Potentiating immune cells function during inflammation and virus infection by modulating metabolism

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Abstract

Natural killer cells are innate lymphocytes that provide antiviral immunity. Resting NK cells oxidize glucose, lipids, and amino acids to generate ATP, which is essential to help the cells maintain their normal functions during immune surveillance. During inflammation and infection, NK cells shift their metabolism and upregulate their nutrient receptors such as glucose, amino acid, and iron transporters, while activating mTORC1 to produce the biosynthetic precursors that are required for cell growth, proliferation, and cytokines secretion. I identified that IL-18 is a potent cytokine which regulates NK cell metabolism and enhances their proliferation by increasing the expression of nutrient receptors and induce leucine-driven mTORC1 activation. During infection, immune cells are subjected to cellular stresses, and these stresses are regulated by the redox state, which influences cell growth, metabolism, and death. Glrx2 is an enzyme of the antioxidant system that controls ROS production. I used *Glrx2*-deficient mice as a model to study the effect of ROS on immune cell development, IFNγ production, cytotoxic potential, and proliferation in the naïve mice and in mice upon acute and persistent MCMV infection. By targeting the antioxidant system and the IL-18 pathway during infection, my study will open up new avenues for therapeutic applications in enhancing natural killer and T cell functions for immunotherapy.

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List of Abbreviations

2-DG2	2-deoxyglucose
α-MeAIB	α-methylamino)isobutyric acid
AAAs	anionic AAs
AAs	amino acids
ACL	ATP citrate lyase
ADCC	antibody-dependent cell-mediated cytotoxicity
AMPK	AMP-activated protein kinase
BCH	heptanecarboxylic acid
CAAs	cationic AAs
CGD	chronic granulomatous disease
DC	dendritic cells
EAA	essential amino acids
ECAR	extracellular acidification rate
ERRα	estrogen-related receptor α
ETC	electron transport chain
FAO	fatty acid oxidation
GAP	GTPase activating protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDH	glutamate dehydrogenase
GPNA	L-γ-Glutamyl-p-nitroanilide
GPX	glutathione peroxidases
Grx2	glutaredoxin 2

GRXs	Glutaredoxins
GSH	Glutathione
H2O2	hydrogen peroxide
HIF2α	hypoxia-inducible factor 2-α
HOCl	hypochlorous acid
IL-18BP	IL-18 binding protein
IRF-3	interferon regulatory factor
ITAMs	immunoreceptors tyrosine-based activation motifs
IL	Interleukin
KIRs	killer-cell immunoglobulin-like receptors
KO	Knockout
LAT1	L-type amino acid transporter 1)
LCMV	lymphocytic choriomeningitis virus
LDH	lactate dehydrogenase
LNAAs	large neutral AAs
LPS	Lipopolysaccharide
LRS	leucyl-tRNA synthetase
MAPK	mitogen-activated protein kinase
MCMV	murine cytomegalovirus
MHC-I	toll- major histocompatibility complex
m-ROS	mitochondrial ROS
mTHF	mitochondrial tetrahydrofolate

mTOR	the mammalian target of rapamycin
mTORC1	mTOR complex 1
NAC	N-acetyl cysteine
NALA	N-acetyl-leucine amide
NAD	nicotinamide
NFAT	nuclear factor of activated T cells
NK	natural killer cells
NOD	non-obese diabetic
OH•	hydroxyl radical
OXPHOS	oxidative phosphorylation
$O_2^{\bullet -}$	super oxide
$O^{\bullet}$	singlet oxygen
PHA	phytohemagglutinin
PKC	protein kinase C
PMA	phorbol 12-myristate-13-acetate
PPP	pentose-phosphate pathway
PRXs	peroxiredoxins
pS6	s6 ribosomal protein
PSSG	proteins or protein thiol mixed disulfide
PTKs	protein tyrosine kinases
PTPs	protein tyrosine phosphatases
ROOH	and lipid peroxidise

ROS	reactive oxygen species
SLC	solute carrier
SNAAs	small neutral AAs
SOD	superoxide dismutase
SRXs	sulfiredoxins
SSG	glutathionylated protein
TCA	tricarboxylic acid
TGF-β	transforming growth factor
TILs	tumor-infiltrating-lymphocytes
TIR	toll-IL-1 receptor
TMDs	transmembrane domains
TLR	like receptor
TNF	tumor necrosis factor
TRAF6	tumor necrosis factor receptor-associated factor 6
Trx1	thioredoxin-1
TRXs	thioredoxins
Txnrd1	thioredoxin reductase-1
UBR1-2	unbranched chain AA receptors 1 and 2
UCP	uncoupling proteion
WT	wild-type mice

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Chapter 1: General introduction

Innate immune response

Human bodies are exposed to many different pathogens daily; therefore, defense mechanisms, such as the innate and adaptive immune responses were developed to provide constant and efficient protection. The innate immune response is the means used by the body to react to pathogens during the first few hours and days of exposure (1). The first lines of defense that protect our body from infections are the skin and the epithelial surfaces in the lung and gut that provide barriers between the inside of the body and the surrounding environment (2). Additionally, the tight junctions between the cells prevent pathogens from traveling between them and the mucus layer that covers the interior epithelial cells protect these surfaces from the microbes. Unlike the adaptive response, the innate response is not specific to the pathogen and involves the activation of phagocytic cells and the release of various cytokines to control the infection (3). During the course of infection, innate immune responses are required to activate the adaptive immune responses, which are necessary for antigen-specific recognition and to establish immunological memory.

In order for the immune system to recognize the pathogens and destroy them without harming the host, it needs the ability to distinguish between the self from non-self. This is achievable through the recognition of specific molecules on the pathogen that are not expressed in the host. The pathogen-associated molecules can stimulate the inflammatory responses and the phagocytosis by myeloid cells (2). There are many types of pathogen-associated molecules, including peptidoglycan of cell walls and the flagella of bacteria, lipopolysaccharide (LPS) on gram-negative bacteria and teichoic acids on gram-positive bacteria. The pathogen-associated molecules can be recognized by different receptors on the cells called pattern recognition receptors (PRRs), and include soluble receptors of the

complement system in the blood and membrane-bound receptors on the cell surface known as Toll-like receptors (TLRs) (2). TLRs are transmembrane proteins with a large extracellular domain consisting of a series of leucine-rich repeats (4). TLRs are expressed on the cell surface of epithelial cells, macrophages, and neutrophils (5). During infection, the intracellular signal transduction pathways downstream of TLRs cause the translocation of the NF-κB protein into the nucleus to activate the transcription of various genes. The activation of TLRs will initiate inflammatory responses, pathogen phagocytosis, and activate both the innate and the adaptive immune responses (6).

