and glutaminolysis, rendering T cells unable to sustain their energetic demands upon activation. GSH-deficient T cells showed an intrinsic inability to control viral infections or to mount an inflammatory response in a model of multiple sclerosis (Mak et al., 2017). On the other hand, mice with a specific deletion of GSH in regulatory T cells, surprisingly, showed a normal abundance of these cells. Despite the fact that GSH does not seem to participate in the regulation of Treg differentiation, their function was impaired and mice developed spontaneous autoimmunity. Contrary to conventional T cells, GSH-deleted Tregs have an increased activation of the mTOR pathway, resulting in boosted serine metabolism (Kurniawan et al., 2020). The subset-specific function of GSH in T cells highlights the importance of deciphering the role of this antioxidant in other immune cell types.

Thus, we propose to decipher the function of GSH in NK cells in the context of viral infection and tumor dvelopment. Therfore, the aims of the project are as follows:

I. Dissect the functions of GSH in homeostasis of natural killer cells.

II. Study the importance of GSH in NK cells metabolic fitness, *in vitro*.

III. Investigate the contribution of GSH for NK cell function with *in vivo* models of viral infection and cancer.

III. Materials and Methods

Mice

Gclc$^{fl/fl}$ mice were generated by Chen et al. as previously published (Chen et al., 2007). Summarily, a neomycin resistance gene (neo^{R}) flanked by *loxP* sites was cloned in intron 3 of the *Gclc* gene, in addition to a *loxP* site clone in intron 6 of the same gene. Gclc$^{fl/fl}$ mice were crossed to *Ncr1*-cre-expressing mice, kindly given by Eric Vivier. Ncr1-Cre-mice were generated by Narni-Mancinelli et al, as described (Narni-Mancinelli et al., 2011). This knock-in mouse line was created by homologous recombination of improved recombinase (icre) at the 3' end of the gene Nkp46 (Ncr1). *Gclc$^{fl/fl}$* mice were crossed with *Ncr1*$^{tm1.1(icre)Viv}$ to obtain a mouse progeny with a NK-specific deletion of Gclc (Gclc$^{fl/fl}$ Ncr1-Cre+). *Gclc $^{fl/fl}$ Ncr1-Cre+ Rag-1$^{-/-}$* mice were obtained by crossing *Gclc $^{fl/fl}$ Ncr1-Cre+* with *Rag-1$^{-/-}$* mice. Rag-$^{1-/-}$ and C57BL/6 mice were purchased from the Jackson Laboratory and Charles River France, respectively. All mice were bred under specific pathogen free conditions at the animal facilities of Luxembourg Institute of Health or University of Luxembourg and kept in a C57BL/6J (B6) background. Sex and age matched (6 to 12 weeks old) mice were used for experiments, unless stated otherwise. Animal experiments were carried out with the authorization of the Veterinary Services Administration (ASV) of the Luxembourgish Ministry of Agriculture, Viticulture and Rural Development and followed the ethical guidelines of the Animal Welfare Structure of Luxembourg Institute of Health.

Tumor models

The cell lines B16F10 and MC-38 were provided by P. Lang and E. Letellier, respectively. B16F10 were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FCS and 1% penicillin/streptomycin. For the culture of MC-38, the same medium was supplemented with 2mM glutamine, 0,1 mM non-essential amino acids and 1mM sodium pyruvate.

For the evaluation of the subcutaneous (s.c.) tumor growth, $2x10^5$ cells B16-F10 or MC-38 cells diluted in PBS were injected into the left flank of the mice, that was shaved prior to tumor inoculation. The weight of the mice was measured daily and the tumor volume (Tv) was calculated using the following formula: $Tv=(length \times diameter^2)/2$. The humane end point was established at 30 days post-injection or tumor volume exceeding 1,2 cm^3. Tumor mass was weighted after dissection.

For the tumor dissemination model, mice were injected $1x10^5$ B16F10 melanoma cells, intravenously, through the tail vein. At day 15, the lung tumor burden was evaluated through the count of macroscopic tumor nodules. During the experiment, mice were scored daily, according to the ethical guidelines.

Tumor Infiltrating Lymphocytes isolation

Tumor infiltrating lymphocytes (TILs) were isolated from the lungs of tumor-bearing mice after perfusion, to exclude potential contamination of the samples with blood circulating cells. The lungs were excised and cut in small pieces with a razor. Thereafter, the Mouse Tumor Dissociation Kit (Miltenyi Biotec) was used according to the manufacturer's instructions. Briefly, the lungs were placed in gentle MACS C Tubes in RPMI medium (not supplemented) with the enzymes A, D and R. The chemical digestion of the samples was enhanced by mechanical disruption with the GentleMACS™ Octo Dissociator with Heaters (program 37C_m_TDK_1). The samples were filtered by passage through a 70µm strainer and washed with RPMI. For an improved selection of CD45+ lymphocytes, the isolation was followed by labelling with CD45 (TIL) microbeads (Miltenyi Biotec) and sorting with autoMACS® Pro Separator (Miltenyi Biotec), according to the manufacturer's protocol. TILs were analyzed by flow cytometry. For intracellular detection of cytokines, cells were treated for 4h with phorbol 12-myristate 13-acetate (PMA), calcium ionophore (Ionomycin) and GolgiPlug™and stained as stated below.

Lymphocytic choriomeningitis virus (LCMV) infection

The viral strain LCMV-WE was propagated in L929 cells and the titers were measured using a plaque-forming assay, as previously described (Welsh and Seedhom, 2008). Briefly, the fibroblast cell line L929 was infected with LCMV-WE (MOI=0,01) and kept in culture conditions that favor virus production. After 48h, the supernatant was harvested and the viral stocks were kept at -80°C. In order to determine the virus titers, $1.6x10^5$ MC57 cells/well were seeded on a 24 well plate and incubated with the viral supernatants (1/10 serial diluted) at 37°C. After 3h, this mixture was overlayed with 1% methylcellulose-containing IMDM. 48h later, the cells were fixed in PBS with 4% formaldehyde (30 minutes), permeabilized (1% Triton 100X in PBS) for 20 minutes and incubated for 1 hour with PBS with 10% FCS to avoid non-specific binding. Subsequently, wells were stained with VL-4 Rat anti-LCMV monoclonal antibody, peroxidase anti-rat antibody and o-phenlyenediamine dihydrochloride dissolved in 50 mM Na_2HPO_4 and 25 mM citric acid (all Sigma-Adlrich). A colorimetric reaction allows the counting of plaque-forming units (PFU) (Lang et al., 2013).

Mice were injected intravenously with $2x10^6$ PFU LCMV-WE (originally obtained from F. Lehmann-Grube, Heinrich Pette Institute, Hamburg, Germany, provided by P. Lang). Following 8 days of infection, LCMV-specific T cells were identified in the spleen and liver of infected mice by flow cytometry. For surface staining, gp33-H-$2D^b$ and np396-H-$2D^{b\ p}$ tetramers (NIH) were incubated at 37°C for 15 minutes, followed by a surface staining of

the remaining cell and activation markers (4°C, 30 minutes). For the intracellular staining of cytokines, cell suspensions of splenocytes and liver were incubated for 1 hour with LCMV-specfic peptides (gp33 or np396) at 37°C, followed by the addition of GolgiPlug™ and additional 5 hours of incubation. The surface staining was followed by fixation with 2% formaldehyde-containing PBS and permeabilization with 0,1% saponin and staining of intracellular antibodies (30 minutes, 4°C).

To determine the viral titers in the organs of infected mice, the plaque forming assay unit assay was performed as described above.

The biochemical analyzer (SPoTCHEM EZ SP-4430 18304, Akray) was used to determine liver damage, by measurement of liver enzymes in the serum of diseased animals. Alanine aminotransferase (ALT or SGPT), aspartate aminotransferase (AST or SGOT) and lactate dehydrogenase (LDH) were evaluated by SPoTCHEM Multiliver-1, a multi-type reagent (Akray).

Preparation of single cell suspensions from murine tissues

For the isolation of peripheral blood cells, blood was withdrawn from the retro-orbital veins of anesthetized mice. The blood cells were subject to red blood cell lysis (Erylyse Puffer), washed twice with medium and resuspended in FACS buffer for futher staining of relevant markers.
To obtain a single cell suspension from the bone marrow, femur of steady-state, non challenged mice were flushed with PBS and the collected cells were treated with red blood cell lysis buffer, washed medium and resuspended in FACS buffer.
Liver and lung were harvested from steady-state mice, cut in small pieces and incubated at 37°C for 20 minutes, in HBSS medium with DNase (20µg/ml) and Liberase (1,7µg/ml). Subsequently, medium supplemented with FBS was used to stop the digestion and organ pieces were smashed with a syringe plunger through a 70µm strainer. After centrifugation, red blood cells were lysed and the remaining cells were washed two times with medium and resuspended in FACS buffer for further analysis.
Spleens were collected in FACS buffer, mashed with a syringe plunger and passed through a 70µm strainer. After red blood cell lysis, splenocytes were washed twice with medium and resuspended in FACS buffer for staining with fluorescent antibodies.

Murine NK cell preparation and culture

Spleens were collected in PBS containing 0,5% BSA and 0,4% EDTA (0,5M, pH=8), mashed with a syringe plunger and passed through a 70µm strainer. Cell suspensions were

centrifuged and labeled with NK Cell Isolation Kit (Miltenyi Biotec), according to the manufacturer's instructions. In order to increase the purity of the isolation, CD3-Biotin beads (Milteny, 2µl/mouse) were added to the solution. Magnetic sorting was performed with autoMACS® Pro Separator (Miltenyi Biotec). NK cells were counted by CASY® cell counter (OMNI Life Science and seeded in RPMI (10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin, 2mM L-Glutamine, 1% Sodium Pyruvate, 1% Glutamax and 55 µM β-mercaptoethanol). Recombinant mouse (rm) IL-15 was added at 50ng/ml, and NK cells were incubated at 37°C for 24 to 72 hours, according to the assay performed. In experiments with NK cells isolated from wild type (C57BL/6) mice, l-buthionine sulfoximine (BSO, 0,5mM) was added to the medium alongside with stimulation.

Murine NK cell culture in nutrient-deprived medium

NK cells were isolated from spleens of C57BL/6 mice, as described above, and seeded in SILAC RPMI Flex Media, supplemented with 10%FBS, 1% Penicillin-Streptomycin, β-mercaptoethanol (55 µM), arginine monohydrochloride (1,15mM) and L-lysinine monohydrochloride (0,22mM). As control, the same medium was supplemented with D-glucose (11mM) and glutamine (2mM) to mimic the standard cell culture conditions. NK cells were stimulated with rm IL-12 (1 ng/ml), rm IL-15 (50ng/ml) and rm-IL-18 (1ng/ml) and incubated at 37°C for 72h.

Cell labeling and *in vitro* proliferation analysis

For the flow cytometry analysis, NK cells were labelled with CellTrace™ CFSE Kit (2,5 µM) for 7 min at 37°C and washed three times with complete media to eliminate the remaining dye. Cells were kept in culture with rmIL-15 (50ng/ml) for 72h and stained as described bellow. Proliferation was measured as percentage of dividing cells, as per lower fluorescence of CellTrace™ CFSE.

In vitro killing assay

The lymphoma cell line YAC-1 (kindly provided by J. Zimmer) was maintained in culture with RPMI supplemented with 10% FCS, 1% Penicillin-Streptomycin, 1% L-Glutamine and 55 µM β-mercaptoethanol.

NK cells were activated *in vitro* for 72h with rmIL-15 (50ng/ml), counted and resuspended at $3x10^5$ cells/ml. YAC-1 cells were labeled with 2,5 µM CellTrace™ Violet Cell Proliferation Kit for 3 minutes, at 37°C, followed by three washing steps in complete medium. Finally, they were resuspended at $3x10^5$ cells/ml. Effector (E) NK cells and target (T) YAC-1 cells were seeded in a 96 well plate at the indicated E:T ratios, briefly centrifuged and kept at

37°C for 18 hours. Cells were washed twice in Annexin Buffer (500 ml of PBS with 3,65g NaCl, 1,19g HEPES, 0,367g CaCl2, pH=7,4) and stained with Annexin V, Zombie NIR™ Fixable Viability Kit and anti-NK1.1 antibody for 20 minutes at room temperature.

Flow cytometry-based cellular energy profiling (SCENITH)

After 72h of stimulation with rm IL-15, NK cells were washed with supplemented RPMI medium and left untreated (control) or incubated with oligomycin (1µM) and/or 2-Deoxy-D-glucose (2-DG, 100mM) for 45 minutes. Upon incubation with metabolic inhibitors, puromycin (final concentration of 10µg/ml) was added to the culture for 45 minutes. Harringtonine (2 µg/ml) was used as a negative control for inhibition of protein translation and added 15 minutes prior to the addition of puromycin. After the treatment with puromycin, cells were washed in cold FACS Buffer and stained for 30 minutes, at 4°C, with a combination of a cell viability marker and anti-NK1.1 antibody. After washing, cells were fixed and permeabilized with the FOXP3 fixation and permeabilization buffer (Thermofisher, eBioscience) according to manufacturer instructions. Cells were stained with anti-puromycin monoclonal antibody diluted in permeabilization buffer and incubated for 30 minutes, at 4°C. After wash, cells were immediately acquired by flow cytometry with NovoCyte Quanteon (Agilent). The geometric mean fluorescence intensity (gMFI) was calculated and used to derive the metabolic parameters, as described (Argüello et al., 2020). In brief, the following formulas were used:

Co = anti-puromcycin (gMFI) upon control treatment (medium only)

DG= anti-puromcycin (gMFI) upon 2-DG treatment

O= anti-puromcycin (gMFI) upon oligomycin treatment

DGO= anti-puromcycin (gMFI) upon oligomycin and 2-DG treatment

Fatty acid and amino acid oxidation capacity=100-(100(Co-DG)/(Co-DGO))

Glycolytic capacity=100-(100(Co-O)/(Co-DGO))

Cell Cycle analysis

NK cells were isolated from *Gclc*$^{fl/fl}$ and *Gclc*$^{fl/fl}$ Ncr1-Cre+ mice and stimulated with IL-15 (50ng/ml) for 72h. Cultured cells were washed with FACS Buffer and stained for surface markers and viability for 30 minutes, at 4°C. Afterwards, cells were immediately fixed with cold ethanol (70%) at -20°C for 2h. After washing, DNA was stained with Hoechst (2 µg/ml) was performed in FACS buffer supplemented with RNase (100µg/ml), at room temperature (RT) for 30 minutes, prior to flow cytometric analysis.

Flow cytometry analysis

For the detection of surface markers expression, NK cells were stained with the fluorochrome-conjugated monoclonal antibodies in FACS Buffer (PBS with 1% FCS, 5mM EDTA, pH=8), for 30 minutes, in the dark, at 4°C. For the analysis of the expression of intracellular nuclear proteins (e.g. Ki67, pSTAT5), cells were fixed and permeabilized with the eBioscience™ FoxP3/Transcription Factor Kit (eBioscience™), according to the manufacturer's instructions. For the detection of intracellular cytokines, the BD Cytofix/Cytoperm™ Fixation/Permeablization Kit (BD Biosciences) was used, following manufacturer's instructions. Phospho (p) mTOR and pS6 were stained intracellularly for 30 minutes, at 4°C, in saponin buffer (0,1% saponin in FACS Buffer) after cell surface staining and fixation with 2% formaldehyde (10 minutes, RT).

Intracellular ROS levels were determined by the incubation of NK cells with dichlorofluorescein diacetate (H_2DCFDA) or MitoSOX™ Red Mitochondrial Superoxide Indicator at 37°C, for 30 minutes, in RPMI medium. Mitotracker™ Deep Red FM (0,1µM) and Mitotracker™ Green FM (10nM) were used to evaluate mitochondrial activity and size, respectively. Cells were incubated at 37°C, for 30 minutes, in RPMI medium. In order to assess intracellular thiols levels, cells were incubated at 37°C, for 10 minutes, in RPMI medium with monobromobimane (MBB). Antibodies for extracellular antigens and viability die (Zombie NIR or DAPI) were added simultaneously. Cells were washed twice with cold PBS prior to acquisition.

Cells were analysed on a BD-LSRFortessa™ (BD Biosciences) or with NovoCyte Quanteon (Agilent) and dead cells were excluded by Live/Dead cell staining. The results were analyzed with FlowJo v10.6.1.

The list of the different antibodies and respective dilutions can be found in Table2.

Glutathione and ATP analysis

Glutathione (GSH) and ATP intracellular levels were quantified with the luminescence kits GSH-Glo™ and CellTiter-Glo® Luminescent Cell Viability Assay (Promega), respectively. In brief, for the measurement of GSH content, NK cells were washed twice in cold PBS and resuspended at the same concentration. After seeding in a 96 or 384 well plate, cells were incubated with GSH-Glo™ Reaction reagent at room temperature for 30 minutes, followed by an incubation of 15 minutes with Luciferin Detection Reagent, as per manufacturer's instructions. For the GSH/GSSG ratio, the Promega GSH/GSSG Glo™ Assay was used following the manufacturers' protocol. GSH/GSSG ratio was calculated according to the

formula: GSH/GSSG=(GSH r.l.u – GSSG r.l.u)/(GSSG r.l.u/2), where r.lu. stands for relative luminescence units and takes into account the fact that two moles of GSH are generated per 1 mole of GSSG.

ATP was measured with the Cell Titer-Glo® assay (Promega). Cells were washed, counted, seeded and incubated for 10 minutes at room temperature with the reagent, as per manufacturer's intructions. The luminescence was measured with Mithras LB 940 (Berthold Technologies).

Extracellular flux analysis

Seahorse extracellular flux analysis was performed using an XFe96 Extracellular Flux Analyzer (Agilent). Seahorse XFe96 cell culture plate (Agilent technologies; 101085-004) was pre-coated overnight with Corning™ Cell-Tak and Tissue Adhesive containing sodium bicarbonate (0,1M). IL-15 activated NK cells, treated with BSO, were washed and seeded at a density of 250.000 cells per well on a. XF Seahorse DMEM medium (Agilent technologies) was used, supplemented with sodium pyruvate (1mM), glutamine (2mM), and glucose (25mM). Oxygen consumption rate (OCR) was measured according to manufacturer's protocol of XF Cell Mito Stress Test (Agilent). Three sequential injections of oligomycin A (1µM), Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 3 µM) and antimycin A/rotenone (1µM) (Sigma-Aldrich) were performed and three measurements were taken after each treatment. Basal OCR represents the raw OCR values just before oligomycin injection. Maximal OCR was calculated from raw OCR values obtained from the three measurements after FCCP injection.

Cytokine Quantification by enzyme-linked immunosorbent assay (ELISA):

Cytokine concentrations of IFN-γ in cell culture supernatants were quantified using the IFN gamma Mouse Uncoated ELISA Kit (Invitrogen™), following the manufacturer's protocol. In brief, the plates were coated with a target specific antibody overnight. The plates were washed with Wash Buffer, followed by a 1h incubation with reagent diluent. After washing, the samples and the respective standards were added to the plate and incubated for 2 hours. The washing was repeated and the IFN-γ detection antibody was added for 2 hours. After washing, a streptavidin molecule coupled to a horseradish peroxidase (HPR) enzyme was added for 20-30 minutes. The plate was washed and a substrate solution containing hydrogen peroxide was added. The reaction with the HPR was stopped and optical density was determined by using a versa max microplate reader (Molecular Devices) and SoftMax

Pro7.1 software set to 450nm. Wavelengths were corrected by subtraction of measurement at 570nm.

Statistical analysis

Data are represented as the mean ±SD (unless stated otherwise), where n represents the number of animals in the experiments. p values ≤0,05 (indicated with *) were considered significant and were determined by the appropriate statistical tests stated in the figure legend. Prism 8.0.1 (GraphPad) were used for graphical illustration and statistical analysis.