During infection, the recognition of the pathogen is immediate and followed by the engulfment of the pathogen by phagocytic cells. The first major family of phagocytic cells is macrophages, which reside in tissues such as the lung, gut, and connective tissues of the spleen and liver. The number of macrophages increases at the site of infection. The second major family is the short-lived neutrophils, which exist in the blood but not in healthy tissue. They are recruited to the area of infection and are activated by macrophages (2) (1). Macrophages and neutrophils express different pattern recognition receptors such as TLR, that attach to ligands on the pathogen's surface allowing the phagocytes' plasma membrane to surround the pathogen and engulf it into the phagosome and destroy it (7). The phagosomes are acidic and fuse with lysosomes which contain lysozyme that can degrade the bacterial cell wall and microbial proteins. If the pathogens are too big, the macrophages and neutrophils will secrete their acidic contents and lysosomal products to kill the pathogen. Many pathogens have the ability to avoid being killed by phagocytes, making these initial defense mechanisms insufficient to clear infection and requiring other innate and adaptive immune response mechanisms to eliminate the pathogen (8).

When an infection occurs, it is always associated with inflammatory responses. These responses appear as fever, pain, redness, and swelling of the area of infection. An inflammatory response is mediated by signaling molecules that recruit lymphocytes, neutrophils, and monocytes to the site of infection to recognize the pathogen and eliminate it (9). Signaling molecules such as cytokines and chemokines are produced in response to the activation of TLRs (6). Chemokines attract different cell types, such as neutrophils which are the first cells to be recruited to the site of the infection; as well as dendritic cells, which pick up antigens from the microbes and carry them to the lymph nodes to be presented to the lymphocytes and activate the adaptive immune response (10).

TLRs are located in the plasma membrane and within endosomal vesicles. TLRs expressed on the plasma membrane recognize hydrophopic lipids and proteins whereas those found in endosomal vesicsles can detect nucleic acids (11). These mechanisms allow the innate cells to recognize the viral envelope on the cell surface upon viral entry, as well as the viral nucleic acid inside the endosome, when the virus uncoats its genome and enters the cytoplasm (11). During viral infection, the innate immune response is triggered through the engagement of PRRs including TLRs, retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), which induce intracellular signaling that upregulate the production of proinflammatory cytokines (12). The host cell can detect the viral genome and destroy it via degradation, while inducing cytokines such as interferon α and β (13). When interferons bind to their receptors on the cell surface, they stimulate specific gene transcription through the JAK/STAT signaling pathway, activate the degradation of viral genome, and shutdown the proteins required of the virus.

Interferons stimulate both innate and adaptive immune responses and enhance the ability of natural killer cells to kill the virus-infected cell (14).

Natural killer cells

Natural killer cells (NK) are lymphocytes that bridge innate and adaptive immunity with the ability to control virus-infected cells and tumor cells. The term natural killer was originally derived from the observation that they are poised to kill target cells without prior sensitization or further differentiation (15). NK cells form the third biggest population of lymphocytes after B cells and T cells, and can be found in the spleen, liver, peritoneal cavity, lung, lymph nodes, and thymus (16). NK cells represent 3-5% of the mouse spleen, 10% of the lungs, and 2-18% of the human peripheral blood. Morphologically, they are large granular lymphocytes (17).

NK cells develop primarily in the bone marrow, lymph nodes, and liver. To generate NK cells from hematopoietic stem cells, NK cell precursors differentiate into NK cell lineages and undergo maturation and homeostasis (18) (19). NK cell development and maturation are regulated by various transcription factors such as Ets-1, Id2, Ikaros, and PU1 (20). The maturation of immature NK cells is regulated by GATA-3 and IRF-2, while CEBP-g, MEF, and MITF regulate the functional differentiation of NK cells. The IL-15 cytokine is required for the development, homeostasis, and survival of NK cells, while IL-2 is essential for the functional maturation of NK cells (21) (22).

Although NK cells lack the antigen specificity of T cells and B cells to recognize the infected cells, they express activating and inhibitory receptors which send signals to activate or inhibit the killing of the target cells (23). NK cells are always ready to kill virus-infected cells or tumor cells, and this immediate response could affect healthy cells when NK cells are

activated (24) (25). However, NK cells are able to distinguish between healthy and infected cells based on two hypotheses. First, the missing-self hypothesis stipulates that NK cells can recognize target cells that are missing class I major histocompatibility complex (MHC-I) molecules (26). Thus, normal levels of MHC-I molecules on the surface of healthy cells will be recognized by the inhibitory receptors on NK cells, which will inhibit the activation of NK cells and prevent the killing of the healthy cells (Fig. 1.1A) (27). Second, the induced-self hypothesis states that NK cells are able to recognize stress ligands that are induced upon infection, such as ULBP and MIC molecules in humans and RAE1, H60 and MULT1 molecules in mice. These ligands are homologs of MHC class I protein that are induced on stressed cells, tumor cells or virus infected cells. Cells infected with viruses such as cytomegalovirus (CMV) will upregulate the transcription of these ligands and become recognized by NK cells activating receptors C-type lectin-like family NKG2D (28) (29) (Fig. 1.1B). Moreover, the activating receptors on NK cells can recognize viral proteins on the surface of virus-infected cells (28) (29) (30). The overall response of NK cells against a target cell is regulated by the balance between the activating and the inhibitory signals (15).

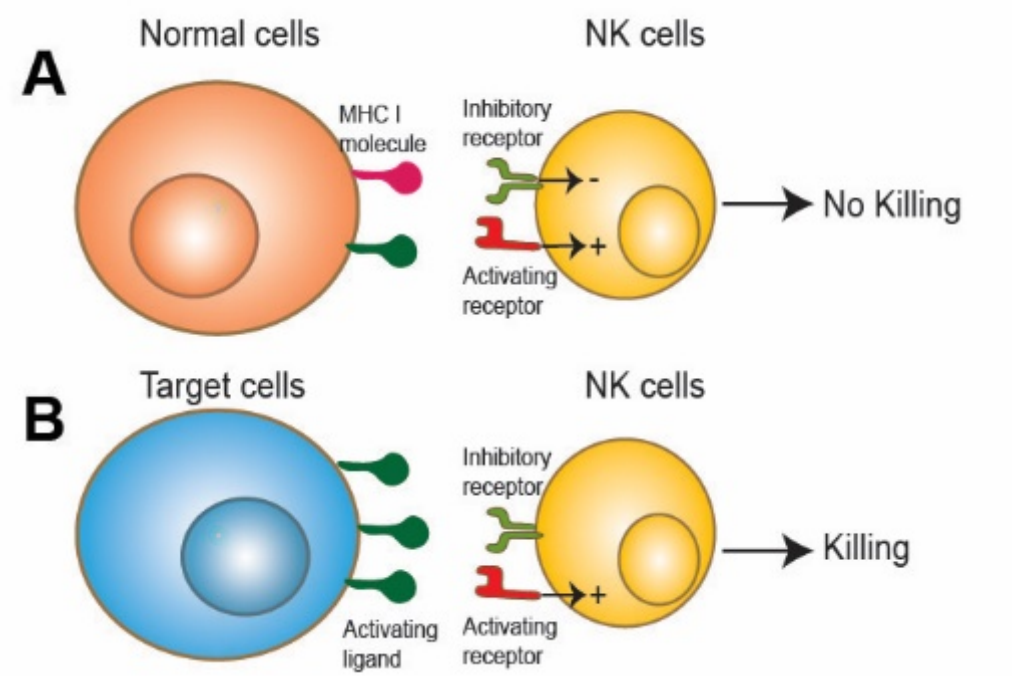

Figure 1.1. The mechanism of NK cell recognition of virus-infected or cancer cells. (A) The NK cell recognizes MHC I through their inhibitory receptors resulting in no killing of the target cell. (B) The lack of MHC I on the target cell and the recognition of the activating ligand through the activating receptors will trigger the killing activity by the NK cell.