Table 2 – Reagents and Resources

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies (anti-mouse)		
NK1.1-APC (1:200)	Biolegend	# 108710
NK1.1-PE (1:200)	Biolegend	# 108708
TER119-APC-eFluor 780 (1:200)	Thermo Fisher Scientific	#15371660
CD19-APC-eFluor 780 (1:200)	Thermo Fisher Scientific	#15331430
CD11b-APC-eFluor 780 (1:200)	Thermo Fisher Scientific	#15391420
CD5-APC-eFluor 780 (1:200)	Thermo Fisher Scientific	#15539506
Ly6G/Ly6C-APC-eFluor 780 (1:200)	Thermo Fisher Scientific	#15391660
CD3-APC-eFluor 780 (1:200)	Thermo Fisher Scientific	#15311420
CD122 (IL-2Rβ), PE (1:200)	Biolegend	#105906
NKp46 (CD335). Alexa Fluor® 647 (1:200)	Biolegend	#137628
CD49b-PE/Cyanine7 (1:200)	Biolegend	#103518
CD11b, Pacific Blue (1:200)	Biolegend	#101224
CD27-FITC (1:200)	Biolegend	#124207
CD3, APC/Cyanine7, CD3ε (1:200)	Biolegend	#100330
CD4-BV785 Clone GK1.5 (1:200)	Biolegend	#100453
CD8α-BUV395 (1:200)	BD Biosciences	#563786
CD19-PE (1:200)	Biolegend	#129708
CD44-PE-Cyanine 7 (1:200)	Thermo Fisher Scientific	#15370920
CD62L-Pacific Blue (1:200)	Biolegend	#104424
CD336 (Tim-3) – Brilliant Violet 785	Biolegend	#119725
TIGIT-PE (1:200)	Biolegend	#142104
PD-1 (CD279)-Brilliant Violet 605 (1:200)	Biolegend	#135220

IFN-γ-APC (1:200)	Biolegend	#505810
CD107a (LAMP1) – PE (1:200)	Biolegend	#15248639
Granzyme B - APC (1:200)	Biolegend	#372203
pS6 ribosomal protein (S235/S236)-APC (1:200)	Thermo Fisher Scientific	#15528216
pmTOR (S2448)-eFluor™ 450 (1:200)	Thermo Fisher Scientific	#15549836
STAT5 (phospho Y694) FITC (1:200)	abcam	#ab278791
Ki67 PE (1:200)	Biolegend	#151209
Annexin V PE (1:100)	Biolegend	#640908
Anti-Puromycin, Alexa Fluor® 488	Merck, Sigma Aldrich	#MABE343-AF488
Chemicals, Peptides, and Recombinant Proteins		
DMEM 4.5 g/L Glucose with L-Glutamine	Westburg	#BE12-604F
RPMI 1640 (without L-Glutamine)	Westburg	#BE12-167F
Gibco™ SILAC RPMI 1640 Flex Media	Thermo Fisher Scientific	#15347143
IMDM with HEPES and L-Glutamine	Westburg	#BE12-722F
Hanks' BSS, with Phenol Red without Ca, Mg	Westburg	#BE10-543F
FBS Superior Lot: 0193F	Biochrom GmbH	#S0615
Penicillin-Streptomycin (10,000 U/mL)	Gibco	#11548876
L-Glutamine	Westburg	#BE17-605E
2-Mercaptoethanol	Sigma Aldrich Merck	#M3148-25ML
Gibco™GlutaMAX™ Supplement	Thermo Fisher Scientific	#13462629
Gibco™ Sodium Pyruvate	Thermo Fisher Scientific	#12539059
Recombinant Murine IL-15	Peprotech	#210-15
Mouse IL-12, research grade	Miltenyi Biotech	#130-096-707
Recombinant Mouse IL-18/IL-1F4 Protein	R&D Systems	#9139-IL-010
PBS (1X) without Ca++, Mg++, 500ml	Westburg	#BE17-516F
Bovine Serum Albumin (BSA)	Sigma-Aldrich	#A9647-500G
Formaldehyde	Sigma-Aldrich	#252549-1L
Saponin	Sigma-Aldrich	#S4521-25G
Monobromobimane (MBB) (1:1000)	Thermo Fisher Scientific	#M1378
H_2DCFDA (1:2000)	Thermo Fisher Scientific	#D399
MitoSOX™ Red Mitochondrial Superoxide Indicator (1:5000)	Thermo Fisher Scientific	#M36008
MitoTracker™ Deep Red FM (0,1µM)	Thermo Fisher Scientific	#M22426
MitoTracker Green FM (10nM)	Thermo Fisher Scientific	#M7514
DAPI (1:2000)	Thermo Fisher Scientific	#D1306
Zombie NIR™ Fixable Viability Kit	Biolegend	#423106
Zombie Green™ Fixable Viability Kit	Biolegend	#423112
7-AAD	Thermo Fisher Scientific	#A1310

Hoechst 33342 solution (20mM)	Thermo Fisher Scientific	#62249
CellTrace™ CFSE Cell Proliferation Kit	Thermo Fisher Scientific	#C34554
CellTrace™ Violet Cell Proliferation Kit	Thermo Fisher Scientific	#C34557
Buthionine Sulfoximine, Cayman chemical	Sanbio.	#14484-250
L-Arginine monohydrochloride	Sigma-Aldrich	#A5131-10G
L-Lysine monohydrochloride	Sigma-Aldrich	#L5626-100g
Oligomycin A	Sigma-Aldrich	#75351-5MG
FCCP	Sigma-Aldrich	#C2920-10MG
Antimycin A	Sigma-Aldrich	#A8674-25MG
Rotenone	Sigma-Aldrich	#R8875-1G
2-Deoxy-D-glucose (2-DG)	Sigma-Aldrich	# D8375-5G
Puromycin	SAS INVIVOGEN	#ant-pr-1
Harringtonine	Abcam	# ab141941
GolgiPlug™ (Protein Transport Inhibitor)	BD Biosciences	#555029
Corning™ Cell-Tak Cell and Tissue Adhesive	Thermo Fisher Scientific	#10317081
Seahorse XF DMEM medium	Agilent Technologies	#103575-100
RNase A	Thermo Fisher Scientific	#R1253
DNAse I, grade II	Sigma-Aldrich	#10104159001
Liberase™ TM Research Grade	Sigma-Aldrich	#5401127001
Erylyse-Puffer pH 7,2 - 7,4	Morphisto GmbH	#1297202500
Critical Commercial Assays and Kits		
NK cell isolation kit, mouse	Miltenyi Biotec	#130-115-818
CD3-Biotin, mouse	Miltenyi Biotec	#130-123-861
Tumor Dissociation Kit, mouse	Miltenyi Biotec	#130-096-730
CD45 (TIL) MicroBeads, mouse	Miltenyi Biotec	#130-110-618
Foxp3/Transcription Factor Staining Buffer Set	Thermo Fisher Scientific	#15151976
BD Cytofix/Cytoperm Fixation/Permeabilization kit	BD Biosciences	#554714
GSH-Glo™ Assay	Promega	# V6911
GSH/GSSG-Glo™ Assay	Promega	#V6612
CellTiter-Glo®	Promega	#G7571
Seahorse XFe96 Fluxpak	Agilent Technologies	#102416-100
SPOTCHEM Multiparametric Test Liver-1	Menarini Benelux	# 3392
Invitrogen™ IFN gamma Mouse Uncoated ELISA Kit	Invitrogen	# 15501107
Experimental Models: Organisms/Strains		
Gclc$^{fl/fl}$: B6	In house, Chen et al., 2007	N/A
Ncr1$^{tm1.1(icre)Viv}$	E. Vivier, Mancinelli, 2011	N/A
Rag1$^{-/-}$: B6	The Jackson laboratory	#002216
Gclc$^{fl/fl}$ *Rag1*$^{-/-}$: B6	-	N/A
C57BL/6J	Charles River, France	#632C57BL/6J

B16-F10	P. Lang, ATCC	#CRL-6475
MC-38	E. Letellier, Kerafast	#ENH204-FP
YAC-1	J. Zimmer, ATCC	#TIB-160
L-929	P. Lang	
Lymphocytic choriomeningitis virus (LCMV)-WE strain	P. Lang	
Software and Algorithms		
FlowJo Software	Tree Star	
Graphpad Prism	GraphPad Software, Inc	
Wave Software	Agilent	
Biorender	https://biorender.com/	

IV. Experimental Data

1. Glutathione is important for NK cell activation

Redox regulation is crucial for cell survival and function (Gorrini et al., 2013). Balanced ROS levels depend on the efficient scavenging by antioxidants, namely GSH (Meister, 1983). GSH is an essential metabolic and functional checkpoint of effector T cells and regulatory T cells (Tregs) (Kurniawan et al., 2020; Mak et al., 2017). To date, it is known that extrinsic sources of ROS are detrimental for natural killer (NK) cell function (Mellqvist et al., 2000) and that distinct antioxidant pathways are upregulated upon activation, including GSH (Siernicka et al., 2015). However, the importance of GSH for NK cell function has not been dissected in detail.

IL-15 is a key cytokine for NK cell activation (Cooper et al., 2002; Fehniger et al., 2001). Henceforth, in order to test the importance of GSH regulation for IL-15-dependent NK cell activation, NK cells were isolated by magnetic cell sorting, and activated with IL-15 for 24h or left unstimulated. As anticipated, activation of murine NK cells with IL-15 resulted in significant upregulation of NK cell activating markers, NCR1/Nkp46, NKG2D and DNAM-1 (Figure 6 A-C). Remarkably, IL-15-mediated NK cell activation resulted in a downregulation of cytosolic and mitochondrial ROS (Figure 6 D, E), compared to NK cells left unstimulated. This was paralleled by an upregulation of thiol levels, as per MBB measurement (Figure 6F). More specifically, IL-15 stimulation drove an upregulation of GSH in NK cells (Figure 6G). These data suggest a key role for redox regulation by GSH in IL-15-mediated NK cell activation.

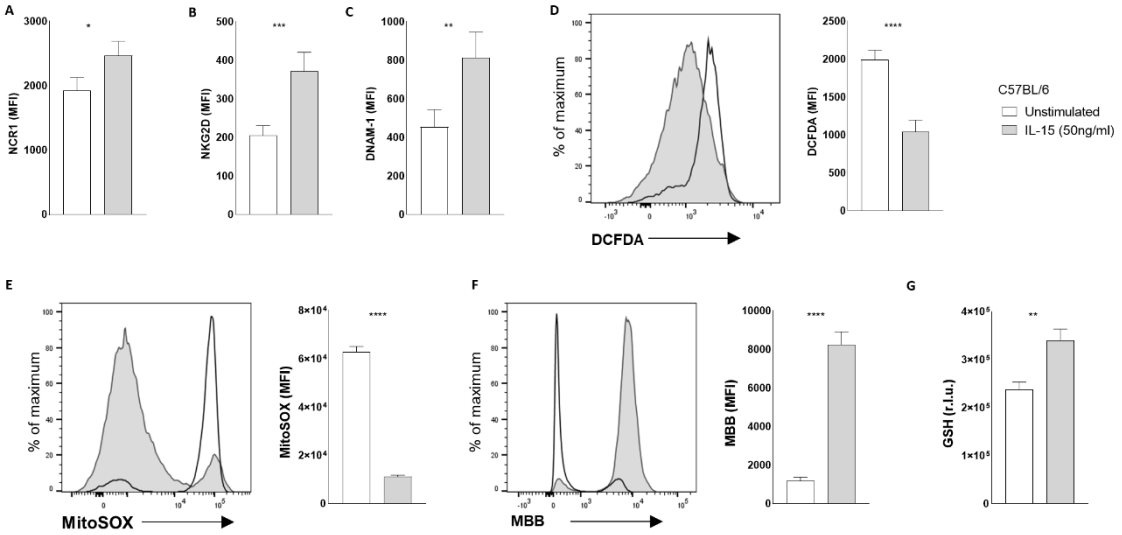

Figure 6 – GSH levels are increased upon IL-15-mediated NK cell activation.
NK cells were isolated from C57BL/6 mice and were left unstimulated or activated with IL-15 overnight. (A-D) Flow cytometric quantification of the expression of the NK cell activating receptors (A) NCR1/NKp46 (B) NKG2D (C) DNAM-1 (D) Left: histogram of representative flow cytometry analysis of intracellular ROS levels, detected with DCFDA. Right: quantification of the data in the left panel. (E) Left: histogram of representative mitochondrial ROS level as per intracellular MitoSOX staining. Right: quantification of the data in the left panel. (F) Left: representative histogram of intracellular thiol levels by MBB staining. Right: quantification of the data in the left panel. **(G)** Quantification of intracellular GSH levels in a luminescence based assay. MFI, mean fluorescence intensity. r.l.u., relative luminescence units. Data are mean ± SD (n=3-4) of two independent experiments. *P<0,05.

2. Absence of GSH does not alter redox control of NK cells at steady state

To investigate in depth the importance of redox metabolism by GSH in NK cells, we used conditional knockout mice in a Cre/loxP system. *Gclc*$^{fl/fl}$ mice (Chen et al., 2007), in which the exons 4 to 6 of the *Gclc* allele are flanked by loxP sites. *Gclc*$^{fl/fl}$ mice were crossed with *Ncr1-Cre*-expressing mice (Narni-Mancinelli et al., 2011) to obtain *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ progeny. This strategy permited the removal of essential coding sequences of *Gclc*, specifically in NK cells. GCLC participates in the first step of GSH production and its deletion results in hindered production of this antioxidant (Figure 5) (Chen et al., 2007). As expected, GSH production was ablated in NK cells of *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice (Figure 7A). Accordingly, given that GSH is the most abundant non-protein thiol in the cell (Dickinson and Forman, 2002), deletion of *Gclc* in NK cells results in significantly reduced levels of intracellular thiols, measured by MBB (Figure 7B). However, cytosolic ROS and mitochondrial superoxide in GSH-ablated NK cells were comparable to wild type (WT) controls at steady state (Figure 7C, D).

GSH exists in a reduced (GSH) and in an oxidized (GSSG) state. With increased levels of oxidative stress, GSSG accumulates and the GSH/GSSG ratio decreases (Zitka et al., 2012). However, in line with the sustained cytosolic and mitochondrial ROS levels, ablation of GSH production did not induce oxidative stress in resting NK cells, and the GSH/GSSG ratio did not decrease, when compared to WT NK cells (Figure 7E). These results indicate that resting NK cells do not depend on GSH to maintain their redox balance.

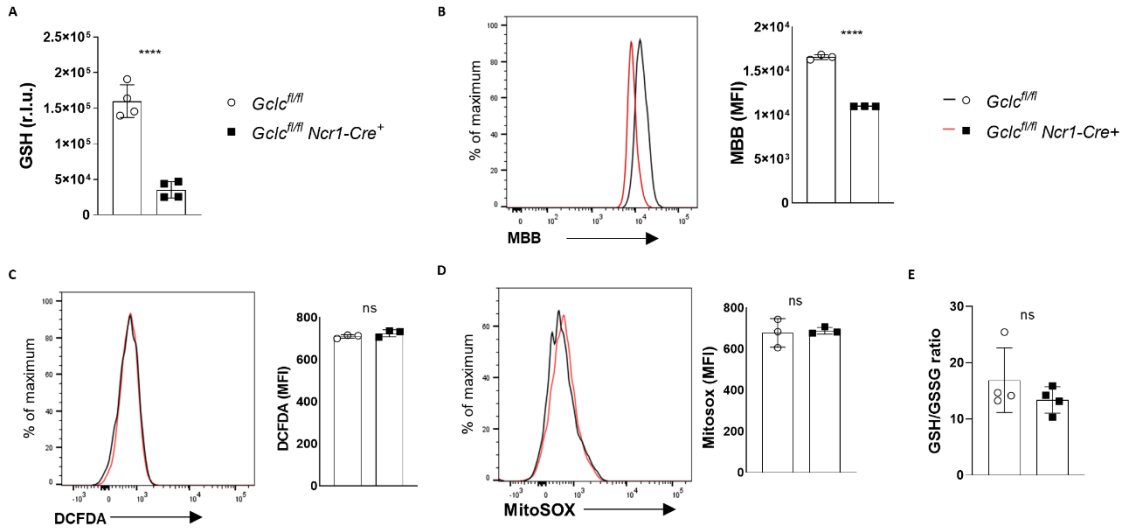

Figure 7 – NK cell specific deletion of GSH does not alter the redox balance in resting NK cells.

(A) Intracellular levels of GSH, expressed by relative luminesce units (r.l.u), measured in NK cells isolated from *Gclc*$^{fl/fl}$ and *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice at steady state. **(B)** Splenocytes from WT and *Gclc*-deficient mice were isolated and incubated with MBB, to assess intracellular thiol levels in NK cells. Left: representative histogram of MBB levels in the two genotypes. Right: quantification of MBB levels. **(C)** Splenocytes incubated with DCFDA and flow cytometry analysis to evaluate cytosolic ROS. Left: representative histogram of DCFDA fluorescence in WT and mutant NK cells. Right: quantification of DCFDA levels. **(D)** Splenocytes were incubated with MitoSOX, for evaluation of mitochondrial ROS. Left: representative plot of MitoSOX fluorescence in NK cells. Right: quantification of the data in the left panel. **(E)** NK cells were *Gclc*$^{fl/fl}$ and *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ and GSH and GSSG were measured by luminescence. Ratio of GSH to GSSG levels, as per representation of oxidative stress. Data are mean ± SD (n=3-4) and are representative of at least 4 independent experiments. MFI, mean fluorescence intensity. *P<0,05. ns, non significant.

3. NK cells peripheral abundance is reduced in *Gclc$^{fl/fl}$ Ncr1-Cre*+ mice

The preservation of the redox balance in GSH-deficient resting NK cells prompted us to investigate their phenotype *ex vivo*. The largest reservoir of NK cells in the mouse is the spleen, where 2 to 3 million NK cells can be found, constituting around 2% of the splenocytes (Grégoire et al., 2007). Upon NK cell-specific *Gclc* deletion, we observed a significant reduction of the frequency and total abundance of CD3$^-$ NK1.1$^+$ NK cells in the spleen (Figure 8 A,B). Furthermore, in the lungs, the non-lympoid organ with the highest abundance of NK cells (Grégoire et al., 2007), we observed that GCLC ablation also impacted NK cell density at steady state (Figure 8C).

The overall reduction in the peripheral abundance of NK cells in *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice led us to examine whether the development of these lymphocytes was impaired upon *Gclc* ablation. NK cell expansion starts in the bone marrow, arising from NK cell precursors (NKP, Lin$^-$CD122$^+$NCR1$^-$CD49b$^-$). Subsequently, the expression of the NK cell receptor NCR1 is increased, and more differentiated immature NK cells (iNK, lin$^-$CD122$^+$NCR1$^+$CD49b$^-$) are formed. The last step requires the acquisition of the integrin CD49b and establishment of mature NK cells (mNK, lin$^-$CD122$^+$Ncr1$^+$CD49b$^+$). In the bone marrow of *Gclc*$^{fl/fl}$ Ncr1-Cre+ mice, we observed a slight reduction in the frequency and density of the mNK cell subset (Figure 8D, E), which, may explain the lower numbers of NK cells in the periphery. Upon lineage commitment in the bone marrow, NK cells undergo further maturation, characterized by the sequential expression of CD27 and CD11b. The most immature subsets of NK cells are CD11b^{lo} CD27^{lo} and CD11b^{lo} CD27^{hi}. Subsequently, CD11b^{hi} CD27^{hi} and CD11b^{hi} CD27^{lo} subsets arise, marking the final stage of maturation and the acquisition of effector functions (Chiossone et al., 2009).