Activating receptors such as, Ly49H and Ly49D and in mice (15), KIR2DL4, KIR2DLS1, KIR2DLS2 and KIR2D3 in humans, and NKG2D and NKp46 in mice/humans (31) have signaling subunits that contain immunoreceptors tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails. Through activating receptors, NK cells recognize their target ligands and the ITAMs on kinase-dependent adapter molecules, such as DAP-12, become phosphorylated on the tyrosine residues, which promote the recruitment and activation of the cytoplasmic protein tyrosine kinases ZAP-70 and Syk (32, 33). The protein tyrosine kinases phosphorylate and activate downstream signal transduction pathways such as PI3K, NF-κB and ERK which lead to the activation of NK cell effector functions (34). Through the stimulation of activating receptors, NK cells can proliferate, produce cytokines such as IFNγ, and kill their target cell via a perforin/granzyme B-dependent mechanism (35). Inhibitory receptors such as KLRG1, Ly49A, Ly49B, Ly49C in mice (15) and KIR2DL1, KIR2DL2, KIR2DL3 and KIR2D5 in human (31), contain immunoreceptors tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails. When they bind to MHC-I molecules, inhibitory receptors become phosphorylated on their tyrosine residues and promote the activation of the cytoplasmic protein tyrosine phosphatase SHP-1 (32). SHP-1 removes the phosphate group from the tyrosine residues of signaling molecules and inhibit the function of ITAMs, which leads to blocking of NK cell effector functions (33).

NK cells also express the low-affinity Fc receptor CD16 which enables them to recognize the antibodies that bind to viral/tumor antigens on the surface of infected/cancer cells and kill it through antibody-dependent cell-mediated cytotoxicity (ADCC) (36). Examples of monoclonal antibodies that are used for ADCC-mediated cancer

immunotherapies are anti-CD20 (Rituximab), anti-Her-2 (Trastuzumab), anti-CD52 (Alemtuzumab) and anti-EGFR (Cetuximab) (37).

The cytokine microenvironment and the interaction with immune cells such as T cells, macrophages, and dendritic cells (DCs) affect the activation of NK cells (38). During infection, many cytokines are produced such as IL-2, IL-15, IL-12, 1L-18, and type I IFNs. IL-15 and IL-2 are known to be potent activators of NK cells, while IL-12 and IL-18 stimulate the proliferation, the cytotoxicity and the release of IFNγ (22). Moreover, macrophages secrete IFNγ which activates NK cells, whereas chemokines such as CCL3, CXCL10, and CXCL9 can regulate the trafficking of NK cells to the site of infection (10). The function of NK cells can be regulated by transforming growth factor (TGF)-β and regulatory T cells. In addition, NK cells can act as regulatory cells by interacting with DCs, macrophages, T cells, B cells and endothelial cells by producing TNF-α and IL-10 as well as growth factors and chemokines (39). NK cells can regulate the homeostasis of DCs and promote their maturation (40) (41). Moreover, NK cells can prime CD4+ T cells through the production of IFNγ and kill the activated T cells unless they express a sufficient number of MHC-I molecules (42). T cells secrete IL-2, which stimulates NK cell proliferation and during tumorigenesis, CD8+ T cells help NK cells infiltrate tumors (43). The MHC-I molecules are often lost or down-regulated in virus-infected and cancer cells, making these cells more susceptible to NK cell-mediated killing (44). However, tumor cells develop several mechanisms to evade NK cell recognition such as downregulating their adhesion molecules, upregulating MHC-I molecules, producing immunosuppressive factors, and inhibiting perforin mediated apoptosis (45, 46).

NK cell-mediated recognition of virus-infected cells

NK cells can control many viruses in mice such as herpes simplex virus-1, influenza virus, ectromelia poxvirus, and murine cytomegalovirus (MCMV) (47). MCMV has been used as a model to study the early response of NK cells against viruses and to show the susceptibility or the resistance to MCMV after NK cell depletion or adoptive transfer. Hosts possessing NK cells deficient in IFNγ production and cytotoxicity are sensitive to MCMV infection (47, 48) and the recognition of MCMV infection by NK cells depends on the mouse strain. In C57BL/6 mice, NK cells can recognize MCMV through the interaction between the viral protein m157 and the activating receptor Ly49H. This activation of NK cells induces perforin-mediated cytotoxicity and specific proliferation of Ly49H+ NK cells (48, 49). The deletion of the genetic locus of Ly49H, *Cmv1*^{r}, or the blockade of Ly49H using antibodies increases the susceptibility of the host to the virus (50). BALB/c mice do not express Ly49H receptors, which make them susceptible to MCMV infection (51). Nonetheless, some mice strains have activating receptors other than Ly49H, which can recognize MCMV. For example, in Ma/My mice, Ly49P, an activating receptor of NK cells, is associated with virus resistance and interacts with H-2D^{k} viral protein (52).

MCMV has many mechanisms to evade NK cell killing such as the downregulation of NKG2D ligands (MCMV m145, m152, and m155) and increasing the expression of MCMV m144 protein which mimics MHC-I molecules that inhibit NK cell activation (53). In 129/J mice, Ly49I, an inhibitory receptor, interacts with m157 molecules and allows MCMV to evade NK cell-mediated immune response (49).

NK cells are able to recognize influenza virus-infected cells through the activating receptor NKp46, which is found on all NK cells, as well as through TLR stimulation (54).

TLR2 activates NK cells and increases their effector function during vaccinia infection (55), through the activation of MyD88-PI3K-ERK pathway. During bacterial infection, NK cells become activated through the stimulation of TLR4 (56).

Besides the stimulation of NK cells through their activating receptors, NK cells can be activated by cytokines, which can influence the activating receptor-mediated NK cell activation (57). Type I IFNs, which include IFNα and IFNβ, and IL-15 play critical role in increasing cell-mediated cytotoxicity during MCMV and lymphocytic choriomeningitis virus (LCMV) infections (58). In order to induce NK cell activation by cytokines, the cytokine receptors signal through the JAK/STAT pathway (58). Type I IFN induces phosphorylation of STAT1 and STAT2 to induce the gene expression that is involved in NK cell cytotoxicity (59). IL-15 causes the phosphorylation of STAT5 and induces NK cell proliferation, whereas IL-12 causes the phosphorylation of STAT4 to induce IFNγ secretion. The absence of STAT molecules can impair the host's ability to control the virus at the early stages of infection (50).

During MCMV, LCMV, mouse hepatitis virus, and vaccinia virus infections, NK cells migrate to the site of infection (60). The four chemokines that play crucial role in NK cell trafficking toward the site of infection are CCR2, CCR5, CXCR3, and CX_3CR1. Among these chemokines, CCR2 and CCR5 are critical for NK cell migration to the liver during MCMV infection (16). Moreover, CCR5 recruits NK cells to the central nervous system in Herpes simplex virus-2 (HSV-2) infection, whereas, CXCR3 is involved in NK cells trafficking to the liver during Dengue virus infection and facilitates the migration of NK cells to the lymph nodes (16, 61). The mechanism of NK cell migration is controlled by PI3K pathway, where the p11γ and p11δ isoforms are critical for leukocytes movement (61).

Upon infection, NK cells employ three main mechanisms to kill the virus-infected cells: the secretion of pro-inflammatory cytokines such as IFNγ, the release of cytotoxic granules, and through FasL and tumor necrosis factor (TNF) family death receptor signaling (47). IFNγ production is one of the critical NK cell effector functions which can directly affect the virus, while it can also activate and recruit other immune cells such as the cytotoxic T cells and CD4+ T helper cells. Also, IFNγ plays a role in controlling MCMV, vaccinia, HSV, and other viral infections (62). Activated NK cells called large granular lymphocytes can directly kill the target cells by forming cytotoxic granules, which contain perforin and granzyme B (63) (47). Perforin is a membrane pore-forming molecule, which can disrupt the cell surface (64). On the other hand, granzyme B is a member of the serine proteases family and affects the cell cycle, resulting in DNA damage. Hence, perforin forms pores in the surface membrane of the target cells, which allows granzyme B to enter the cells and induce apoptosis. Consequently, perforin knockout mice are not able to clear MCMV, influenza virus, and other viral infection (47, 65). In FasL and TNF-related apoptosis-inducing ligand (TRAIL) mechanisms, NK cells are able to kill the target cell through activating the death receptors on the infected cells, thus inducing apoptosis. This mechanism is beneficial for killing by immature NK cells when they are not able to use the perforin-dependent mechanism (66).

Interleukin 18

Upon infection, IL-18 is one of the proinflammatory cytokines that is produced by activated macrophages, dendritic cells, Kupffer cells, and epithelial cells (67). IL-18 was identified in 1989 as an IFNγ inducing factor in liver of mice that were injected with lipopolysaccharide and heat-killed *Propionibacterium acne* (68). IL-18 is also produced by macrophages during human HIV-1 infections and is produced by pancreatic islets upon

cyclophosphamide-induced insulitis in non-obese diabetic (NOD) mice (69). Moreover, after stimulation with contact sensitizer, keratinocytes produce IL-18, which may suggest a role of IL-18 in allergen-induced inflammation (70). High levels of IL-18 are found in patients with acute lymphoblastic leukemia and chronic myeloid leukemia (71). Also, during stress, IL-18 is produced in the adrenal cortex and in the neurohypophysis (72). IL-18 plays a pivotal role in inducing TNF-α, IL-1β as well as CXC and CC chemokines (73). IL-18 also upregulates Fas ligand-mediated cytotoxicity by NK cells and T cells and induces nuclear translocation of NF−κB (74). In addition, IL-18 is a potent cytokine that plays a critical role during infection by inducing the secretion of IFNγ and cytotoxicity in NK cells and T cells and increasing the response of Th1 cells (75). IL-18 can stimulate the production of IFNγ by Th1 cells as well as enhance the production of IL-2 and IL-2Rα, and induce cellular proliferation. In addition, IL-18 stimulates Th2 cells to produce IL-4 and IL-13 which will directly induce allergic inflammatory responses (76). Splenic NK cells from IL-18Rα deficient mice have defects in their cytotoxicity against YAC-1 target cells (77).

IL-18 belongs to the IL-1 cytokine family that is conserved in mammals and consists of IL-1α, IL-1β, IL-1R antagonist, IL-18 (IL-1F4), IL-1F5-F10 and IL-33 (78). IL-18 binds to a heterodimer receptor complex which is composed of primary binding chain IL-18Rα (IL-1R Rp1) and a coreceptor IL-18Rβ (79). When IL-18 binds to IL-18Rα, it has a low binding affinity, however when bound to the receptor complex, it has a high binding affinity and results in signaling (80). After the formation of the heterodimer complex, Toll-IL-1 receptor (TIR) domains will recruit MyD88 that leads to the phosphorylation of IRAKs, TRAF-6 and the activation of NF-κB, which results in the induction of proinflammatory signals (Fig. 1.2) (81). IL-18Rα is ubiquitously expressed on various cell types, however, IL-18Rβ has been

known to be expressed on T cells and dendritic cells but not on mesenchymal cells (82). In the absence of IL-18Rβ, IL-18 binds to IL-18Rα without inducing proinflammatory signals (76).

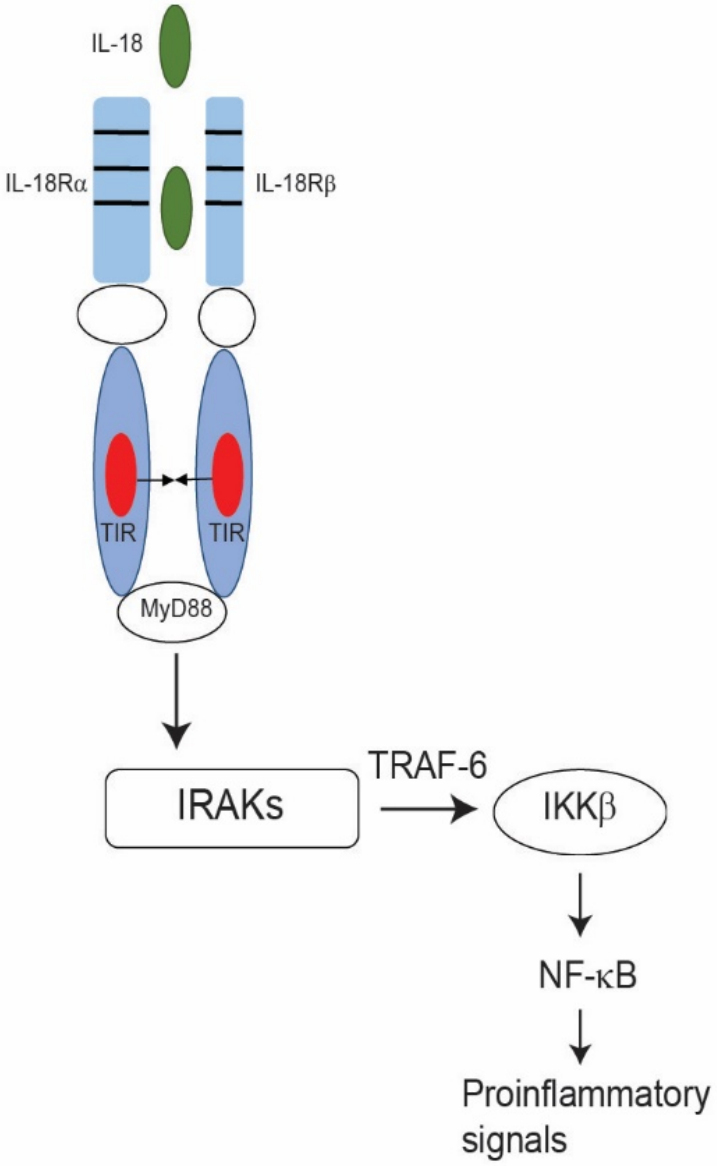

Figure 1.2. IL-18 signaling transduction. IL-18 forms a signaling complex by binding to IL-18Rα. The co-receptor IL-18Rβ is recruited to form a high-affinity complex. Following the formation of the heterodimer, the Toll-IL-1 receptor (TIR) domains come closer to each other, triggering the binding of MyD88, phosphorylation of the four IRAKs, TRAF-6, and activation of NF-κB resulting in the expression of proinflammatory genes.