From the bone marrow, NK cells home to lymphoid and non-lymphoid organs (Hayakawa and Smyth, 2006). Ablation of GSH production does not affect profoundly the maturation process of NK cells. The frequency of the different NK cell subsets in the spleen is comparable between *Gclc*$^{fl/fl}$ Ncr1-Cre+ mice and WT controls (Figure 8F). The reduced number of highly mature CD11b^{hi} CD27^{lo} NK cells upon *Gclc* deletion (Figure 8G) can be explained by the lower number of splenic NK cells in these mice (Figure 8G). Comparable frequencies of mature CD11b^{hi} CD27^{lo} NK cells were also observed in the lungs, liver and peripheral blood of Gclc$^{fl/fl}$ and Gclc$^{fl/fl}$ Ncr1-Cre+ mice. (Figure 8H). Thus, these results indicate that the absence of GSH affects the peripheral abundance of steady state NK cells, but not their overall maturation state in the periphery.

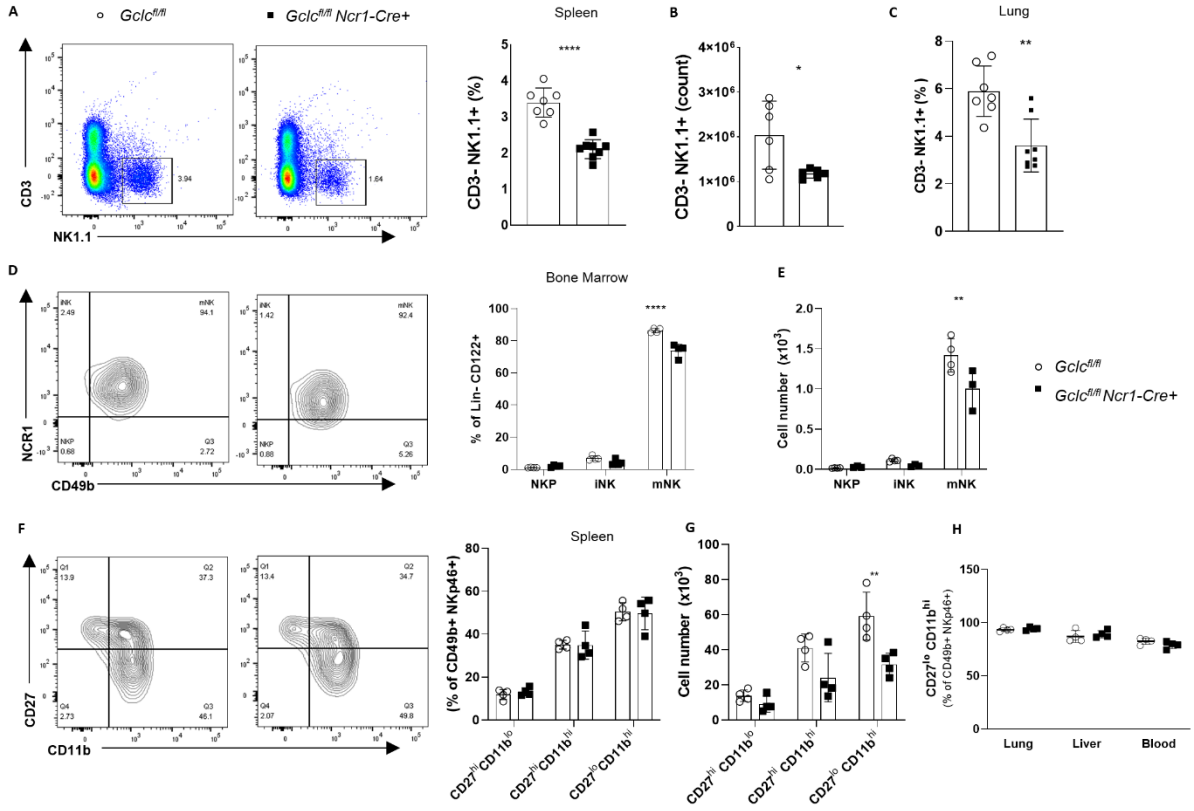

Figure 8 – NK cell peripheral abundance, but not their maturation status, is compromised in *Gclc*$^{fl/fl}$ *Ncr1-Cre+* mice.

(A-B) Splenocytes from Gclc$^{fl/fl}$ and *Gclc*$^{fl/fl}$ Ncr1-Cre+ mice were isolated to assess NK cell abundance by flow cytometry. **(A)** Left: representative dot plot of frequencies of splenic CD3^{-} NK1.1^{+} NK cells. Right: quantification of the data in the left panel. **(B)** Absolute count of CD3- NK1.1+ NK cells in the spleen of WT and *Gclc*-deleted mice. **(C)** Frequency of CD3^{-} NK1.1^{+} NK cells in the lungs of *Gclc*$^{fl/fl}$ and *Gclc*$^{fl/fl}$ Ncr1-Cre+ mice, as assessed by flow cytometry. **(D-E)** Bone marrow cells from WT and NK-specific *Gclc*-deficient mice were analyzed by flow cytometry to assess NK cell development. **(D)** Left: representative dot plots of Ncr1 and CD49b expression of Lin^{-} CD122^{+} progenitor NK cells. Right: frequencies of NK cell precursors (NKP, Ncr1^{-} CD49b^{-}). intermediate NK cells (iNK, Ncr1^{+} CD49b^{-}) and mature NK cells (mNK, Ncr1^{+} CD49b^{+}). **(E)** Absolute count of bone marrow-resident NKP, iNK and mNK as seen in **(D)**. **(F-H)** Flow cytometry analysis of maturation status of NK cells, based on the surface expression of CD27 and CD11b. **(F)** Left: representative scatter plot of splenic NK cells maturation cycle. Right: frequencies of CD27^{hi} CD11b^{lo}> CD27^{hi} CD11b^{hi}> CD11b^{hi} CD27^{lo} splenic NK cells, based on the data of the left panel. **(G)** Absolute count of the different splenic NK cell subsets, based on flow cytometry data, as seen in **(F)**. **(H)** Frequencies of highly mature CD11b^{hi} NK cells in the lungs, liver and peripheral blood of NK cell-specific *Gclc*-deficient mice and respective controls. Data are mean ± SD (n=4-6) and are representative of at least 4 independent experiments. MFI, mean fluorescence intensity. *P<0,05. ns, non significant.

4. Absence of glutathione in NK cells alters their response to IL-15 stimulation

Prior evidence suggested that redox regulation mechanisms are indispensable for activation of NK cells (Siernicka et al., 2015; Yamauchi and Bloom, 1997; Yang et al., 2020). Thus, we examined the impact of GSH deficiency on NK cell activation. When stimulated with IL-15, wild type (WT) *Gclc*$^{fl/fl}$ NK cells increase their size (FSC-A) and granularity (SSC-A), which was not observed in GSH-deficient NK cells (Figure 9A). Furthermore, ablation of GSH synthesis was associated with a significant defect in NK cell proliferation, depicted by a lower dilution of Cell Trace^{TM} CFSE (Figure 9B) and reduced expression of the nuclear protein Ki67 (Figure 9C). In brief, GSH is required for IL-15-induced activation of NK cells. The reduced size and proliferation upon *Gclc* ablation indicate that GSH may be needed for NK cell function.

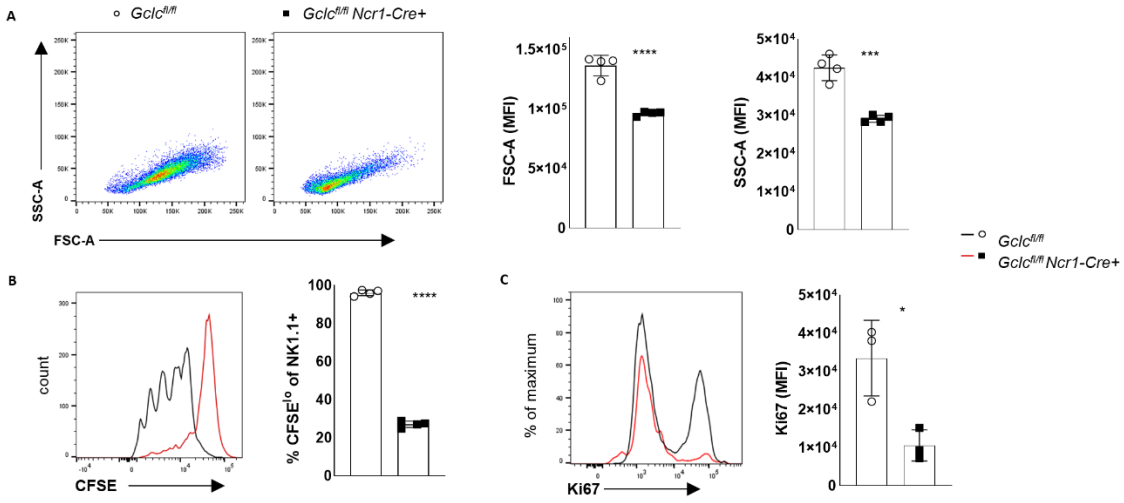

Figure 9 – *Gclc* deletion in NK cells results in reduced blasting and proliferation upon activation.
NK cells isolated from spleens of Gclc$^{fl/fl}$ and *Gclc*$^{fl/fl}$ Ncr1-Cre+ mice were activated with IL-15 for 72h. **(A)** Left: representation of flow cytometry analysis of size (FSC-A) and granulosity (SSC-A) of live CD3- NK1.1+ activated NK cells of the indicated genotype. Right: quantification of the data represented in the left panel. **(B)** Proliferation-induced CFSE dilution was assessed by flow cytometry. Left: representative histogram of CFSE dilution of WT and *Gclc*-deficient NK cells. Right: proportion of NK cells with a higher dilution of CFSE (CFSE^{lo}), representing high proliferative capacity. **(C)** Left: representative histogram of intracellular levels of the nuclear protein Ki67 in NK cells. Right: quantification of the data from the let panel. Data are mean ± SD (n=3-4) and representative of at least 3 independent experiments. MFI, mean fluorescence intensity. *P<0,05

5. *Gclc* ablation impairs NK cell cytotoxic function

Besides stimulating NK cell proliferation, priming with IL-15 elicits a cytotoxic response, characterized by IFN-γ production and degranulation (Fehniger et al., 2000; Zhang et al., 2018a). We observed that, in contrast to GSH-sufficient NK cells, GSH deficiency leads to an abrogation in the production of the regulatory cytokine IFN-γ (Figure 10A, B), the cytotoxic molecule Granzyme B (Figure 10B) and the degranulation marker CD107a (Figure 10C) upon activation with IL-15 for 72h. Furthermore, we evaluated the impact of GSH on NK cell-mediated cytotoxicity by co-culturing IL-15 *in vitro* activated NK cells with the lymphoma cell line YAC-1. YAC-1 cells are sensitive to NK cell-cytotoxic mechanisms through the expression of the NKG2D ligand RAE-1 (Kim et al., 2000). In line with the downregulation of the cytotoxic machinery, *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ NK cells were not as efficient in lysing the target YAC-1 cells, as their WT counterparts (Figure 10D). In summary, NK cell-specific GCLC ablation induces the generation of NK cells that are hyporesponsive to IL-15 stimulation and show a reduced cell-targeted cytotoxicity.

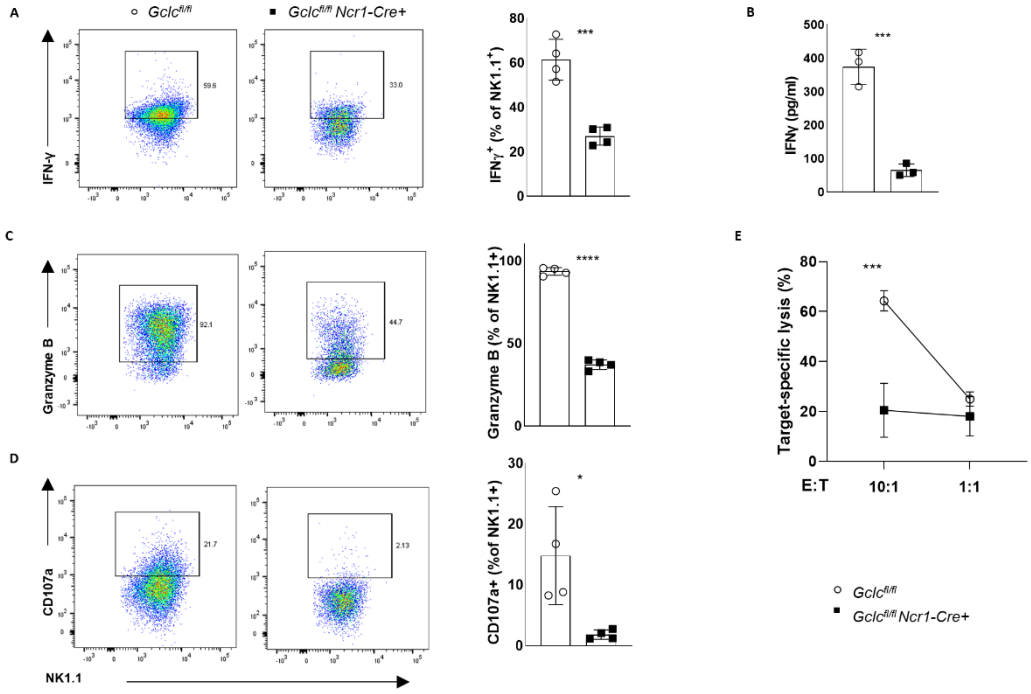

Figure 10 – Cytokine production and cytotoxic activity in response to IL-15 stimulation is severely impacted in glutathione-deficient NK cells.
NK cells isolated from *Gclc*$^{fl/fl}$ and *Gclc*$^{fl/fl}$ Ncr1-Cre+ mice were activated with IL-15 for 72h. Intracellular presence of cytokines and degranulation markers was analysed by flow cytometry in (A, C, D). **(A)** Left: representative dot plots of IFNγ production by NK cells of the indicated genotype. Right: quantification of the IFN-γ+ NK cells in *Gclc*-sufficient and deficient mice. **(B)** Quantification by ELISA of IFN-γ in supernatant of NK cells cultured with IL-15 for 72h. **(C-D)** Representative flow cytometry dot plot (left) and quantification (right) of **(C)** Granzyme B and **(D)** CD107a. **(E)** IL-15 primed NK cells (effectors, E) were co-cultured with previously labeled YAC-1 cells (targets, T). Lysis of YAC-1 cells was assessed by flow cytometry by Annexin V+ staining. Data are mean±SD (n=3-4), representative of at least 2 independent trials. *P<0,05

6. *Gclc*-deficient NK cells accumulate mitochondrial ROS and show impaired mitochondrial fitness

Abrogating glutathione production does not seem to induce oxidative stress in resting NK cells (Figure 9C, D). However, our data indicate that the presence of this antioxidant is particularly important upon NK cell activation. When compared to *Gclc*-sufficient NK cells, *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ NK cells did not increase cytosolic ROS levels upon IL-15-dependent activation, as measured by DCFDA (Figure 11A). In contrast, mitochondrial ROS, measured by MitoSOX, were significantly upregulated in NK cells lacking GSH. (Figure 11 B).

Heightened levels of superoxide in the mitochondria are closely associated with electron leakage from the electron transport chain during oxidative phosphorylation. Local increase of ROS is detrimental to mitochondrial function due to heightened oxidative stress (Murphy, 2009). Therefore, in order to investigate whether mitochondrial dynamics were affected in NK cells upon *Gclc* deletion, we stained IL-15 activated NK cells from *Gclc*$^{fl/fl}$ and *Gclc*$^{fl/fl}$ *Ncr1*-Cre+ with Mitotracker™ Deep Red (MTDR) and Mitrotracker™ Green (MTG) to examine mitochondrial membrane potential and mass, respectively. Ablation of *Gclc* led to a decreased mitochondrial membrane potential (Mitotracker Deep Red, MTDR) (Figure 11C), suggesting an impaired mitochondrial activity. In line, mitochondrial mass (Mitotracker Green, MTG) was increased in activated NK cells from Gclc$^{fl/fl}$ *Ncr1-Cre*+ (Figure 11D), indicating that mitochondria structurally change in order to compensate for the deficient mitochondrial activity (Sun et al., 2016; Yu et al., 2020). Furthermore, the ratio of MTDR to MTG, depicting mitochondrial activity per mitochondrial mass, was reduced in Gclc-deficient NK cells (Figure 11E), which defective mitochondrial activity. In sum, these results indicate that glutathione deficiency induces accumulation of mitochondrial ROS and impairs mitochondrial activity of NK cells.

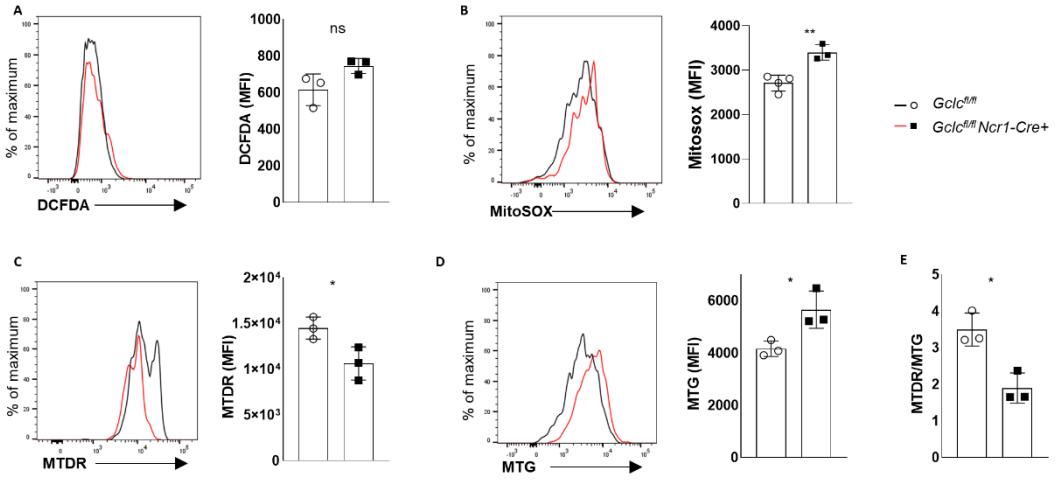

Figure 11 –*Gclc$^{fl/fl}$ Ncr1*+ NK cells accumulate mitochondrial ROS, which associated with a defective mitochondrial function.

NK cells from Gclc$^{fl/fl}$ and Gclc$^{fl/fl}$ Ncr1-Cre+ mice were isolated from spleens and activated with IL-15 for 72h. **(A)** Left: histogram of representative flow cytometry analysis of intracellular ROS levels, detected with DCFDA. Right: quantification of the data in the left panel. **(B)** Left: histogram of representative levels of mitochondrial ROS in NK cells, as per intracellular staining with MitoSOX. Right: quantification of the data in the left panel. **(C)** Flow cytometry analysis of NK cells treated with Mitotracker™ Deep Red (MTDR) to assess mitochondrial activity. Left: representative histogram. Right: quantification of MTRD. **(D)** Left: flow cytometry analysis of NK cells incubated with Mitotracker™ Green (MTG) to evaluate mitochondrial mass. Right: quantification of the data in the left panel. **(E)** Calculation of MTDR7MTG ratio, as a measure of mitochondrial activity per mitochondrial mass. Data are mean±SD (n=3) and are representative of at least 3 independent experiments. MFI, mean fluorescence intensity. *P<0,05. ns, non significant

7. Absence of glutathione curtails metabolic fitness of NK cells

Cytokine-primed NK cells rely on oxidative phosphorylation (OXPHOS) and glycolysis to maintain their survival and functional capacity (Donnelly et al., 2014; Marçais et al., 2014). Given their defective activation and mitochondrial fitness, we hypothesized that GSH insufficiency may affect NK cell metabolism. We used a flow-cytometry based method to unravel the energetic profile of *Gclc*-deficient NK cells, and use protein translation as a readout of energy consumption, as described previously by (Argüello et al., 2020). In brief, half of the energy produced by mammalian cells through the degradation of glucose, amino acids and/or fatty acids is instantly used for protein synthesis (Buttgereit and Brand, 1995) and, therefore, the latter can be a measure of global metabolic activity (Argüello et al., 2020). Puromycin inhibits protein synthesis and has a similar structure to 3' end of aminoacyl-tRNA carrier, which is rapidly incorporated into newly synthesized amino acids, indicating the rate of mRNA translation (Schmidt et al., 2009). The parallel use of metabolic inhibitors allows to study the impact of one specific metabolic pathway on the overall energetic profile of the cell, which is monitored by an anti-puromycin antibody by flow cytometry (Argüello et al., 2020).