IL-18 serves as a substrate of caspase-1, which is the intracellular cysteine protease that becomes activated by inflammasomes whose activation is triggered by extracellular ATP, phagocytosis, or infection. Active IL-18 can also be released by non-caspase proteases such as the neutrophil protease PR-3, mast cell chymase, meprin, and granzyme B. The molecular weight of the IL-18 precursor is 24 kDa, which undergoes cleavage by activated caspase-1 into an active mature form of 18 kDa. Inactive pro-caspase-1 is activated into an active form by the nucleotide-binding domain and leucine-rich repeat pyrin containing protein-3 (NLRP3) inflammasome (69). Approximately 80% of the precursor form of IL-18 stays unprocessed inside the cells and within the intracellular compartment of mesenchymal cells. Endotoxins in caspase-1-deficient mice do not induce IFNγ production due to the absence of IL-18 processing (83).

IL-18 binding protein (IL-18BP) is a secreted protein with a high affinity to IL-18 and it was shown to downregulate the Th1 response through binding to IL-18 and inhibiting IFNγ production (69). IL-18BP is regulated at the gene expression level by IFNγ, which increases the gene expression and synthesis of IL-18BP. Since IL-18 plays a role in Th2 response, IL-18BP regulates Th2 cytokine production (76). The level of IL-18BP in the sera of healthy individuals is 2,000-3,000pg/ml, while the level of IL-18 in the same serum is 80-100pg/ml and one molecule of IL-18BP binds to one molecule of IL-18 (69) (84). In autoimmune diseases, the levels of both IL-18BP and IL-18 are high but the level of IL-18BP is not high enough to neutralize IL-18 (85). During viral infections, the viral IL-18BP acts as natural neutralization and reduces IL-18 which leads to the reduction of the inflammatory response by immune cells in the infected tissue (86).

IL-18 induces IFNγ production which plays a detrimental role in NK cell pathogenesis and autoimmune diseases. The combination of IL-18 and IL-12 is known to induce enhanced IFNγ, and injecting mice with IL-18 and IL-12 will increase IFNγ production in the mice and cause death from hypoglycemia, intestinal inflammation, and inanition (87). IL-18, together with IL-12, induces acute pancreatitis in leptin-deficient mice (88). The elevated levels of IL-18 and IFNγ are involved in several human autoimmune diseases such as type-1 diabetes, systemic lupus erythematosus, rheumatoid arthritis, Crohn's disease, and psoriasis (69). The hepatic damage in macrophages activation syndrome is due to FasL induction by IL-18 (89). Blocking IL-18 production in autoimmune disease can be therapeutically applied, as in Crohn's disease; anti-IL-18 decreases the severity of the disease (90).

IL-18 and IL-18Rα deficient mice become obese and show lipid abnormalities, which cause arthrosclerosis, insulin resistance, diabetes and metabolic syndrome (91). Also, IL-18-deficient mice exhibit an increase by 100% of the adipose tissue with fat deposition in the arterial wall, as well as insulin resistance and these mice show a decrease in insulin resistance through the injection of recombinant IL-18. IL-18-deficient mice also have a higher food intake compared to wild-type mice, and this is due to the loss of appetite control (69)

Metabolic regulation of immune responses

The cellular programs such as development, differentiation, proliferation and effector functions depend on the metabolism of the immune cell, which is necessary to fulfill the bioenergetic demands and provide the building blocks that are required for the cell's survival and function (92). All cellular metabolic pathways are controlled by extracellular signals which regulate nutrient uptake (glucose, amino acids, and fatty acids), storage and usage (93). In order for immune cells to secrete cytokines, chemokines, proliferate and undergo clonal

expansion to control microbial infections, they need to increase their metabolism. Since immune cells lack the ability to store nutrients, they increase their nutrient receptors to increase the uptake of nutrients from their microenvironment (92). The response of immune cells depends on the uptake of glucose and glutamine that are important for generating adenosine 5'-triphosphate (ATP) as well as the building blocks that are necessary for the synthesis of RNA, DNA, and proteins, which support the proliferation and activation of immune cells. The reprogramming of cellular metabolism is also an important step for immune cell maturation (94, 95).During infection, immune cells shift from a quiescent state to an activated state in which they increase the production of cytokines, lipid mediators, tissue remolding enzymes, and migrate to the site of infection. The transition from a quiescent to an activated state depends on the different nutrient pathways, and how these metabolic pathways support the functional changes (96).

Glucose, the most abundant carbohydrate and the principal energy source, can fuel the production of ATP through different pathways. The canonical pathways are glycolysis, which occurs in the cytoplasm, as well as the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) which occur in the mitochondria. The cells uptake glucose through glucose transporters and convert it to glucose-6-phosphate using hexokinases. Glucose-6-phosphate is the starting point for glycolysis and the pentose-phosphate pathway (PPP) that generates riboses, the backbone of RNA and DNA synthesis, and NADPH which is essential for fatty acid synthesis. After glucose is converted to glucose-6-phosphate, the latter is metabolized into two molecules of pyruvate by reducing NAD^+ to NADH, consequently generating two molecules of ATP (Fig. 1.3) (97).

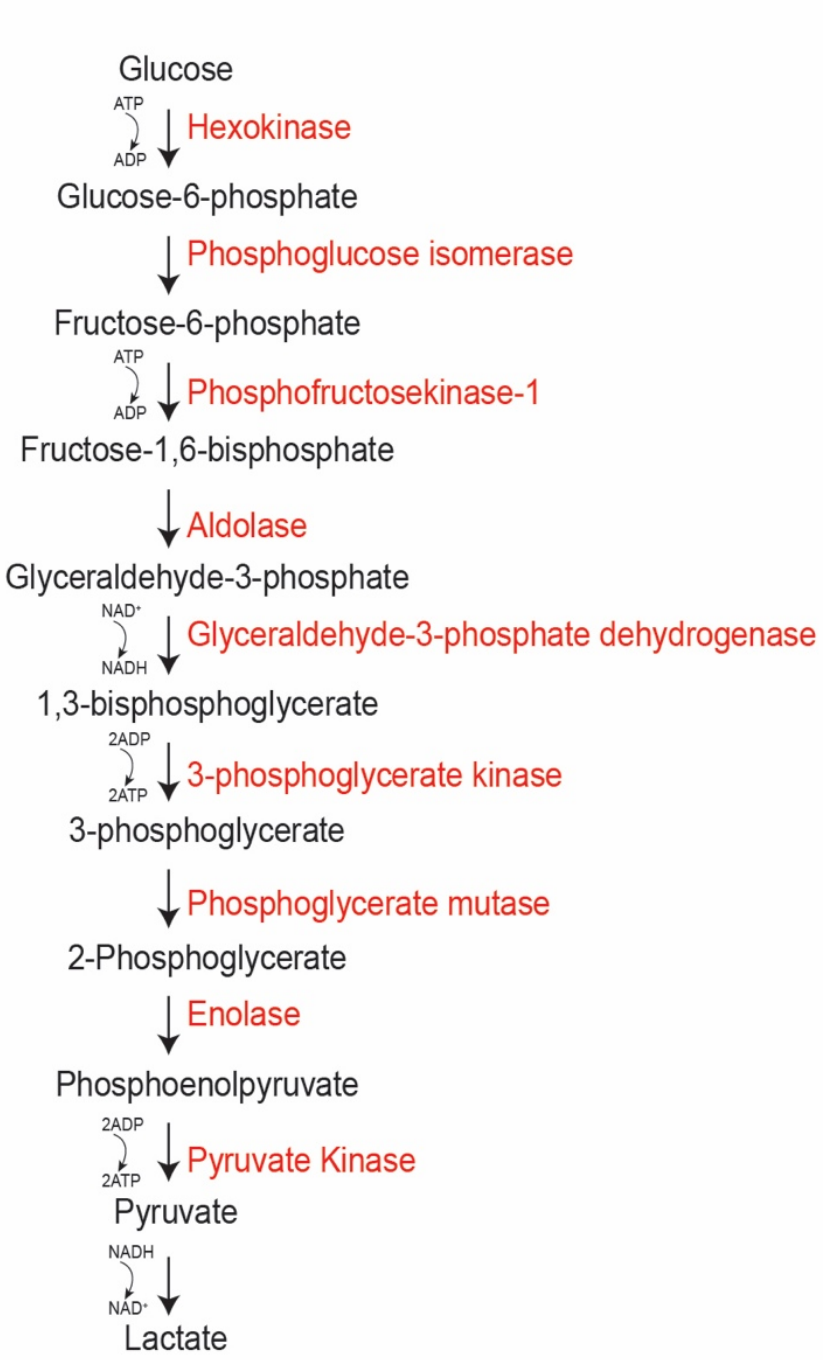

Figure 1.3. Glycolysis pathway.

In normoxic conditions, pyruvate is metabolized to CO_2 , H_2O and GTP/ATP in the TCA cycle to generate NADH which is oxidized by OXPHOS to generate ATP and NAD^+, whereas, in hypoxic conditions, pyruvate is reduced to lactate and NAD^+ (92). In the TCA cycle the oxidation of nutrients as well as the generation of NADH contribute to ATP synthesis. Carbohydrates, fatty acids, and proteins are metabolized to acetyl-coenzyme A (acetyl-CoA) in the TCA cycle and provide the substrates for isoprenoids, cholesterol, flavonoids, and fatty acids synthesis that is important for the post-translational modifications of histones and proteins (98). Acetyl-CoA is oxidized in the TCA cycle and generates CO_2, NADH, and succinate. In the process of OXPHOS, NADH fuel the electron transport chain through complex I, while succinate is a substrate of complex II and thereby is involved in the generation of the mitochondrial proton gradient required for ATP synthesis. Mitochondrial OXPHOS generates 30-36 ATPs per molecule of glucose compared to 2 ATPs per molecule of glucose via glycolysis (92). Through the PPP pathway, the glycolysis pathway and TCA cycle generate the required intermediates for the biosynthesis of riboses and fatty acids. TCA cycle also generates acetyl-CoA by using citrate in the presence of ATP citrate lyase. The glycolysis intermediate, 3-phosphoglycerate, and pyruvate are essential for the synthesis of nonessential amino acids such as serine, cysteine, glycine, and alanine, whereas the TCA cycle intermediates, oxaloacetate and α-ketoglutarate, are used for the synthesis of aspartate, asparagine, proline, and arginine (99). In order for the biosynthetic reactions to occur, immune cells need to replenish the intermediates from the glycolysis pathway and TCA cycle to sustain these metabolic pathways (100). To replenish the required glycolysis intermediates, activated innate and adaptive immune cells increase their metabolic flux through the glycolytic

pathway. There are two reactions by which the intermediates of the TCA cycle are replenished, first converting pyruvate to oxaloacetate in the presence of pyruvate carboxylase and second converting glutamate to α-ketoglutarate by glutamate dehydrogenase (95).

Activated immune cells need to increase their glucose uptake to generate pyruvate and increase their glutamine uptake to generate glutamate as these increases are required to sustain the anaplerotic reactions (95). Most cancer cells and activated immune cells produce an elevated level of lactate regardless of the availability of oxygen (aerobic glycolysis), and this is called the Warburg effect (101). Normal proliferating cells undergo cell cycle arrest and switch to the catabolic metabolism when their ability to generate ATP from glucose is compromised. To overcome this problem, cells will activate adenylate kinases which keep ATP from decreasing by converting two ADPs to one ATP and one (adenosine 5′-monophosphate) AMP (102). On the other hand, AMP will accumulate inside the cells and activate AMP-activated protein kinase (AMPK). The activation of AMPK increases catabolic pathways such as glucose uptake and fatty acid oxidation to promote ATP production (103).

Glucose and glutamine are the main sources of carbon, nitrogen and free energy for cell growth and division. Most of the glucose is involved in the synthesis of macromolecule precursors such as acetyl-CoA to generate fatty acids, glycolytic intermediates for nonessential amino acids synthesis, as well as ribose for nucleotides. Glioblastoma cells in culture convert as much as 90% of glucose and 60% of glutamine to lactate to generate NADPH to support cell proliferation (101). The conversion of glucose and glutamine into lactate occurs in the presence of lactate dehydrogenase (LDH) and the inhibition of LDH activity disrupts cell proliferation (99).

The source of carbon involved in fatty acid synthesis is glucose that is converted into acetyl-CoA in the mitochondrial matrix, used to generate citrate for the TCA cycle. Proliferating cells show high levels of ATP/ADP as well as $NADH/NADH^{+}$, which direct the citrate back into the cytosol to generate lipids (104). The acetyl-CoA is converted back from citrate and is used as a carbon source to generate acyl chains. Its synthesis requires ATP citrate lyase (ACL) and the inhibition of ACL impairs cell growth (105). Glutamine uptake provides carbons for lipid synthesis in the form of mitochondrial oxaloacetate to maintain citrate production. Taken together, the metabolism of glucose and glutamine is important for the production of acetyl-CoA and NADPH that are required for fatty acid synthesis (99).