IL-15 activated *Gclc*$^{fl/fl}$ and *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ NK cells were left untreated or treated with oligomycin, which inhibits mitochondrial complex V ATP synthase and/or 2-deoxyglucose (2-DG), an analogue of glucose that suppresses glycolysis. Interestingly, non-treated *Gclc*-deficient NK cells showed a drastically lower protein translation and, therefore, reduced basal energetic state, when compared to wild type controls (Figure 7A). Furthermore, independently of the pathway inhibition, the energetic capacity of GSH-deficient NK cells was significantly lower than their *Gclc*-sufficient counterparts. Inhibition of glycolysis resulted in a significant reduction in ATP levels in WT NK cells, in line with previous results (Felices et al., 2018). Nevertheless, only a combination of oligomycin and 2-DG, or complete inhibition of protein synthesis by harringtonine, reduced energetic levels in WT NK cells to the ones observed in GSH-deficient cells (Figure 12A). This indicates that the metabolic dysfunction of *Gclc*-deficient NK cells compares to the energetic state of complete inhibition of major metabolic pathways in WT NK cells. In addition, *Gclc* deletion led to a reduction in fatty acid and amino acid oxidation capacity (the maximum capacity to maintain protein translation when OXPHOS is inhibited) in NK cells (Figure 7B). In addition, ablation of GSH production resulted in a defective glycolytic capacity (Figure 7C), since the capacity to maintain protein translation, upon glycolysis inhibition, was much lower than in their WT counterparts. The lower energetic capacity induced by glutathione ablation was further analyzed by direct measurement of intracellular ATP levels of Gclc$^{fl/fl}$ and Gclc$^{fl/fl}$ Ncr1-Cre+

activated NK cells (Figure 12D). These results indicate that GSH plays an essential role in regulating metabolic activity and energetic capacity of activated NK cells.

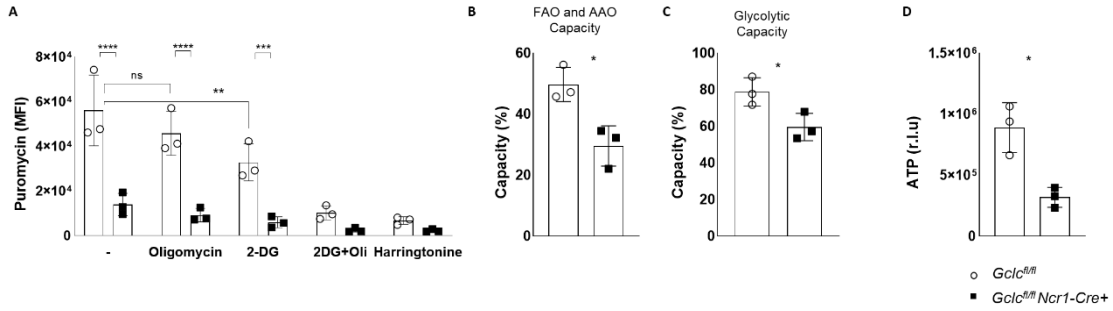

Figure 12 – NK cell-specific *Gclc* deficient mice show deficient metabolic activity upon activation.
(A-C) *Gclc*$^{fl/fl}$ and *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ NK cells were stimulated with IL-15 for 72h and subsequently treated with different metabolic inhibitors: oligomycin and/or 2-DG and puromycin. Harringtonine was used as a positive control for protein synthesis inhibition. Protein translation was followed by incorporation of puromycin, detected by an anti-puromycin antibody by flow cytometry. Puromycin fluorescence is used as a surrogate for protein synthesis and energetic levels of the cells. **(A)** Flow cytometry analysis of protein synthesis, as detected per intracellular detection of puromycin in NK cells previously treated with the different metabolic inhibitors. MFI, geometric mean fluorescence intensity. **(B)** Fatty acid oxidation (FAO) and amino acid oxidation (AAO) capacity were calculated according to the formulas described in Materials and Methods, upon treatment of cells with oligomycin or a combination of 2DG and oligomycin. **(C)** Glycolytic capacity can be calculated from the MFI of puromycin under 2DG or a combination of 2DG and oligomycin treatment, following the formulas in Materials and Methods. **(D)** NK cells from WT and mutant mice were primed with IL-15 for 72h and ATP levels were measured by luminescence in relative luminescence units (r.l.u). Data are mean ± SD (n=3) of at least 2 independent experiments. *P<0,05. ns, non significant

8. Pharmacological inhibition of GCLC impairs NK cell function and metabolism

The method used above to assess the cellular energetic profile by flow cytometry has the advantage of needing lower cell numbers to be applied, when compared to the metabolic profile that can be determined by extracellular flux analysis with Seahorse (Argüello et al., 2020). The flow cytometric method allowed us to circumvent the limitations originating from the lower numbers of splenic NK cells that we can obtain from *Gclc*$^{fl/fl}$ *Ncr1-Cre*+, associated with a lower proliferative capacity. Nevertheless, it is of great significance to validate our metabolic profile results with Seahorse analysis.

In order to bypass the low number of cells from *Gclc*$^{fl/fl}$ *Ncr1-Cre*+, we used WT NK cells from C57BL/6 mice and treated them with l-buthionine-sulfoximine (BSO), to pharmacologically inhibit GCLC (Meister, 1983). The treatment WT NK cells with BSO allowed us, at the same time, to investigate the effects of acute inhibition of GCLC in NK cells. Similar to *Gclc*-deficient NK cells, BSO-treated WT NK cells displayed reduction in thiols upon IL-15-induced activation (Figure 13A), with a low impact on their viability (Figure 13B). In line with our genetic model, BSO-treated NK cells also showed reduced proliferation (Figure 13C) and lower production of IFN-γ, Granzyme B and CD107a, depicting a defective functional capacity induced by the absence of GSH (Figure 13D-F). Finally, the Seahorse-based extracellular flux analysis revealed that BSO-treated NK cells have an impaired OXPHOS capacity, represented by an almost null oxygen consumption rate (OCR) (Figure 13F). Specifically, acute GSH depletion with BSO led to a significant reduction in basal and maximal OCR in NK cells (Figure 13H, I). Overall, these results indicate that pharmacological depletion of GSH in activated NK cells mimics the genetic deletion of *Gclc* and both approaches severely impact the metabolic capacity of activated NK cells.

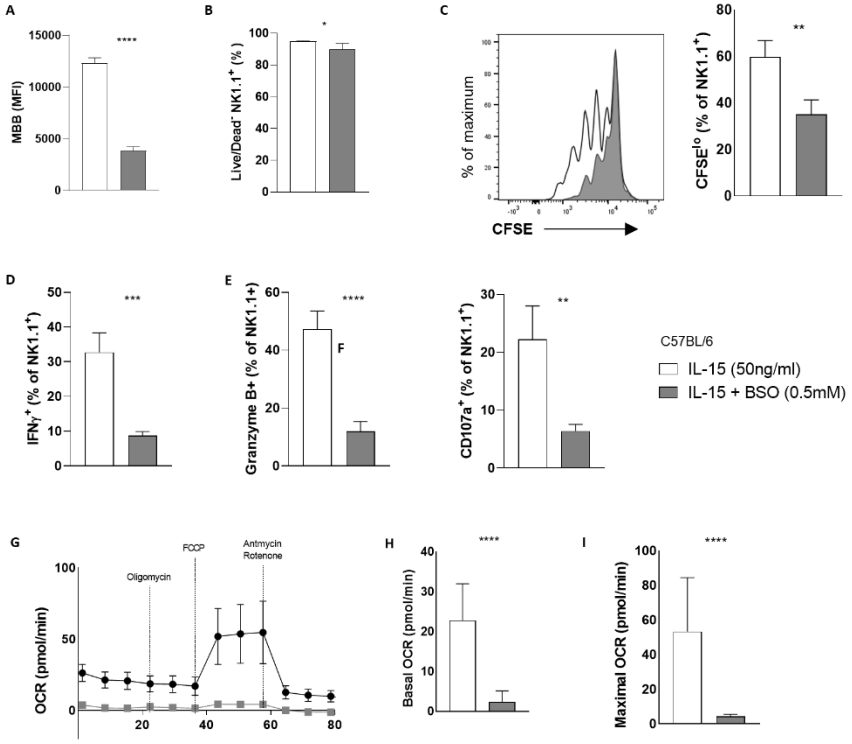

Figure 13 – BSO prevents GSH production and recapitulates the functional deficits induced by genetic deletion of *Gclc* in NK cells.

(A-H) NK cells isolated from C57BL/6 mice were activated with IL-15 and treated with BSO for 72h. **(A)** Quantification of intracellular thiol levels, determined by MBB by flow cytometry. **(B)** Frequency of viable NK1.1$^+$ NK cells after 72h of culture with IL-15, as measured by lower fluorescence of the LIVE/DEAD™ Fixable Green viability die. **(C)** Left: representative histogram of CFSE-labeled NK cells at 72h time point. Right: Frequency of dividing NK cells (CFSE^{lo}), determined by CFSE dilution. **(D-E)** Flow cytometry analysis of intracellular cytokines. Frequency of NK cells expressing: **(D)** IFN-γ. **(E)** Granzyme B and **(F)** CD107a. **(G-I)** Oxygen consumption rate (OCR) measured by Seahorse assay. Quantifiation of basal OCR **(H)** and maximal OCR **(I).** Data are mean±SD (except F, mean±SEM) (n=3-4) and are representative of at least 3 independent experiments. MFI, mean fluorescence intensity. *P<0,05.

9. GSH regulates mTOR signaling and expression of nutrient transporters in NK cells

The metabolic profile of NK cells is sustained by the kinase mammalian target of rapamycin (mTOR) (Marçais et al., 2014). In IL-15 activated NK cells, mTOR signaling promotes NK cell growth and nutrient uptake and is essential for proliferation and acquisition of cytotoxic capacity, under inflammatory conditions or upon viral infection (Marçais et al., 2014). In line with their reduced size, proliferative capacity (Figure 9) and deficient activation (Figure 10), IL-15-activated NK cells from *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice showed reduced phosphorylation of S6 (pS6), the downstream target of mTOR (Figure 14A). These results were confirmed with BSO-treated NK cells (Figure 14B, C). mTORC1 senses and integrates intracellular availability of nutrients, such as glucose, amino acids, lipids and ATP. Those nutrients sustain mTORC1 activation, which, in turn, stimulates further nutrient uptake to sustain cell growth (Goberdhan et al., 2016; Weichhart et al., 2015). In line with a lower mTOR activity, genetic deletion of *Gclc* in NK cells resulted in a reduced expression of the glucose transporter GLUT1 (Figure 14D), the amino acid transporter CD98 (Figure 14E) and the transferrin receptor CD71 (Figure 14F). The lower levels of pS6 and nutrient transporters are in line with an impaired metabolic fitness of NK cells in the absence of GSH.

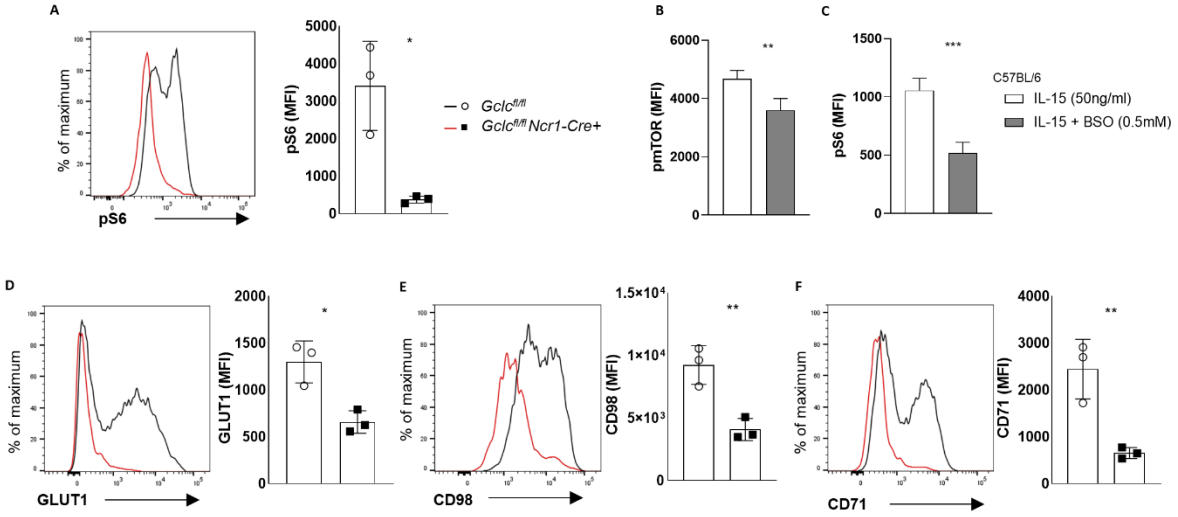

Figure 14 – Gclc-deficient NK cells show reduced mTOR signaling activation and lower expression of nutrient receptors.
(A) Gclc$^{fl/fl}$ and Gclc$^{fl/fl}$ Ncr1-Cre+ NK cells were stimulated with IL-15 for 72h and intracellular expression levels of phospho-S6 (pS6) was examined by flow cytometry. Left: histogram of a representative difference in the expression of pS6 between WT and mutant mice. Right: quantification of the data in the left panel. **(B-C)** NK cells isolated from C57BL/6 mice were activated with IL-15 ± BSO for 72h. Flow cytometry analysis was performed to assess intracellular levels of **(B)** pmTOR and **(C)** pS6. **(D-F)** Gclc$^{fl/fl}$ and Gclc$^{fl/fl}$ Ncr1-Cre+ NK cells were stimulated with IL-15 for 72h, as in (A). Surface expression of different nutrient receptors was determined by flow cytometry. Representative histograms (left) and quantification (right) of **(D)** Glut1, **(E)** CD98 and **(F)** CD71 in WT and *Gclc*-depeleted NK cells. Data are mean ± SD (n=3) and representative of at least 2 independent experiments. * P<0,05. MFI, mean fluorescence intensity.

10. GSH deficiency alters STAT5 signaling pathway in NK cells

mTOR activation is paralleled by a dependence on STAT5 phosphorylation for NK cell survival (Marçais et al., 2014; Wang et al., 2018). NK cell-specific STAT5 ablation leads to developmental defects that drastically impact NK cell presence in peripheral lymphoid organs (Eckelhart et al., 2011). Indeed, as observed for mTOR signaling, ablation of GSH production resulted in reduced phosphorylation of pSTAT5 upon activation with IL-15 (Figure 15A).

In cancer cells, T cells and NK cells, STAT5 signaling has been associated with cell cycle progression by upregulation of c-MYC or D-type cyclins (Gotthardt et al., 2016; Moriggl et al., 1999; Xiong et al., 2009). Indeed, in Gclc$^{fl/fl}$ Ncr1-Cre+ mice, we observed an increased frequency of NK cells in the G0/G1 phase of the cell cycle (Figure 15B), paralleled by a significant reduction in the G2/M phase (Figure 15C), upon IL-15 priming *in vitro*. In sum, these results indicate that the low proliferation and functionality of GSH-deficient NK cells is associated with a reduced mTOR and STAT5 signaling upon activation, which may impair their capacity to control viral infections or tumor growth.

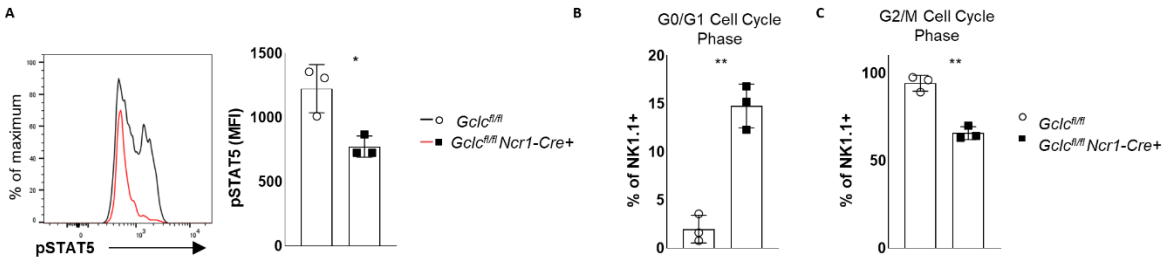

Figure 15 – STAT5 signaling is reduced in NK cells from *Gclc$^{fl/fl}$ Ncr1-Cre+* mice and cell cycle progression is arrested.
NK cells isolated from the spleen of *Gclc$^{fl/fl}$* and *Gclc$^{fl/fl}$ Ncr1-Cre+* were stimulated with IL-15 for 72h. **(A)** Left: representative histogram of flow cytometry analysis of intracellular pSTAT5. Right: quantification of the data in the left panel. **(B and C)** IL-15 primed NK cells were stained with Hoechst to assess cell cycle progression by flow cytometry. **(B)** Quantification of NK1.1+ cells in G0/G1 phase. **(C)** Quantification of NK1.1+ cells in G2/M phase. Data are mean ± SD (n=3) and representative of at least 2 independent experiments. * P<0,05. MFI, mean fluorescence intensity

11. NK cells deficient in GSH are not able to regulate LCMV-specific T cell responses

In order to assess the capacity of GSH-deficient NK cells to surveil intracellular infections, we challenged *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ and WT controls with lymphocytic choriomeningitis virus (LCMV). NK cells can respond quickly to viral infections by directly lysing virus-exposed cells (Daniels et al., 2001). However, in the specific case of LCMV infection in mice, NK cells do not exert direct antiviral effects of NK cells (Bukowski et al., 1983), despite their high activity and cytotoxicity (Welsh et al., 1991). Indeed, NK cells present an immunoregulatory role by modulating the activity of DCs and T cells during the course of an LCMV infection. Selective depletion of NK cells using anti-NK1.1 monoclonal antibodies enhances antigen presentation and subsequent IFN-γ production by CD4+ and CD8+ T cells (Lang et al., 2012; Su et al., 2001).

To establish the role of GSH in NK cell-dependent anti-viral responses, we infected Gclc$^{fl/fl}$ and *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice infected with a high dose (2x10^6 p.f.u.) of LCMV-WE. Given the importance of T cell-dependent immunity upon LCMV infection, we evaluated the T cell response in the two groups of mice 8 days post-infection (p.i.). LCMV-specific T cells were recognized by LCMV-specific fluorescently-stained MHC tetramers, presenting the viral glycoprotein gp33 and the nucleoprotein np396. Gp33 and np396 are predominant epitopes for CD8 T cell LCMV-specific response. Interestingly, an increased frequency and abundance of gp-33-tretamer$^+$ and np396-tetramer$^+$ T cells was observed in the spleen (Figure 16A) and liver (Figure 16B) of *Gclc*$^{fl/fl}$ *Ncr1-Cre*+, when compared to WT littermate controls. Moreover, when re-stimulated *ex vivo* with the LCMV peptides gp33 and np396, T cells from mutant mice showed an increased production of IFN-γ in the spleen (Figure 16C) and in the liver (Figure 16D). These results indicate that, in the absence of GSH, NK cells are not able to exert their regulatory role of controlling T cell-dependent antiviral function.