The control center of cellular metabolism is the mechanistic/mammalian target of rapamycin (mTOR), which is a serine/threonine kinase that is highly conserved from yeast to humans (106). Rapamycin, an mTOR inhibitor, was discovered in 1970 in the soil of Rapa Nui island and was found to have antifungal activity. Now, mTOR has many functions in the mammalian cells and acts as a metabolism regulator (107, 108). There are many signals that can control mTOR response to regulate cell growth and proliferation. Examples include hormones (insulin), growth factors (IGF-1), antigen-specific receptors (TCR and BCR), pattern recognition receptors (TLR), cytokines, and nutrients such as amino acids (109). mTOR can form two complexes in the cell: the mTOR complex 1 (mTORC1) and the mTOR complex 2 (mTORC2). The two complexes have different signaling pathways, although they can mediate overlapping cellular functions (110). The distinct structure of both complexes mediates their specificity with Raptor being the target for mTORC1 and Rictor being that for mTORC2. In a study investigating the function of each complex in immune cells, acute treatment with rapamycin in mice inhibited mTORC1 activity and increased the activity of

mTORC2, whereas Torin, an active site mTOR inhibitor, inhibited both complexes (111). The main function of mTORC1 is regulating the cellular growth and proliferation by targeting the anabolic pathways including mRNA translation; while the main function of mTORC2 is to control the downstream signal transduction through AKT, SGK1 as well as the actin cytoskeleton (112).

Both mTORC1 and mTORC2 influence cellular metabolism. PI3K signaling leads to the phosphorylation of AKT and the activation of mTORC1 results in the phosphorylation of ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E (eIF4E) binding protein (4E-BP) (Fig. 1.4). These processes promote the translation of the metabolic enzymes that are important for cell proliferation, such as the nucleotide biosynthesis enzymes and the mitochondrial proteins that are involved in mitochondria homeostasis and electron transport (TFAM, complex I and V intermediates) (109). They also promote the synthesis of transcriptional factors required for the metabolic reprogramming such as Myc and HIF-1α (113). mTORC1 also can regulate metabolism through S6K to stimulate the biosynthesis of pyrimidine and stimulate the mitochondrial tetrahydrofolate (mTHF) cycle to increase purine biosynthesis. Moreover, mTORC1 stimulates the biosynthesis of lipids and sterols by the activation of sterol regulatory binding element proteins (SREBPs) (112).

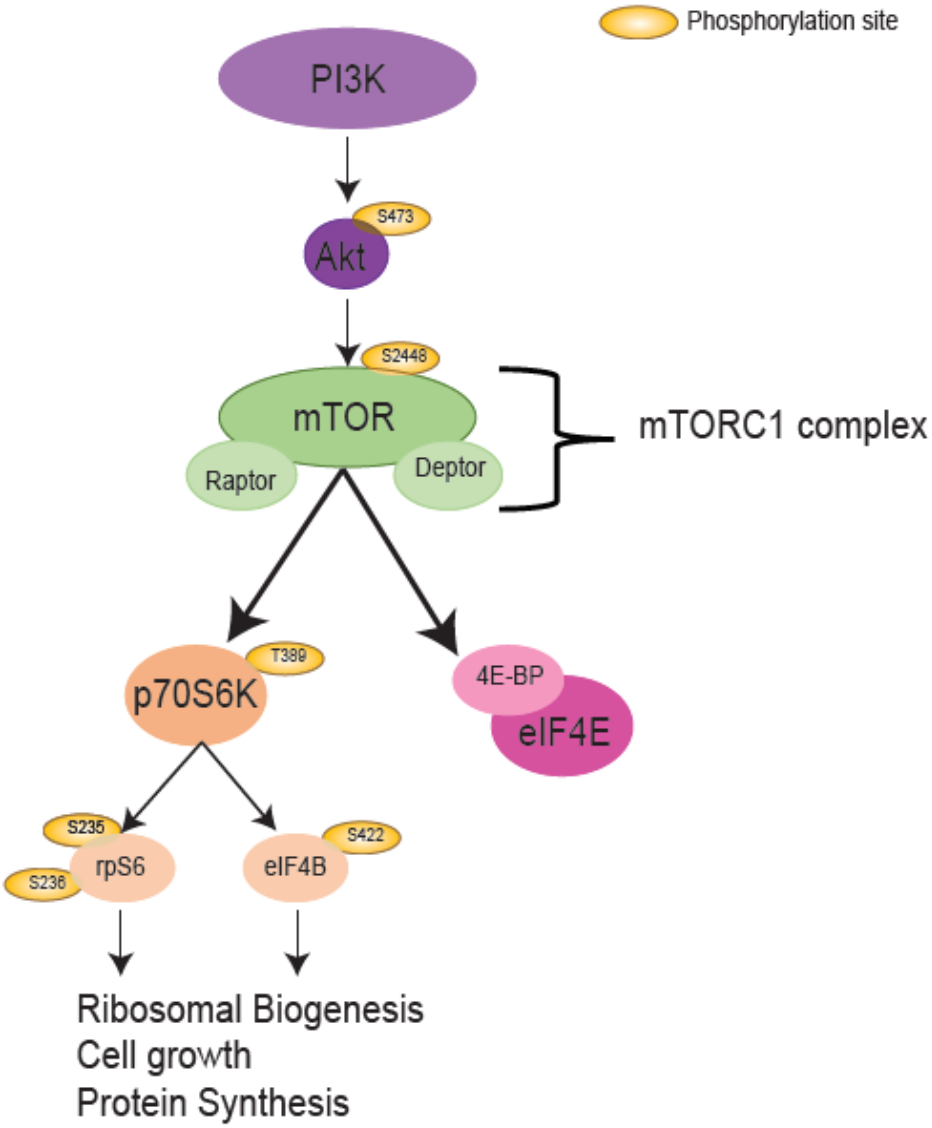

Figure 1.4. mTORC1 pathway and translation initiation. Cellular mTORC1 activity is regulated by phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) signaling. Phosphorylation of mTOR on residue serine 2448 leads to the activation of mTOR and phosphorylation of ribosomal protein S6 kinase 1 (p70S6K) and eukaryotic initiation factor 4E binding protein (4E-BP). Phosphorylation of p70S6K phosphorylates ribosomal protein S6 (rpS6) and eukaryotic initiation factor 4B (eIF4B).

mTORC2 has a role in enhancing glycolytic metabolism by activating AKT through Ser473 phosphorylation. The mTORC2-AKT signaling activates hexokinase 2 (HK2), which is required for the glycolysis pathway (114). The transcription factor Foxo1 can inhibit Myc, which results in decreasing the Myc-mediated metabolism as well as the suppression of metabolism through both glycolysis and oxidative phosphorylation. mTORC2 can phosphorylate Foxo1 and inhibit its role in suppressing Myc activity which is required for cell growth (115).