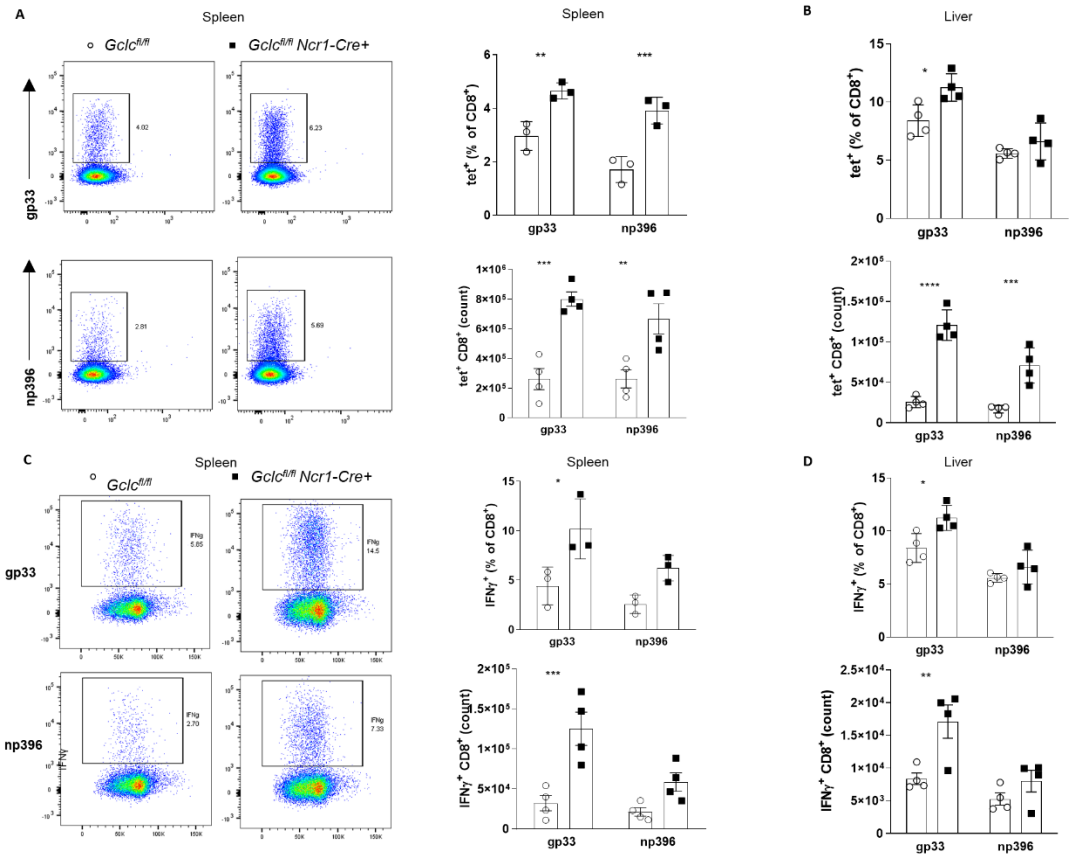

Figure 16– Genetic ablation of *Gclc* hampers NK cell-dependent regulation of anti-viral T cell response.

Gclc$^{fl/fl}$ *or Gclc*$^{fl/fl}$ *Ncr1-Cre+* mice were inoculated with 2x10^6 plaque forming units (p.f.u) of LCMV-WE. Flow cytometry analysis was performed in spleen and liver of these mice, 8 days post-infection (p.i.) **(A)** Left: flow cytometric analysis of gp33 (top) and np396 (bottom) tetramer$^+$ CD8$^+$ T cells in splenocytes of mice from the indicated genotype. Right: frequencies (top) and absolute count (bottom) of the indicated cell subpopulations from the data from the left panel. **(B)** Frequencies (top) and absolute count (bottom) of flow cytometric analysis of gp33 and np396 tetramer$^+$ CD8$^+$ T cells in the liver. **(C)** Left: Flow cytometry analysis of IFN-γ+ CD8+ T cells after restimulation of splenocytes with the LCMV epitopes gp33 (top) and np396 (bottom). Right: frequencies (top) and absolute count (bottom) of the indicated cell subpopulations from the data from the left panel. **(D)** Frequencies (top) and absolute count (bottom) of flow cytometry analysis of IFN-γ+ CD8+ T cells after restimulation of hepatocytes with the LCMV epitopes gp33 and np396. Data are mean ± SD (n=4) and are representative of 2 independent trials. *P<0,05

12. *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice show an improved LCMV clearance, when compared to wild type littermates

As innate immune effectors, the peak of NK cell activation and T cell killing upon LCMV challenge occurs during the initiation phase of the infection, within the first few days (Welsh et al., 1991). Therefore, we assessed NK cells from *Gclc*$^{fl/fl}$ and *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice 1 day after LCMV-WE infection. Co-culture of splenocytes of LCMV-infected mice with the target cell line YAC-1, showed that, at high effector to target ratio, NK cells deficient in GSH production were less efficient at killing the YAC-1 target cells (Figure 17 A). *Gclc*-deficient NK cells exhibited significantly lower levels of the activating receptor NCR1/NKp46 (Figure 17B), which was shown to be crucial for elimination of LCMV-specific CD8+ T cells (Duhan et al., 2019; Pallmer et al., 2019). These data point towards an intrinsic inability of GSH-deficient NK cells to exert their cytotoxic function, hindering their immunoregulatory role in the context of LCMV infection.

Indeed, at day 8 p.i., analysis of viral load in the spleen, liver, lungs and kidney of LCMV-infected mice, showed that the absence of GSH in NK cells unleashed T-cell dependent viral clearance, significantly reducing the viral titers in the spleen of *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice, compared with *Gclc*$^{fl/fl}$ mice (Figure 17C). Although GSH-deficient mice showed a tendency for lower viral load in the liver, lung and kidney, this was not significant. Acute and chronic infection of LCMV induces T cell-mediated liver damage. Hepatitis can be monitored by the activity of liver enzymes in the serum, such as aspartate transaminase (AST), aspartate transaminase (ALT) and lactate dehydrogenase (LDH). In line with an enhanced T cell response and better viral clearance, NK cell-specific *Gclc* deletion led to milder hepatitis caused by LCMV-WE, as exemplified by lower serological levels of AST, ALT and LDH (Figure 17 D). Taken together, in the absence of GSH, NK cells of virus-infected mice, are unable to suppress CD8+ T cell-mediated immune response, which leads to an improved anti-viral immunity.

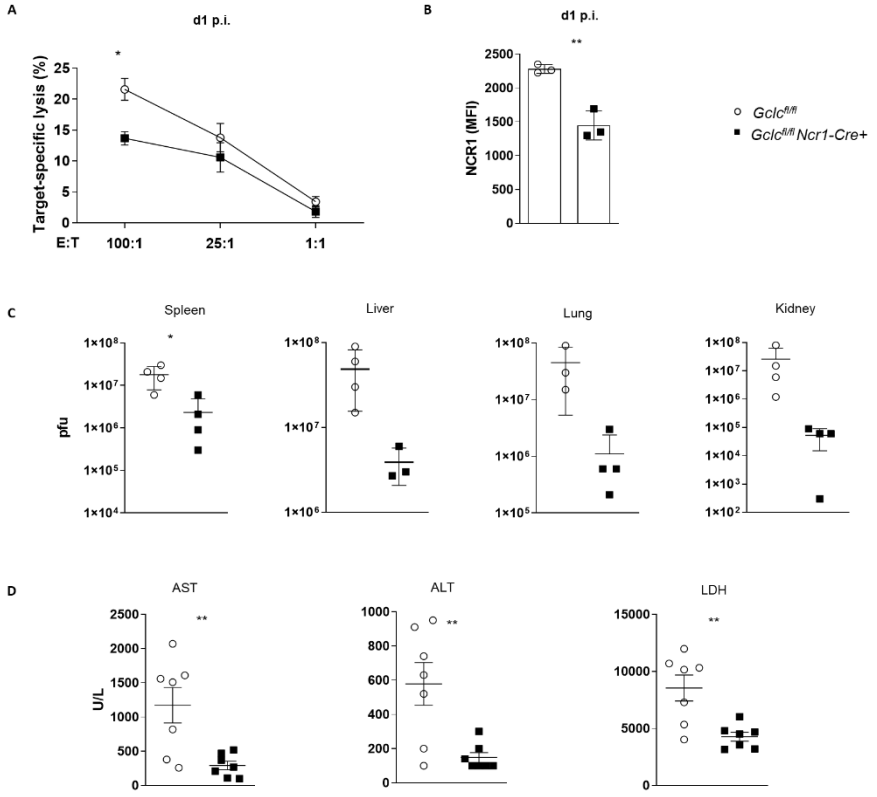

Figure 17 – NK cell-specific *Gclc* deletion leads to decreased cytotoxic capacity of NK cells but better viral clearance upon LCMV infection.
Gclc$^{fl/fl}$ *and Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice were inoculated intravenously with 2x10^6 plaque forming units (p.f.u) LCMV-WE. **(A-B)** 1 day post-infection (p.i.), mice were sacrificed and spleens were isolated for further analysis. **(A)** NK-cell mediated cytotoxicity was measured by co-culture of splenocytes of LCMV-infected mice with CFSE-labeled YAC-1 cells at the indicated E:T ratios. Data are mean ± SEM (n=4) and are representative of at least 2 independent trials. **(B)** Surface expression of Ncr1/Nkp46 in NK cells from LCMV infected mice was determined by flow cytometry. Data are mean ± SD (n=3) and are representative of at least 2 independent trials. **(C-D)** LCMV-WE infected mice were sacrificed 8 days p.i. **(C)** Virus titers were determined in the spleen, liver, lung and kidney. p.f.u., plaque forming units. Data ate mean ± SD (n=4) and are representative of at least 2 independent trials. **(D)** Serum of infected mice from the indicated genotypes was collected and liver damage induction was assessed by measuring activity of: left, aspartate aminotransferase (AST), middle, alanine aminotransferase (ALT) and, right, lactate dehydrogenase (LDH). Data are mean ± SEM (n=6) and are pooled from 2 independent trials. *P<0,05

13. NK cell-dependent primary tumor control is not affected by the absence of GSH

Given the low functional capacity and metabolic fitness of *Gclc*-deficient NK cells, we decided to investigate the role of GSH in NK cell-dependent tumor control. We injected $2x10^5$ B16-F10 cells subcutaneously (s.c.) and assessed tumor growth. Surprisingly, there was no difference in the tumor growth rate between *Gclc*$^{fl/fl}$ and *Gclc*^{flfl} *Ncr1-Cre*+ mice (Figure 18A). At day 18, when subcutaneous melanomas were resected, the weight of the tumors did not differ between WT and GSH-deficient mice (Figure 18B). However, the injection of B16-F10 melanoma cells induces poorly immunogenic tumors, with low immune infiltration ("*cold tumor*") (Lechner et al., 2013; Yu et al., 2018) and the importance of GSH in NK cells could have been masked by a lower infiltration of NK cells into the TME. Therefore, we used MC-38, a murine-derived colon adenocarcinoma cell line, described to be more immunogenic (Efremova et al., 2018). MC-38 cells were injected s.c. and tumor growth was measured for 30 days. In line with the results from the B16-F10 melanoma model, GSH deficiency in NK cells did not alter growth of the primary tumor (Figure 18C). These results indicate that GSH is not important for NK cell-mediated control of primary tumors.

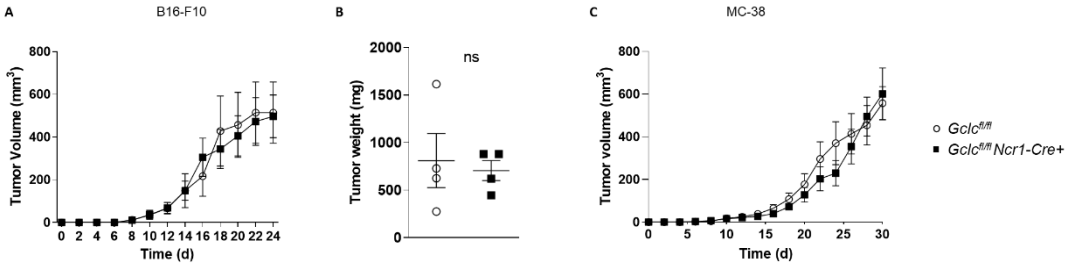

Figure 18 – Primary tumor control is not affected by the absence of GSH in NK cells.
(A-B) B16-F10 cells were injected subcutaneously into *Gclc*$^{fl/fl}$ *and Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice **(A)** Tumor growth was monitored. Data are mean ± SEM (n=5) and are representative of at least 3 independent trials. **(B)** Tumor weight was measured upon tumor resection 18 days post-inoculation. **(C)** MC-38 cells were injected subcutaneously into *Gclc*$^{fl/fl}$ *and Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice and tumor growth was monitored. Data are mean ± SEM (n=10) and are pooled from 2 independent trials. *P<0,05

14. GSH-deficient NK cells are not able to control tumor metastatic dissemination

In vitro, NK cells lyse cancer cells irrespective of their primary or metastatic origin or stemness (Ames et al., 2015), and *in vivo* depletion of NK cells increases tumor burden (Smyth et al., 2000). NK cells participate in the control of hematological malignancies (Ilander et al., 2017; Street et al., 2004) but the extent of NK cell-mediated primary tumor control remains a matter of debate (López-Soto et al., 2017). Thus, we hypothesized that although GSH does not interfere with primary tumor growth, the ablation of this antioxidant may be important for NK cell-mediated suppression of tumor metastasis. Therefore, we injected WT and NK cell-specific *Gclc*-deficient mice with B16-F10 through the tail vain to mimic metastatic dissemination. 15 days after tumor inoculation, we assessed the number of macroscopic metastatic *foci* formed in the lungs (Figure 19A). In line with a defective NK cell functionality, *Gclc*$^{fl/fl}$ *Ncr1-Cre+* mice had twice the number of tumor nodules as their WT littermates (Figure 19B). This was associated with a marked reduction in NK cell infiltration in the lungs (Figure 19C).

Closer investigation of tumor infiltrating NK cells revealed increased abundance of TIGIT+ NK cells upon *Gclc* ablation (Figure 19D). GSH-deficient NK cells also showed a significantly higher expression of the inhibitory receptors PD-1 (Figure 19E) and TIM3 (Figure 19F), both associated with NK cell exhaustion and dysfunction in tumor settings (Niu et al., 2020; Xu et al., 2015). Furthermore, the expression of the NK cell activating receptors NCR1 and NKG2D was drastically reduced in tumor-NK cells of *Gclc*$^{fl/fl}$ *Ncr1-Cre+* mice (Figure 19G, H). Finally, consistent with a poor activation phenotype, GSH deficiency resulted in reduced production of granzyme B by tumor NK cells (Figure 19I). In sum, these results indicate that the GSH antioxidant system is a functional requirement for NK cell-dependent control of metastatic dissemination. Furthermore, the finding that GSH is crucial for prevention of NK cell exhaustion in the TME adds a new level in the regulation of effector cell function in the tumor, which may be crucial for improvement of immune checkpoint blockade therapies. Nevertheless, NK cells are not sole players in anti-tumor responses and their activity is essential for shaping adaptive immune response (Martín-Fontecha et al., 2004; Moretta et al., 2008). Hence, it is key to investigate whether GSH deficiency in NK cells may affect T cell function, as described in the context of LCMV infection.

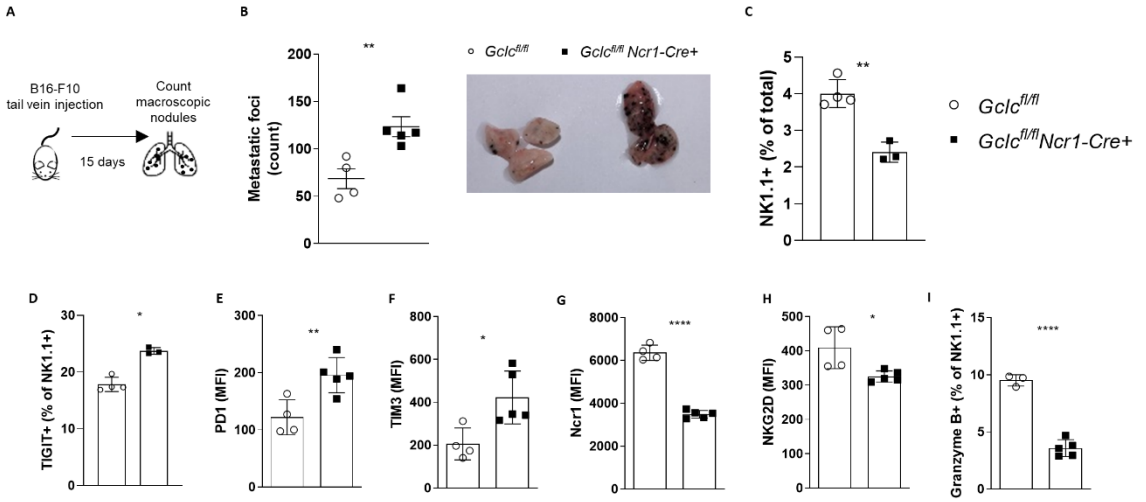

Figure 19 – NK cell-dependent control of metastatic surveillance is reduced in *Gclc$^{fl/fl}$ Ncr1-Cre+ mice.*

B16-F10 cells were injected intravenously into *Gclc$^{fl/fl}$ and Gclc$^{fl/fl}$ Ncr1-Cre+* mice **(A)** Schematic representation of the B16-F10 dissemination model, in which mice are sacrificed 15 days post-inoculation and macroscopic metastatic nodules are counted per lung. **(B)**. Left: Number of metastatic foci per lung of *Gclc$^{fl/fl}$ and Gclc$^{fl/fl}$ Ncr1-Cre+* mice. Right: one representative example of an infiltrated lung of the indicated genotype is shown. **(C-I)** Tumor infiltrating lymphocytes (TILs) isolated from the lungs of tumor-bearing mice and immediately treated for flow cytometry analysis. **(C)** Quantification frequencies of total CD3$^-$ NK1.1$^+$ NK cells from tumor. **(D)** Quantification of frequencies of total CD3$^-$ NK1.1$^+$ TIGIT$^+$ NK cells. Flow cytometric analysis of the expression of **(E)** PD-1 **(F)** TIM3 **(G)** Ncr1/NKp46 **(H)** NKG2D, where MFI represents mean fluorescence intensity. **(I)** Quantification of flow cytometry analysis of Granzyme B$^+$ CD3$^-$ NK1.1$^+$ NK cells isolated from TILs of the lung of tumor bearing mice and treated with PMA/Ionomycin and GolgiPlug for 4h. Data are mean ± SD (n=3-5) and are representative of at least 2 independent trials.*P<0,05

15. Inability to control tumor dissemination is an intrinsic characteristic of NK cells lacking of GSH

The adaptive immune system plays a fundamental role in governing anti-tumor control (Shankaran et al., 2001; Smyth et al., 2001c) and NK cells can regulate T and B cell function (Böttcher et al., 2018; Rydyznski et al., 2015; Schuster et al., 2014). Therefore, we investigated whether the defective NK cell function observed in the absence of GSH would affect T and B cell presence in the tumor. Lymphocytes isolated from lungs of tumor-bearing *Gclc*$^{fl/fl}$ and *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ showed a similar frequency of CD3^{+} T cells (Figure 20A). Furthermore, no significant difference was observed in the proportion of the different subsets of CD4+ and CD8+ T cells: naïve (single positive CD62L^{+}), effector/effector memory (single positive CD44^{+}) and central memory (CD62L^{+} CD44^{+}) (Figure 20B, C). Similar results were observed for the tumor-infiltrating CD19^{+} B cells (Figure 20D).

In order to assess more thoroughly the NK cell-mediated response against metastatic tumors, we crossed *Rag-1*-deficient mice, that lack functional T and B cells, with *Gclc*$^{fl/fl}$ (*Rag-1*$^{-/-}$ *Gclc*$^{fl/fl}$) and *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ (*Rag-1*$^{-/-}$ *Gclc*$^{fl/fl}$ *Ncr1-Cre*+). These mice were challenged with i.v. inoculation of B16-F10. Rag-1$^{-/-}$ Gclc$^{fl/fl}$ had extremely low numbers of metastatic foci (Figure 20E), in line with what has been described in previous studies for Rag-1$^{-/-}$ mice (Cuff et al., 2010). However, *Rag-1*$^{-/-}$ *Gclc*$^{fl/fl}$ *Ncr1-Cre*+, lacking both an adaptive immune system and GSH-sufficient NK cells, showed a drastic increase in tumor burden (Figure 20E).

Collectively, our data reveal that, in contrast to LCMV infection, tumor inoculation in *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice does not elicit a differential T cell response, when compared to WT controls. TILs isolated from mice lacking *Gclc* specifically in NK cells, have a similar frequency of T cells. Most importantly, in the absence of the adaptive immune system, *Rag-1*$^{-/-}$ *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ showed an increased metastatic burden, suggesting an intrinsic role for GSH for the regulation NK cell-mediated anti-tumor response. This is specially important in the light of the TME constraints for immune effector function, where abundant levels of ROS and/or nutrient depletion, amongst other factors, can affect GSH availability and redox regulation of NK cells, interfering with their functionality.

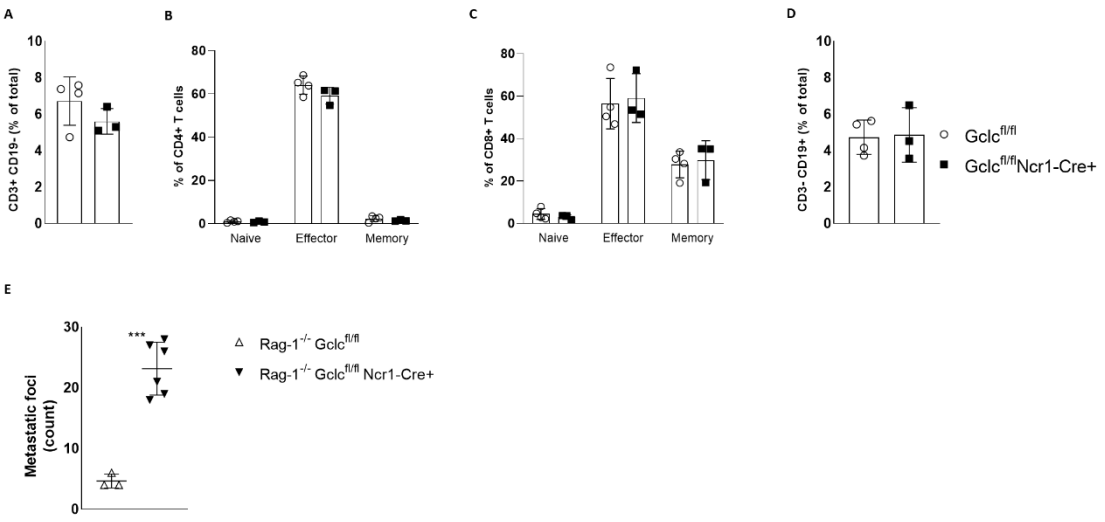

Figure 20 – The incapacity of *Gclc$^{fl/fl}$ Ncr1-Cre*+ mice to control metastatic dissemination is intrinsic to NK cells.

(A-D) Tumor infiltrating lymphocytes (TILs) were isolated from the lungs of *Gclc$^{fl/fl}$ and Gclc$^{fl/fl}$ Ncr1-Cre*+ mice 15 days after intravenous injection with B16-F10 melanoma cells. Flow cytometry analysis of **(A)** Frequencies of total CD3$^+$ CD19$^-$ T cells from TILs. **(B)** Frequencies of naïve (CD62L$^+$), effector (CD44$^+$) and memory (CD62L$^+$ CD44$^+$) within CD4$^+$ T cells and **(C)** within CD8$^+$ T cells from TILs. **(D)** Frequencies of total CD3$^-$ CD19$^+$ B cells in TILs.

(E) B16-F10 cells were injected intravenously into *Rag-1$^{-/-}$ Gclc$^{fl/fl}$ and Rag-1$^{-/-}$ Gclc$^{fl/fl}$ Ncr1-Cre*+ mice post-inoculation. Numbers of macroscopic metastatic foci per lung were counted after 15 days. Data are mean ± SD (n=3-4) and are representative of at least 2 independent trials.

16. Nutrient deprivation of NK cells results in impaired GSH production and reduced function

It is known that high metabolic demands of cancer cells elicit dysfunctional angiogenesis and restrict the availability of essential nutrients in solid tumors (Bertout et al., 2008). Low oxygen availability induces the activation of glycolysis and glutaminolysis pathways in cancer cells (Elstrom et al., 2004; Sun and Denko, 2014), draining glucose and glutamine from the interstitial fluid and depriving effector immune cells of glucose and glutamine (Ho et al., 2015; Son et al., 2013). Low nutrient availability and TME acidification, due to increased glycolysis and lactate production, have shown to negatively impact T and NK cell antitumor capacity (Brand et al., 2016; Cham et al., 2008; Chang et al., 2015; Leone et al., 2019). Indeed, TME nutrient deprivation correlates with poor prognosis and lower patient survival (Le et al., 2006). Low glutamine levels also affect cellular redox regulation since glutamine-derived glutamate is required for *de novo* GSH synthesis, in an ATP consuming process (Lu, 2013; Yuneva et al., 2007). Therefore, we hypothesized that nutrient deprivation of tumor infiltrating NK cells may interfere with GSH production and, hence, reduce their metabolic fitness and functional capacity in the TME.

To test this hypothesis, we cultured WT NK cells isolated from C57BL/6 mice in glucose- and glutamine-free medium and activated them with IL-12, IL-15 and IL-18 for 3 days. Nutrient-deprived activated NK cells had significantly reduced levels of GSH, when compared to their counterparts cultured in glucose and glutamine-sufficient medium (Figure 21A). In line with our observations for *Gclc*$^{fl/fl}$ *Ncr1-Cre+* mice, nutrient-deprived NK cells showed decreased energetic capacity, depicted by the lower intracellular ATP levels (Figure 21B) and impaired activation of the mTOR signaling pathway, defined by pS6 levels (Figure 21C). Furthermore, we observed that activation of NK cells in glucose and glutamine-deprived medium leads to decreased production of IFN-γ (Figure 21D). Collectively, these results demonstrate that nutrient deprivation in the TME can interfere with redox regulation of NK cells by lowering their capacity of GSH production, which culminates in the impairment of NK cell metabolic regulation, by reduction of mTOR activity, and antitumor function, by lower IFN-γ production. These preliminary findings merit further investigation, particularly in what concerns the impact of the TME in GSH deficiency in NK cells. As evidenced by our genetic model, NK cells from *Gclc*$^{fl/fl}$ *Ncr1-Cre+* mice show metabolic defects that affect their activation, proliferation and functional capacity, which drive NK cell exhaustion and hinder tumor control. A combinatorial strategy involving metabolic modulation of the TME could improve redox regulation of NK cells, enhance GSH availability and contribute to NK cell-targeted immunotherapeutic approaches (André et al., 2018; Guerra et al., 2020; Zhang et al., 2018b).

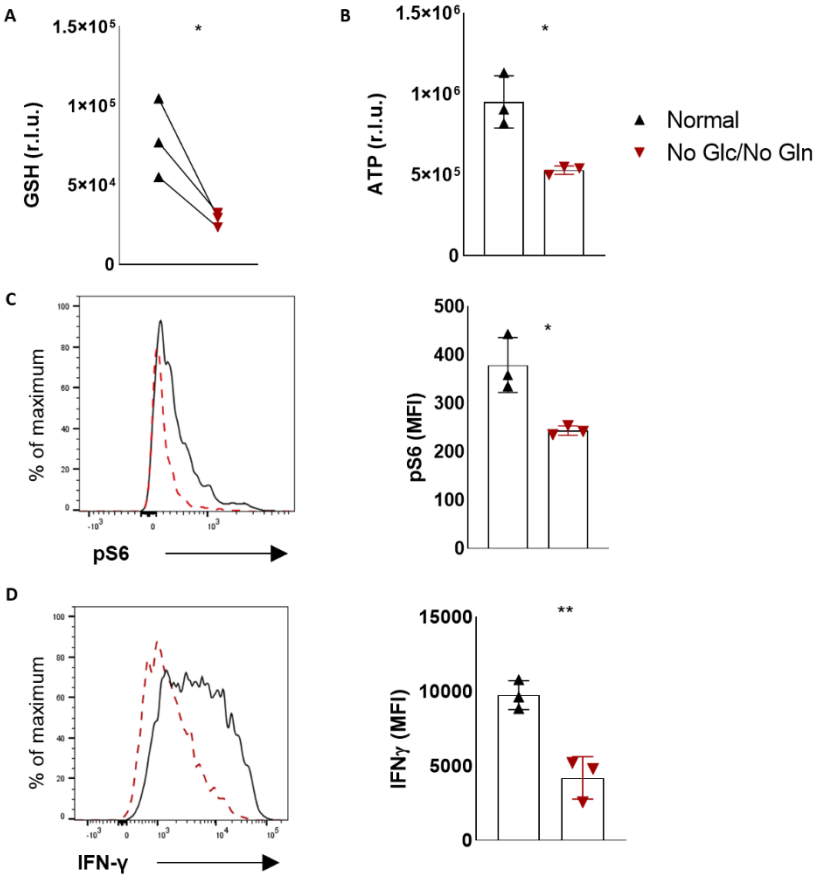

Figure 21 – Glucose and glutamine deprivation deplete GSH pool of NK cells and affect mTOR signaling and IFN-γ production.

Splenic NK cells isolated from C57BL/6 mice were cultured in supplemented (Normal) and glucose (Glc) and glutamine (Gln)-deficient (No Glc/No Gln) media. **(A)** Quantification of GSH by luminescence-based assay. Data are expressed in r.l.u. **(B)** Quantification of ATP by luminescence-based assay. Data are expressed in r.l.u. **(C)** Left: representative histogram of intracellular pS6 levels assessed by flow cytometry. Right: quantification of the data in the left panel. (D) Left: representative histogram of IFN-γ levels by flow cytometry analysis. Right: quantification of the data in the left panel. Data are mean ± SD (n=3) and are representative of at least 2 independent trials.

V. Discussion and Perspectives

NK cells are innate lymphocytes that participate in the control of viral infections and tumors (Vivier et al., 2008), which constitute highly oxidative environments (Piskounova et al., 2015; Schwarz, 1996). It is known that ROS mediate signaling events that are essential for B and T cell activation, proliferation and differentiation (Devadas et al., 2002; Singh et al., 2005). Nevertheless, to date, ROS signaling was not directly associated with NK cell activation. On the contrary, NK cell cytotoxic capacity is suppressed in the presence of exogenous ROS (Aydin et al., 2017; Mellqvist et al., 2000; Seaman et al., 1982; Viora et al., 2001). ROS-mediated inhibition of IFN-γ or TNF-α production was reversed by treatment of NK cells with antioxidants, such as catalase, histamine or GSH (Kono et al., 1996; Mellqvist et al., 2000). In order to buffer the increasing intracellular ROS levels upon activation, derived, for example, from mitochondrial metabolism, NK cells upregulate distinct antioxidant pathways upon activation (Siernicka et al., 2015; Yang et al., 2020). GSH is one of the most abundant intracellular thiols and it was shown to be important for T cell activation (Kurniawan et al., 2020; Mak et al., 2017). Despite an early association of reduced GSH levels in HIV infection and a reduced NK cell-dependent *M. tuberculosis* clearance in these patients (Millman et al., 2008), the current understanding of the GSH pathway in NK cells is limited. The present study unraveled a major contribution of GSH for NK cell function and homeostasis in a genetic model of NK cell-specific *Gclc* ablation. We observed that, upon abrogation of GSH production, NK cells adopted a hyporesponsive phenotype, characterized by reduced activation and a generalized metabolic failure, culminating in a defective control of metastatic response *in vivo*.

- **GSH is a checkpoint for NK cell activation**

At steady state, *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice showed reduced frequency of NK cells in the spleen and lungs, possibly due to lower numbers of mature NK cells arising from the bone marrow. Nevertheless, the maturation status of NK cells in the peripheral organs of these mice was not affected. The distribution of the distinct subsets, based on the expression of the markers CD27 and CD11b, was comparable to their WT counterparts.

Most notably, prior to activation, *Gclc* deletion in splenic NK cells, did not result in increased ROS levels. However, upon IL-15-induced activation, *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ NK cells, when compared to Gclc$^{fl/fl}$ controls, showed reduced size and defective proliferation. Ablation of GSH production resulted in a defective cytotoxic capacity against YAC-1 target cells, characterized by a lower production of IFN-γ and Granzyme B.

It should be noted that our investigation did not comprise the effects of early signaling driven by the downstream effectors of the IL-15 receptor. We are aware of the limitations of studying the outcome of this cytokine on NK cell functionality after 72h of culture. Currently,

we cannot rule out that the effects of mitochondrial ROS accumulation may underlie an impaired intermeadiate early IL-15 signaling, in the absence of GSH. It was shown that the priming of NK cells with IL-15 could enhance their function as short as 5 minutes after the start of stimulation (Luu et al., 2021). Notably, the increased degranulation and IFN-γ production was only observed upon crosslinking of NK cell activating receptors or IL-12/IL-18 stimulation. The influence of short term IL-15 priming is mainly based on the amplification of other activating signaling pathways. Nevertheless, there is augmented activity of the downstream signaling mediators mTOR, STAT5 and ERK, essential for maintaining NK cell function. Remarkably, despite the early activation of downstream targets of mTOR, S6 and 4EBP1, transitory priming with IL-15 does not increment the oxidative phosphorylation capacity nor the glycolytic rate of murine NK cells (Luu et al., 2021). Thus, we would assume that, although it would be interesting to dissect the early signaling of IL-15 priming, the considerable effects of the absence of GSH in NK cell metabolism would not be so evident.

Surprisingly, absence of GSH did not induce the accumulation of cytosolic ROS in activated NK cells. This suggests the presence of alternative antioxidant mechanisms in NK cells that can maintain redox balance, compensating for the absence of GSH. Specifically, peroxiredoxin or thioredoxin, both upregulated upon activation, were shown to be relevant for maintenance of NK cell function (Neumann et al., 2003; Siernicka et al., 2015; Yang et al., 2020). Nonetheless, mitochondria are the major source of intracellular ROS. ATP generation during OXPHOS can result in leakage of a small fraction of electrons from the electron transport chain (ETC). Due to damaging effects of accumulation of oxygen radicals, the compartmentalization of GSH in mitochondria is critical for maintenance of their function and cell survival (Ribas et al., 2014). Indeed, activation of GSH-deficient NK cells resulted in a significant accumulation of mitochondrial ROS, when compared to *Gclc*$^{fl/fl}$ NK cells. These results reveal an organelle-specific redox regulation in NK cells, where GSH is particularly important for maintaining mitochondrial ROS in check.

- **GSH controls NK cell mitochondrial fitness, metabolism and mTOR**

Accumulation of ROS in mitochondria can result in defects in mitochondrial function, given ROS reactivity against lipids, proteins and nucleic acids (Ribas et al., 2014). Accordingly, NK cells from *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice showed reduced mitochondrial activity, when compared to their WT counterparts. The decreased activity was associated with an increased mitochondrial size. The shift towards mitochondrial biogenesis is a compensatory mechanism for the loss of function, in order to maintain ATP levels adequate to cellular homeostasis (Chen et al., 2010). Despite the compensatory mitochondrial proliferation, *Gclc* deletion in NK cells resulted in decreased ratio of activity per mass of mitochondria, which has been described in exhausted tumor-infiltrating T cells (Yu et al., 2020). These results

highlight the importance of GSH for the maintenance of mitochondrial fitness in NK cells. Furthermore, they are in line with the exhaustion phenotype observed in *Gclc*$^{fl/fl}$ *Ncr1*+ NK cells infiltrating the TME, where absence of GSH led to an increased expression of the inhibitory receptors TIGIT, PD-1 and TIM-3.

Paralleled with reduced mitochondrial fitness, *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice showed a reduced fatty acid and amino acid oxidation capacity and glycolytic capacity upon IL-15 activation. As NK cells rely on both OXPHOS and glycolysis for functional activity upon cytokine-induced activation (Donnelly et al., 2014; Marçais et al., 2014), *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ NK cells had a significantly reduced ATP pool. Interestingly, the energetic profile of NK cells deficient for GSH is comparable to the simultaneous inhibition of OXPHOS and glycolysis in WT controls. In sum, our results unravel GSH as a key metabolic checkpoint for the metabolic maintenance of NK cells upon cytokine stimulation, as it was described for T cells (Mak et al., 2017).

In line with a defective metabolic capacity, NK cells lacking GSH showed reduced phosphorylation of S6 kinase, a downstream target of mTOR. The latter is a serine/threonine kinase that integrates distinct extracellular cues: its activation is dependent on the environmental availability of nutrients and growth factors, as well as antigenic and inflammatory signals. mTOR is part of two distinct complexes, mTORC1 and mTORC2. Whereas mTORC2 is involved in Akt phosphorylation and controls cytoskeleton organization, mTORC1 regulates protein synthesis by signaling through 4EBP1 and pS6. Furthermore, mTORC1 induces the expression of HIF-1α and c-MYC, which are mediators of glycolysis induction (Liu and Sabatini, 2020). In *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ NK cells, lower levels of pS6 were associated to a significantly reduced protein translation, measured by puromycin incorporation. These results are in line with previous studies unravelling the essential role for mTOR in IL-15-induced NK cell activation (Marçais et al., 2014). Similar to *Mtor*-deficient cells, ablation of GSH resulted in lower expression of transporters of glucose, amino acids and transferrin, Glut1, CD98 and CD71, respectively. Additionally, genetic deletion of mTOR or *Gclc* affected NK cell proliferative capacity and effective expression of Granzyme B and IFN-γ (Marçais et al., 2014). These results highlight an alternative level of mTOR regulation in NK cells through GSH activity.

It is important to mention that IL-15 stimulation of NK cells drives the activation of extracellular signal-regulated kinase (ERK) signaling cascade, through the Ras/Raf/MAPK pathway (Jiang et al., 2000; Niogret et al., 2019). In cancer cells, it has been proposed that ERK can promote phosphorylation of the kinase S6, in an mTOR-independent manner (Roux et al., 2004; Roux et al., 2007). We have not measured ERK activation and, thus, cannot disregard the possibility of a lower phosphorylation of S6 in *Gclc*-deficient NK cells

driven by lower ERK signaling. Given that pharmacological inhibition of GCLC with BSO induces a significant reduction of mTOR itself, we would hypothesize that, if confirmed, ERK-mediated regulation of S6 would be an additional mechanism of GSH-dependent modulation of NK cell function.

Neverheless, the results obtained in activated NK cells recapitulate the effects of *Gclc* deletion in effector T cells, where lower expression of pS6 resulted in a reduction of glycolysis and glutaminolysis (Mak et al., 2017). In contrast, in Tregs, GSH deficiency induced mTOR activation and increased metabolic activity, which was linked to a defective suppressive capacity and lower Foxp3 expression (Kurniawan et al., 2020). These results highlight the importance of the antioxidant GSH for NK cell's energetic capacity and give further insight into the cell-specific regulation of the GSH/mTOR axis.

- **GSH fosters STAT5 activation, which is essential for NK cell homeostasis**

In addition to mTOR, STAT5 constitutes an alternative and non-redundant level of regulation of NK cell homeostasis (Eckelhart et al., 2011; Gotthardt et al., 2016). In fact, at high concentrations of IL-15, STAT5 and S6 phosphorylation are upregulated in a similar manner. Interestingly, STAT5 phosphorylation was decreased in the absence of mTOR (Marçais et al., 2014). Furthermore, it was shown that simultaneous mTOR and STAT5 inhibition was necessary to reduce the cytotoxic capacity of IL-15 activated human NK cells against K-562 (Mao et al., 2016). According to the regulator activity of mTOR and STAT5 in NK cells, we observed that, *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ NK cells showed a decreased phosphorylation of STAT5.

Wang et al. characterized a direct link between mTOR and STAT5 regulation, which is essential for NK cell maturation and effector function. Genetic deletion of mTORC2 in NK cells resulted in induction of mTORC1 signaling, which was followed by an increased pSTAT5 activity and heightened abundance of the amino acid transporter SLC7A5 (Wang et al., 2018). In our study, we have not investigated the role of mTORC2 signaling in *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice. Thus, it would be of great interest to investigate a GSH-dependent regulation of mTORC2/mTORC1/pSTAT5 axis in NK cells.

Nevertheless, we observed that *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ NK cells had arrested cell cycle progression in the G0/G1 phase. This is in line with different studies linking IL15, mTOR and STAT5 to NK cell cycle progression, in agreement with their role in enhancing cell growth and proliferation (Mao et al., 2016; Marçais et al., 2014). The STAT5-dependent cell cycle regulation does not seem to be exclusive for NK cells and participates in IL-2-induced cell cycle progression of T cells, for example (Moriggl et al., 1999).

In *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ NK cells, the halt in cell cycle progression may be linked to a lower capacity for protein synthesis seen in these GSH-deficient NK cells, measured by puromycin incorporation. mRNA translation is regulated by the downstream target of mTOR, 4E-BP1 and it influences cell proliferation (Fingar et al., 2004; Liu and Sabatini, 2020). The absence of GSH inhibits the mTOR/STAT5 signaling in NK cells and arrests in proliferation and cell growth. These results could explain the lower numbers of mature NK cells in the bone marrow and reduced NK cell frequency in the peripheral organs of *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice. Immature NK cells proliferate to give rise to more mature NK cells and, therefore, show enriched transcription of genes related with cell cycle and ribosome (Chiossone et al., 2009; Marçais et al., 2014). Hence, GSH absence may hinder cell cycle progression of more immature NK cells that cannot proliferate in the bone marrow, depleting the peripheral organs from NK cells. The quiescent state of *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ NK cells may also contribute to the lower functional capacity of these effector cells observed in the LCMV infection and tumor dissemination models.

- **The molecular regulation of GSH/mTOR/STAT5 axis in NK cells is still yet to be determined**

How GSH can specifically regulate the mTOR/STAT5 axis in NK cells and whether it is a ROS-dependent or independent mechanism remains unsolved. In effector T cells, GSH deficiency negatively affected c-Myc expression which rendered them unable to perform a metabolic switch towards a catabolic phenotype (Mak et al., 2017). Indeed, low levels of mTOR in NK cells are linked to lower c-Myc expression, which is essential for NK cell metabolic and functional responses (Loftus et al., 2018). In NK cells, mTOR signaling activates the endoplasmic reticulum stress sensor inositol-requiring enzyme 1 (IRE1α) and X-box-binding protein 1 (XBP1), which induces c-Myc translation (Dong et al., 2019). c-Myc activation is sustained by transport of glutamine through SLC7A5 (Loftus et al., 2018), which would activate mTORC1. As NK cells derived from *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice have a decreased activation status, SLC7A5 may be downregulated, which would explain the defective mTOR/STAT5 signaling (Jensen et al., 2017; Keating et al., 2016; Wang et al., 2018).

Furthermore, the increased mitochondrial ROS induced by lack of GSH may also participate in the energetic regulation of NK cells. Mitochondrial ROS may trigger AMPK activation and inhibit mTOR activity and further protein translation, similar to what has been described in other cells during hypoxia (Liu et al., 2006). Alternatively, lower ATP levels due to mitochondrial dysfunction could induce AMPK activation, which subsequently inactivates mTORC1 activity, through the inhibition of TSC2 or RAPTOR (Gwinn et al., 2008; Inoki et al., 2003). AMPK also activates the transcription factor forkhead box O 3 FOXO3 (Greer et

al., 2007), which was associated with a lower NK cell function (Huntington et al., 2007a). In general terms, oxidative stress activates FOXO-mediated transcription (Hedrick, 2009), linked to negative regulation of NK cell maturation and function (Deng et al., 2015b; Huang et al., 2019).

Additionally, mitochondrial ROS were implicated in the dysregulation of the disulfide proteome and in the inhibition of phosphatases, both essential regulators for general cell signaling and homeostasis (Kamata et al., 2005; Yang et al., 2007). For example, the phosphatase Shp-2 was deemed essential for IL-15-mediated NK cell activation, through the activation of ERK and mTOR signaling cascades and engagement of glycolysis and OXPHOS (Niogret et al., 2019). Interestingly, NK cell-specific deletion of Shp2 did not alter Jak-STAT5 signaling (Niogret et al., 2019). However, we have not tested Shp-2 activity in GSH-deficient NK cells.

Either through ROS-dependent or –independent mechanisms, GSH seems to be a key metabolic regulator of NK cell homeostasis and function. The ablation of this antioxidant pathway is implicated in a dysregulation of the mTOR/STAT5 axis in NK cells, which induces a profound metabolic impairment, characterized by the inhibition of OXPHOS and glycolysis.

- **GSH deficiency abrogates the NK cell-mediated regulation of LCMV-specific T cell response**

In vivo, the hyporesponsiveness of GSH-deleted NK cells was corroborated in a model of viral infection. Upon challenge with LCMV-WE, *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice showed an unleashed anti-viral T cell response. The immune response against LCMV is T cell-dependent. Nonetheless, NK cells have been shown to play an important regulatory role on the T cell response. During the first days of chronic LCMV infection, the absence of NK cells resulted in an enhanced ability of APCs to stimulate CD8+ T cell proliferation ex vivo (Cook and Whitmire, 2013). Interestingly, the authors attributed this phenotype to a potential NK cell-dependent cytotoxic on infected APCs (Cook and Whitmire, 2013), and a similar mechanism was suggested for MCMV infection (Andrews et al., 2010). Upon challenge with LCMV, NK cells directly eliminate $CD4^+$ T and $CD8^+$ T cells, modulating the T cell-driven immunopathological effects of the virus and, most importantly, preventing chronic viral infection (Lang et al., 2012; Lu et al., 2007; Waggoner et al., 2012). NK cell specific ablation of GSH increased the frequency of LCMV-tetramer+ CD8+ T cells, which also had an increased production of IFN-γ. NK cells from LCMV-challenged *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice showed a lower YAC-1 killing capacity, when compared to WT. This could explain the rise of highly active anti-viral T cells. In addition, GSH deficiency in NK cells resulted in lower

expression of the receptor NCR1/Nkp46, which negatively modulates CD8+ T cell response in this viral model (Duhan et al., 2019; Pallmer et al., 2019). Furthermore, NK cells were also shown to regulate antibody production by B cells trough regulation of T follicular helpers(T_{FH}) in the germinal center (Cook et al., 2015), in a perforin-dependent manner (Rydyznski et al., 2015). It would be interesting to measure antibody levels in the serum of LCMV-infected mice, in order to investigate whether GSH in NK cells could also affect T_{FH} response and, consequently, humoral immunity. In sum, we speculate that reduced GSH levels in NK cells impede them from exerting their cytotoxicity against infected APCs and/or highly activated CD8+ T cells upon LCMV challenge. These results highlight the importance of GSH NK cell-dependent T cell regulation in the context of LCMV infection.

Interestingly, in CMV seropositive individuals, resistance to oxidative stress was associated with persistence and functional maintenance of adaptive NK cells. CD38, a NADase that degrades NAD+, a necessary cofactor for ROS scavenging proteins, is downregulated in adaptive NKG2C+ NK cells from CMV seropositive donors, which were shown to be more resistant to oxidative stress (Cichocki et al., 2019). It would be of great interest to study if GSH activity is important to regulate the presence of adaptive NK cells and creation of memory in these innate lymphocytes.

- **Our results suggest a role of NK-mediated ROS in autoimmunity**

The question that imposes is: why is it beneficial for the host NK cells to hinder anti-viral T cell response and humoral immunity? During LCMV infections, NK cells are able to prevent an exacerbated T cell response and diminish T cell-mediated immunopathology, which is key to prevent T cell exhaustion and chronic infection (Cook et al., 2015; Lang et al., 2012; Waggoner et al., 2012). However, the regulatory role of NK cells is not limited to the mouse model of LCMV infection. Namely, upon MCMV infection, where NK cells can directly exert cytotoxicity against MCMV-infected cells (Daniels et al., 2001), it was also described that NK cells can specifically eliminate activated $CD4^+$ T cells in a TRAIL-dependent manner, reducing autoimmunity (Schuster et al., 2014).

NK cell-dependent regulation of immune tolerance and autoimmunity prevention has also been evidenced in other contexts. For example, during stem cell transplantation, increased numbers of IL10-producing NK cells inversely correlated with the establishment of T cell-mediated graft versus host disease (GvHD) (Chan et al., 2018; Ruggeri et al., 2002). Additionally, in EAE, the experimental model for multiple sclerosis (MS), impaired recruitment of NK cells into the central nervous system was associated with increased disease burden and mortality (Huang et al., 2006). Notably, daclizumab, a monoclonal antibody targeting CD25 (IL-2Rα chain), used in the treatment of MS, seems to induce a

population of regulatory NK cells that inhibit T cell survival (Bielekova et al., 2006) and, therefore, ameliorate the disease.

In MS, one of the bona fide treatments for relapsing-remitting patients is dimethyl fumarate (DMF). DMF's mechanism of action is based on the activation of the transcription factor NRF2, which is essential in redox homeostasis and response to ROS (Linker et al., 2011; Suzuki et al., 2013). Indeed, treatment with DMF increased NK cell count in MS patients. We hypothesize that, in line with our findings, the activation of ROS scavenging programs in NK cells may induce their capacity of regulating highly activated T cells in MS. The elimination of auto-reactive T cells ameliorates the symptoms of the disease and contributes to the efficacy of DMF in MS patients. In sum, clinical data underlines the importance of antioxidant-dependent mechanisms for NK cell-mediated regulation of T cell response in autoimmunity and may be applied to viral chronic infections.

- **Increased ROS in NK cells promote metastatic dissemination**

Concerning the role of GSH in NK-mediated anti-tumor response, the primary tumor models generated unexpected evidence. Despite the impaired energetic and functional profile of NK cells from *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice, the subcutaneous injection of the melanoma cell-line B16-F10 did not result in increased tumor burden, when compared to their WT controls. A similar outcome was observed upon challenge with a more immunogenic tumor, the adenocarcinoma MC-38 (Efremova et al., 2018). Yet, when we used an experimental model for metastatic dissemination, through the intravenous injection of B16-F10, *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice had a significant increase in lung metastatic lesions, when compared to *Gclc*$^{fl/fl}$ controls. Metastasis account for around 90% of cancer-related deaths (Chaffer and Weinberg, 2011) and our results highlight the importance of redox homeostasis for NK cell function and metastatic control and raise questions concerning the modulation of NK cell-mediated anti-tumor control.

NK cell intrinsic mechanisms

First, it is key to investigate whether NK cells are essential for the control of primary tumors (López-Soto et al., 2017). These innate lymphocytes mediate the immune response against hematopoietic neoplasia (Ilander et al., 2017; Street et al., 2004) and have risen as crucial for new therapeutic approaches in hematological malignancies (Lamb et al., 2021). *In vitro,* NK cells are also able to establish targeted cytotoxicity against virtually any cancer cell and *in vivo* NK cell depletion results in heightened tumor incidence (Smyth et al., 2000). Additionally, NK cell-mediated anti-tumor response is evidenced by the positive prognostic value of NK cell infiltration in certain solid tumors (Imai et al., 2000). However, there are results akin to ours, where NK cells have distinct responses against primary and metastatic

tumors in mouse models of tumor development. Namely, NKG2D-deficient mice show a higher incidence of lymphoma and prostate carcinoma, but are as sensitive to chemical-induced carcinogenesis as their wild-type counterparts (Guerra et al., 2008). Likewise, Toll like receptor 3 (Tlr3) and Ncr1 knock out mice present an increased metastatic burden, although they do not differ in their sensitivity to primary tumor incidence, when compared to WT (Glasner et al., 2012; Guillerey et al., 2015). Interestingly, it was also described that upon NK cell-specific deletion of STAT5, tumor burden was increased in an experimental model of i.v. injection of B16F10, but not upon s.c. injection of MC-38 (Eckelhart et al., 2011). Our study adds to this set of previously published results, where NK cell-specific GSH absence affected only the control of metastatic dissemination, but not primary tumor growth. The reason behind these differences has not yet been unraveled and more studies are necessary to decipher the role of NK cells in the control of primary vs. metastatic cancer.

Notably, at steady state, we observe a deficient number of NK cells in the lungs of *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice. Thus, we cannot rule out the possibility that the increased tumor burden of GSH-deficient mice arises from a decreased NK cell presence. This would reduce the ratio of effector to target cells and affect the elimination of tumor cells. This premise could only be proven by transferring the same number of NK cells from *Gclc*$^{fl/f}$ and *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice to NK cell-depleted wild type hosts, which would be challenged with intravenous inoculation of B16-F10. Nevertheless, given our *in vitro* observations, we hypothesize that the low expansion and reduced cytotoxic capacity of GSH-deficient NK cells would outweigh the effect of their reduced presence in peripheral organs.

It is important to note that the divergent outputs between primary and metastatic tumors models in *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice can be reasoned by the existence of a distinct microenvironment in the two sites.

Lymphocyte infiltration and ROS

First, lymphocyte infiltration seems to differ between primary and metastatic tumors (Hendry et al., 2017), although with controversial observations, depending on the type of tumor. As an illustrative example, in studies of breast carcinoma, lower levels of CD4+ and CD8+ TILS are present at metastatic sites than in primary tumors, which supports the concepts of *immune escape* and *immune editing* in tumor progression (Ogiya et al., 2016). In contrast, in melanoma patients, lung metastasis showed a significantly higher infiltration of TILs, when compared to non-lung tissue (Ben-Avi et al., 2016). In our experimental model, higher lymphocytic infiltration in the lungs permits the emphasis on NK cell dysfunction induced by

GSH deficiency. This is particularly evident in the i.v. inoculation of B16-F10 cells in *Rag*-deficient *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice, that displayed an increased tumor burden.

Of outmost importance, it was recently shown that higher levels of the antioxidant thioredoxin conferred NK cells greater capacity to infiltrate the core of the tumor (Yang et al., 2020). Analysis of datasets from non small cell lung cancer, where ROS levels in the TME are directly related to a smoking signature, showed that NK cells accumulate in the periphery of tumor from patients with smoking history, worsening the disease prognosis (Yang et al., 2020). Therefore, we can speculate that a reduced capacity to regulate redox balance hinders *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ NK cells to infiltrate the primary subcutaneous tumor, unleashing T cell effector function.

Kynurenine, lactate and ROS

Furthermore, glutathione ablation may induce a higher susceptibility of NK cells to different TME factors that culminate in higher levels of ROS. Namely, kynurenine, can induce apoptosis of NK cells in a ROS-dependent manner (Song et al., 2011). NK cells are depleted from liver metastasis of colorectal cancer patients due to an accumulation of lactate in the TME, which causes mitochondrial ROS accumulation and apoptosis of NK cells (Harmon et al., 2019). In line with these findings, previous studies have shown that the direct release of ROS by myeloid cells can inhibit NK cell function (Aydin et al., 2017; Hellstrand et al., 1994; Kono et al., 1996; Mellqvist et al., 2000). Interestingly, Piskounova and colleagues observed that, in human melanomas, ROS levels were significantly increased in circulating melanoma cells and metastatic nodules as compared to subcutaneous tumors (Piskounova et al., 2015). Therefore, *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice, which lack GSH, a major ROS scavenger, can be more susceptible to the higher levels of ROS in circulating and lung melanoma cells, upon i.v. injection. On the contrary, in the primary, subcutaneous tumor, lower ROS levels are not able to induce such strong NK cell dysfunction, explaining the primary tumor growth rate of *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice comparable to WT controls.

Nutrient availability and ROS

In addition to a distinct ROS modulation, the metabolic environment may vary between the primary tumor and metastasis (Bergers and Fendt, 2021). Gene expression profiles of primary and corresponding metastatic lesions evidence great heterogeneity between the sites of tumor origin (Sullivan et al., 2019). For example, it was shown that invasive ovarian cancer and melanoma cells heavily rely on glutamine for their survival (Rodrigues et al., 2016; Yang et al., 2014). This supports the notion that nutrient availability and metabolism affect the nesting of metastatic cancer cells in distant organs and the outcome of the immune response (Fischer et al., 2019; Sullivan et al., 2019).

In the tumor, lower levels of glucose and glutamine drive T cell metabolic insufficiency and dysfunction (Ho et al., 2015; Leone et al., 2019; Scharping et al., 2016; Siska et al., 2017). In the same way, for NK cells, nutrient availability seems to be pivotal for their anti-tumor capacity (Loftus et al., 2018). In different murine models of lung metastasis, it was shown that ILC2-mediated eosinophilia leads to glucose restriction in the TME and exacerbated production of lactate. This results in restrained NK cell activity, paralleled by a lower expression of IFN-γ, Granzyme B and mTOR signaling (Schuijs et al., 2020). Furthermore, the TME can hamper NK cell glycolytic metabolism due to induction of fructose-1,6-biphosphatase (FBP1) expression in NK cells, which impairs their viability and anti-tumor response. Pharmacological inhibition of FBP1 enhanced NK cell cytotoxic response, while decreasing ROS generation in the TME. Adoptive transfer of NK cells treated with FBP1 inhibitor significantly slowed tumor growth (Cong et al., 2018). Furthermore, inhibiting glutaminolysis by cancer cells promotes glucose consumption in NK cells, boosting their cytotoxic function (Cichocki et al., 2017; Loftus et al., 2018; Parameswaran et al., 2016) (NCT03081780, NCT01632306). Thus, we can speculate that distinct metabolic availability of the primary and metastatic tumors can differentially influence NK cells from *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice and affect, or not, their tumor control.

Further, our results regarding nutrient deprivation provide an additional level for understanding metabolic and functional regulation of NK cells by antioxidants. Withdrawal of glutamine and glucose, upon cytokine-induced NK cell activation, resulted in decreased intracellular levels of GSH. Importantly, nutrient-deprived NK cells had a reduced ATP pool and decreased IFN-γ production, paralleled by a lower mTORC1 activity. Interestingly, the phenotype induced by nutrient deprivation mimics the genetic deletion of GSH in NK cells. These results link nutrient availability in the TME, redox regulation of NK cells and their functional capacity. Our observations are in line with a recent study on cancer patient samples demonstrating that NK cell dysfunction in the TME arises from inhibition of glycolysis and OXPHOS induced by oxidative stress. Pharmacological activation of NRF2, a central regulator of antioxidant defense, restored NK cell anti-tumor activity (Poznanski et al., 2021). Interestingly, culturing NK cells from healthy individuals with a IL-21-expressing feeder cell line, drives the expansion of STAT3+ NK cells, with increased expression of proteins involved in serine synthesis, one-carbon and folate metabolism, contributing to an reduced oxidative stress. Exposure of these expanded cells to the TME leads to the upregulation of enzymes regulating GSH-dependent antioxidant defenses, which confers them greater metabolic flexibility in order to, afterwards, sustain the hostile tumor environment (Poznanski et al., 2021). These observations highlight the significance of redox regulation and metabolic modulation of NK cell function in the TME.

Exhaustion and ROS

It is also important to note that tumor infiltrating NK cells from *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice showed an increased expression of inhibitory receptors, such as TIGIT, PD-1 and TIM-3, which have been associated with NK cell exhaustion and dysfunction (André et al., 2018; Niu et al., 2020; Xu et al., 2015; Zhang et al., 2018b).

In the light of the results previously discussed, nutrient deprivation in the TME can drive GSH depletion and promote NK cell exhaustion and functional impairment. Similar observations were made in cancer cells, where inhibition of glutamine reduced GSH levels and drove the expression of PD-L1 through overactivation of the NF-kB signaling pathway (Byun et al., 2020). In T cells from renal carcinoma, decreased glucose uptake has also been associated with fragmented mitochondria, which accumulated high levels of ROS. Supplementation with pyruvate or ROS scavengers could restore the activation of these T cells (Siska et al., 2017). Additionally, low oxygen availability and continuous stimulation in the TME leads to inhibition of PCG-1α-dependent mitochondrial reprogramming in T cells, resulting in high levels of ROS and exhaustion (Scharping et al., 2021). Reducing hypoxia in the tumor or inhibiting ROS by GPx overexpression reverts T cell exhaustion and allows retention of functional capacity (Scharping et al., 2021).

Curiously, exhausted tumor-infiltrating $CD8^+$ TILs show significantly increased levels of mitochondrial, but not cytosolic ROS (Yu et al., 2020). The phenotype observed in intratumoral CD8+ T cells mimics the reduced mitochondrial fitness of *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ NK cells mice. Concordantly, IL-15-induced activation of GSH-deficient NK cells does not result in increased cytosolic ROS levels, but increased superoxide levels in the mitochondria (Figure 11A, B).

In line with our findings, mitochondrial fitness seems to be crucial for NK cells. Namely, genetic deletion of PGC-1α in NK cells was associated to functional defects induced by mitochondrial dysfunction (Gerbec et al., 2020; Miranda et al., 2016). As previously mentioned, inhibition of PGC-1 α is associated with T cell exhaustion in the TME (Scharping et al., 2021). Furthermore, tumor hypoxia leads also leads to mitochondrial fragmentation in NK cells, increased mitochondrial ROS levels and OXPHOS suppression, inhibiting NK cell-mediated immune surveillance (Zheng et al., 2019). These data highlight the importance of redox regulation in mitochondrial fitness and inhibition of exhaustion in NK cells. These observations also support a GSH-mediated control of exhaustion in NK cells, dependent on ROS buffering for maintenance of mitochondrial fitness.

Furthermore, nutrient availability in the TME seems to be key for GSH-mediated NK cell homeostasis and tumor surveillance, which is crucial for the improvement and design of therapeutic strategies against cancer.

- **Controlling redox status in NK cells is promising as a therapeutic strategy**

Conventional tumor-targeting therapies

The impact of conventional cancer strategies, such as chemo- and radiotherapy in the functionality of immune cells must be weighed carefully. The fundamental knowledge we have accumulated is the result of studies in cancer cell lines, rather than whole tissue samples (Kim et al., 2016; O'Sullivan et al., 2019). Especially in what concerns the pro-oxidative nature of these therapies, the detrimental effects of ROS for NK cells and other immune effectors are rarely considered or anticipated and may constitute the basis of drug resistance (Aydin et al., 2017; Kono et al., 1996; Mak et al., 2017; Mellqvist et al., 2000; Siska et al., 2017). Our results are in line with previously published evidence in T cells, which demonstrate that accumulation of mitochondrial ROS is associated with an exhaustion phenotype and deficient functionality in the tumor (Scharping et al., 2021; Siska et al., 2017; Yu et al., 2020). For example, it was recently shown that histone demethylase LSD1 inhibitors, in clinical trials for treatment of leukemia, can impair NK cell homeostasis. Treatment of NK cells with LSD1 inhibitors deplete the GSH pool and sustain high mitochondrial ROS, interfering with NK cell metabolism and cytotoxic function (Bailey et al., 2020). These observations are in line with our findings and highlight the importance of studying the impact of new anti-tumor strategies in the immune effector function.

Metabolic modulation of the TME and Immunotherapy

Nevertheless, therapies inhibiting malignant cell metabolism can increase the availability of glucose, glutamine or oxygen in the TME, boosting effector immune cells (Leone et al., 2019; Renner et al., 2019). These strategies are particularly effective when used in combination with immune checkpoint blockade (ICB) (Leone et al., 2019; Renner et al., 2019). ICB revolutionized cancer treatment with the use of monoclonal antibodies blocking the T cell inhibitory receptors. The combination of anti-PD1 and anti-CTLA significantly prolonged survival of patients in metastatic melanoma (Wolchok et al., 2017) and monotherapy with anti-CTLA4 treatment (Ipilimumab) reported survival up to 10 years (Schadendorf et al., 2015). However, only 20% of the patients benefit from this treatment and several tumors are refractory (Gauci et al., 2019; Schadendorf et al., 2015), indicating the need for alternative approaches. Namely, by targeting other immune cells. Besides the recognized effect of anti-PD-1/PD-L1 therapy in NK cells (Hsu et al., 2018), alternative immune checkpoints have been designed to specifically enhance NK cell function, such as

NKG2A and TIGIT, both under clinical trials (André et al., 2018; Zhang et al., 2018b) (NCT03563716, NCT02921685).

We have observed that genetic ablation of *Gclc* in NK cells resulted in increased expression of the inhibitory receptors TIGIT, PD-1 and TIM-3. Furthermore, we have preliminary results evidencing that glutamine and glucose depletion lead to reduced levels of GSH in these cells. Hence, it is clear the potential of enhancing GSH activity in NK cells in combination with other immunotherapeutic approaches to reduce exhaustion.

On one hand, ICB could potentially be combined with antioxidants, simultaneously reverting exhaustion and ROS accumulation, enhancing NK cell effector function. For example, supplementation of GSH or its precursors, N-acetyl-cysteine, were proven to boost NK cell cytotoxicity against target cells (Kawada et al., 2010; Millman et al., 2008; Sinha et al., 2018). On the other hand, this approach must be carefully investigated given the dependence of cancer cell proliferation on antioxidant pathways (Harris et al., 2015; Le Gal et al., 2015). Nonetheless, this hindrance can be circumvented by the utilization of adoptive cell therapy (ACT). In ACT, autologous, allogenic or *off-the-shelf* NK cells are expanded to be subsequently injected into the cancer patient (Miller and Lanier, 2019; Shimasaki et al., 2020). Expansion and activation of these cells with cytokines, such as IL-15, would increase intracellular levels of GSH and other antioxidants, protecting them from the oxidative TME (Yang et al., 2020). The culture of NK cells could also be done in combination with ROS scavengers, such as GSH and its precursors or other antioxidants. This has been explored for adoptive therapy of T cells (Pilipow et al., 2018) and has proven to boost NK cell cytotoxicity (Lee and Kim, 2020; Millman et al., 2008). Alternatively, as described recently by Poznanski and colleagues, NK cells can be expanded with the NRF2 activator omaveloxolone. This drug increases NK cell metabolic flexibility and improves cytotoxic response and tumor control (Poznanski et al., 2021). Other drugs, such as monomethyl fumarate or DMF, which induce Nrf2 activity and were shown to augment NK cell cytotoxicity (Vego et al., 2016), can be used in combination with ACT.

- **Outlook**

Mechanisms of GSH-dependent NK cell metabolic regulation

In the future, in order to further dissect the role of GSH and redox regulation in NK cell function, more studies should focus on the direct links between GSH and mTOR. This would allow us to understand whether this is a ROS-dependent or independent mechanism and identify additional levels of regulation of NK cell function and metabolism.

GSH regulation of NK cells upon distinct viral challenges

It would be interesting to challenge *Gclc*$^{fl/fl}$ *Ncr1-Cre*+ with a virus whose clearance is directly mediated by NK cells, such as MCMV, to unravel the importance of redox regulation on NK cell-dependent viral clearance.

Tumor specific GSH-dependent regulation of NK cell function

It would also be of great value to dissect the differences in GSH-mediated regulation of NK cells in the control of primary vs. metastatic tumors. This could be achieved by investigating the development of spontaneous metastasis in *Gclc*-deficient mice, either by surgical removal of the primary subcutaneous melanoma or by orthotopic injection of colon carcinoma cells, which normally colonize the liver.

Metabolic modulation of GSH abundance

To test the hypothesis that different TME metabolic compositions affect, in distinct manners, GSH-dependent NK cell regulation, different approaches could be followed. First, genetically engineered tumor cell lines, with specific nutrient utilization could be applied. This would allow us to investigate if the depletion of a single nutrient in the TME would impact GSH levels in NK cells and their functionality. Alternatively, metabolic studies could be performed *ex vivo* in tumor samples to investigate nutrient availability in the TME. This could be related to the redox status of NK cells.

Effect of GSH in NK cell exhaustion and ICB

Moreover, it would be interesting to further understand the role of ROS on NK cell exhaustion and the potential of GSH-regulation in NK cell-targeted ICB. We have planned in the future to test whether the efficacy of ICB of TIGIT and PD-1 are dependent on the activity of GSH in NK cells. This could be investigated using our mouse model, where the outcome of ICB would be evaluated in *Gclc*$^{fl/fl}$ *and Gclc*$^{fl/fl}$ *Ncr1-Cre*+ mice.

GSH-dependent regulation of human NK cell function

Finally, we have not validated our data in human NK cells. One approach would be, *in silico*, to use validated data sets from cancer patients from single cell RNA sequencing or bulk RNA sequencing data and analyze the transcriptional signature of NK cells. The expression of genes from the GSH pathway could be associated with the expression of other functional genes, such as *IFNG*. With this information, we could correlate redox regulation/functional activity and patient survival or response to different therapies. Alternatively, *in vitro*, the pharmacological inhibitor of GCLC, BSO, could be used in NK cells derived from peripheral blood of healthy donors. This adds the potential of exploring the differential roles of GSH in distinct human NK cell subsets. Although the distribution of NK cells between the different maturation subsets was not disturbed in mice upon GSH deficiency, we cannot exclude a different regulation of GSH in human NK cells. If any difference would arise, it would mirror the distinct sensibility to ROS experienced by $CD56^{bright}$ and $CD56^{dim}$ NK cells (Harlin et al., 2007; Thorén et al., 2007). It would also be interesting to validate our results in patient-derived tumor infiltrating NK cells *ex vivo*, measuring GSH levels, metabolic and cytotoxic capacity and elucidate any correlation with the tumor status of the patient. Finally, testing if GSH supplementation would increase functional response of human NK cells would be of great significance for novel approaches in the treatment of chronic infection, autoimmunity or cancer.

In summary, with this study we highlighted the role of GSH on NK cell function and activation. IL-15 induced activation requires upregulation of GSH levels on NK cells and GSH deficiency resulted in mitochondrial ROS accumulation and lower signaling of mTOR and STAT5, both essential checkpoints for NK cell homeostasis. Ablation of GSH led to a general metabolic shutdown of NK cells, low proliferative and cytotoxic capacity, which, *in vivo*, resulted in hindered capacity to regulate T cell function, upon viral challenge, and defective tumor response.

VI. References

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VII. Appendix

Relevant Publications

- **Guerra L**, Bonetti L, Brenner D. Metabolic Modulation of Immunity: A New Concept in Cancer Immunotherapy. Cell Rep. 2020 Jul 7;32(1):107848. doi: 10.1016/j.celrep.2020.107848. PMID: 32640218

- Kurniawan H, Franchina DG, **Guerra L**, Bonetti L, -Baguet LS, Grusdat M, Schlicker L, Hunewald O, Dostert C, Merz MP, Binsfeld C, Duncan GS, Farinelle S, Nonnenmacher Y, Haight J, Das Gupta D, Ewen A, Taskesen R, Halder R, Chen Y, Jäger C, Ollert M, Wilmes P, Vasiliou V, Harris IS, Knobbe-Thomsen CB, Turner JD, Mak TW, Lohoff M, Meiser J, Hiller K, Brenner D. (2020) Glutathione Restricts Serine Metabolism to Preserve Regulatory T Cell Function. Cell Metabolism. May 5;31(5):920-936.e7. doi: 10.1016/j.cmet.2020.03.004. PMID: 32213345

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Review

Metabolic Modulation of Immunity: A New Concept in Cancer Immunotherapy

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Immunotherapy shifted the paradigm of cancer treatment. The clinical approval of immune checkpoint blockade and adoptive cell transfer led to considerable success in several tumor types. However, for a significant number of patients, these therapies have proven ineffective. Growing evidence shows that the metabolic requirements of immune cells in the tumor microenvironment (TME) greatly influence the success of immunotherapy. It is well established that the TME influences energy consumption and metabolic reprogramming of immune cells, often inducing them to become tolerogenic and inefficient in cancer cell eradication. Increasing nutrient availability using pharmacological modulators of metabolism or antibodies targeting specific immune receptors are strategies that support energetic rewiring of immune cells and boost their anti-tumor capacity. In this review, we describe the metabolic features of the diverse immune cell types in the context of the TME and discuss how these immunomodulatory strategies could synergize with immunotherapy to circumvent its current limitations.

Immune checkpoint blockade (ICB) has transformed cancer treatment. Antibodies targeting the inhibitory T cell receptors CTLA-4 and PD-1 have significantly improved patient survival in many cases (Schadendorf et al., 2015), but numerous cancers remain refractory to this approach (Gauci et al., 2019). Some tumors lack immune cell infiltrates ("cold tumors"), rendering them unaffected by ICB (Fares et al., 2019). Other "hot tumors" contain immune cell infiltrates that support "immunoediting," in which cancer cells expressing neoepitopes are selected and resist the antigen-specific anti-tumor effector T cell (Teff cell) response. This resistance culminates in Teff cell exhaustion, which is closely related to metabolic changes in the tumor microenvironment (TME). The TME influences energy consumption and metabolic reprogramming in immune cells, often inducing them to become tolerogenic and inefficient in cancer cell eradication. In this review, we discuss how modulating the metabolism of immune cells ("immunometabolism") can improve the efficacy and durability of anti-tumor responses, boosting the success of anti-cancer immunotherapy.

Tumor Metabolism Shapes Anti-tumor Immune Responses

The TME contains cancer cells, immune cells, fibroblasts, blood vessels, extracellular matrix, and signaling molecules. The interplay among TME cell populations and their differing energetic needs shapes tumor development (Hanahan and Weinberg, 2011). To cope with their rapid proliferation, cancer cells implement a metabolic switch characterized by increased consumption of glucose and amino acids (AAs). Indeed, although they retain functional mitochondria and the capacity to engage oxidative phosphorylation (OXPHOS), cancer cells use glucose as their main energy source (Morais et al., 1994; Tan et al., 2015). Glucose and AAs are catabolized into carbon intermediates needed to assemble macromolecules, fuel ATP production in the electron transport chain (ETC), and help to maintain cellular redox capacity (Pavlova and Thompson, 2016).

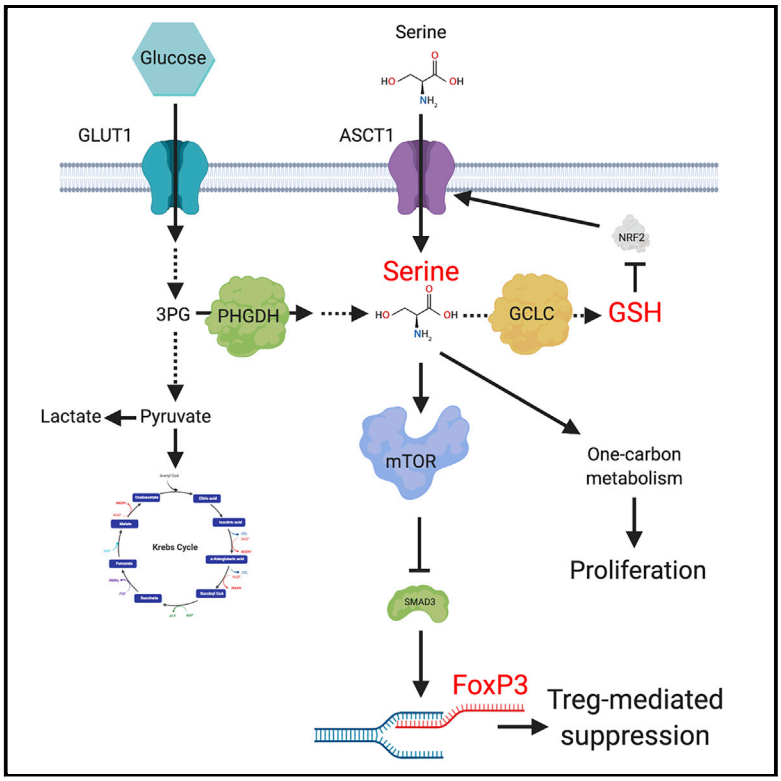
A cancer cell's ability to increase its metabolic rate independently of external growth stimuli rests on its high mutational burden. Heterogeneity in the mutations exhibited by different cancer cells accounts for their distinct metabolic adaptations. Interestingly, excessive nutrient uptake by a tumor imposes metabolic stress on immune cells infiltrating the TME (Franchina et al., 2018b) (Figure 1). Glucose and glutamine deprivation prevent immune cells from switching from OXPHOS to glycolysis, compromising their function (see below). In addition, even in the presence of oxygen, glycolysis-derived pyruvate is not used to fuel the tricarboxylic acid (TCA) cycle in a tumor cell but is converted to lactate by lactate dehydrogenase (LDHA), a principle known as the Warburg effect (Warburg, 1956; Shim et al., 1997). The excess lactate produced by the proliferating cancer cells is exported by monocarboxylate transporters (MCTs) and increases the acidity of the TME (Halestrap, 2012), further interfering with immune cell metabolism (Renner et al., 2019). In melanoma patients, increased LDHA and lactate levels correlate with reduced survival (Brand et al., 2016). Moreover, lactate promotes tumor angiogenesis because it stabilizes

Article

Cell Metabolism

Glutathione Restricts Serine Metabolism to Preserve Regulatory T Cell Function

Graphical Abstract



Highlights

- Ablation of *Gclc* in Tregs causes autoimmunity and increases anti-tumor responses
- *Gclc*-derived GSH is needed for the suppressive function of Tregs *in vitro* and *in vivo*
- GSH in Tregs regulates serine concentrations and metabolism. which impact mTOR and FoxP3
- Serine- and glycine-deficient diet rescues mutant mice from lethal inflammation

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In Brief

Regulatory T cells (Tregs) rely on oxidative metabolism, which triggers the generation of reactive oxygen species (ROS). Accumulating ROS are controlled by the antioxidant glutathione (GSH). Kurniawan et al. reveal an unexpected subset-specific role of GSH in serine metabolism and Treg function.

Kurniawan et al., 2020, Cell Metabolism *31*, 920–936
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