The activation of mTORC1 occurs through AKT mediated phosphorylation of tuberous sclerosis complex (TSC1-TSC2-TBC1D7) (116). TSC2 is a GTPase activating protein (GAP) that targets the Ras family GTPase Rheb. TSC2 inhibits Rheb and results in the inactivation of mTORC1 (Fig. 1.5) (117). The recruitment of mTORC1 to the lysosomes through amino acids such as leucine and the dissociation of TSC2 from the lysosome after the phosphorylation of TSC2 will activate mTORC1 by Rheb (118).

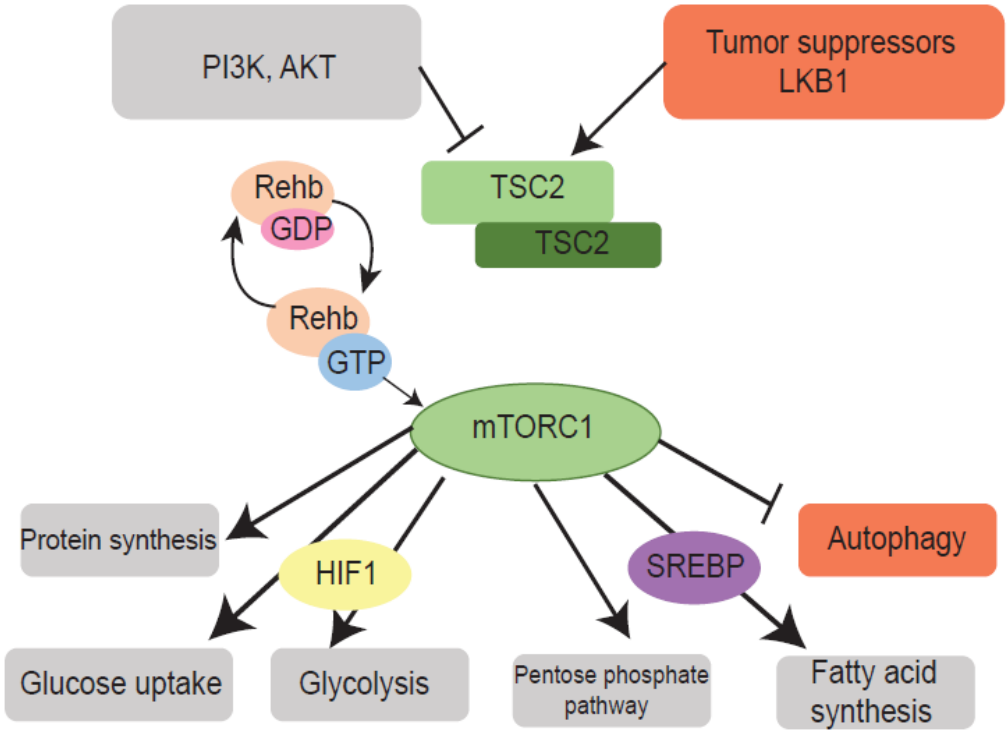

Figure 1.5. Model for mTORC1 activation. mTORC1 activity is regulated by the tuberous sclerosis complex TSC1-TSC2. PI3K and AKT inhibit the TSC1-TSC2 complex to promote the accumulation of Rheb-GTP and activate mTORC1. Tumor suppressors such as LKB1 stimulate the TSC1-TSC2 complex to inhibit the activation of mTORC1 by Rheb. mTORC1 activation can block autophagy, regulate glucose uptake and glycolysis through hypoxia-inducible factor 1(HIF1), activate the pentose phosphate pathway, and induce fatty acid biosynthesis through sterol regulatory element binding protein (SREBP).

mTORC1 plays an important role in promoting or inhibiting inflammation, as well as regulating the balance of pro- and anti-inflammatory signals. For example, blocking mTORC1 with rapamycin in macrophages will promote the production of pro-inflammatory cytokines while inhibiting anti-inflammatory cytokines such as IL-10, resulting in increasing the cell's ability to inhibit angiogenesis in tumors (119). The deletion of TSC2 in macrophages will result in chronic mTORC1 signal, promoting macrophage hypertrophy and proliferation, leading to an increase in granuloma formation *in vivo*. This shows that an increase in the activity of mTORC1 in macrophages can lead to immunopathology (120). mTORC1 affects the production of IL-10 indirectly by increasing the phosphorylation of STAT3, as well as by increasing the degradation of programmed cell death protein 4 (Pdcd4), leading to the release of the twist family transcription factor 2 (Twist2) and promoting Maf-dependent expression of IL-10 (121).

Bone marrow-derived macrophages deficient in TSC1 show increased activity of mTORC1, which increases the inflammatory response upon LPS treatment (122). mTORC1 plays an important role in regulating metabolism. The activation of immune cells is associated with an increase in the glycolysis level that is regulated by mTORC1. mTORC1 is required for NK cell effector function. Upon activation of NK cells with poly (I:C) or cytokines IL-2 and IL-12, their proliferation and the glycolysis level will increase; while treatment with rapamycin will inhibit their proliferation and decreases glycolysis (123, 124). Moreover, the rapamycin-sensitive mTOR-dependent pathway regulates the survival and development of NK cells through IL-15. IL-15 secretion is regulated by TSC1 to prevent the excessive activation of NK cells as well as cell death (125).

Cytokine receptors act as growth factors and are regulated by PI3K-Akt signaling pathway to increase the activation of Rheb on the surface of lysosome. The localization of mTORC1 in the lysosome is required to sense the availability of the nutrients and support anabolic growth. Activation signals such as TCR need to be combined with signals that indicate nutrient availability. During infection when the TCR is triggered, mTORC1 initiates anabolic metabolism to support cell proliferation, growth and effector function (112). mTORC1 is important for the activation of CD8+ T cell as well as the activation and differentiation of a subset of Th cells. In Raptor-deficient T cells, proteomic analysis revealed the expression of different transcription factors that are involved in metabolic reprogramming such as MYC, YY1, GABPA and SREBF1. (126). Also, inhibiting mTORC1 using rapamycin in CD8+ T cells results in a reduction in cell size, protein content, glucose transporters, glycolytic enzymes, and cholesterol synthesis enzymes. A limitation in effector proteins such as IFNγ, perforin-granzyme, and TNFα were also observed upon rapamycin treatment (127). There are additional intracellular sensors that detect when nutrients are scarce and which can shorten the circuit of mTORC1. For example, when glucose levels are low, AMPKα1 suppresses the mRNA translation of IFNγ in T cells (128).

Amino acids transporters

Cells are able to respond to changes in the availability of nutrients by changing their function to adapt to the extracellular milieu. For example, they can modify their growth and proliferation rates as well as their energy consumption. When nutrient availability is low, cells can increase their nutrient uptake by increasing their nutrient transporters (129). Since the cell surface membrane is selective and aminto acides (AAs) are not able to diffuse across the lipid membrane, the cells uptake AAs through membrane-spanning transporter proteins (130)

(131). AA transporters are also able to move ions, including Na+, H+, K+ and Cl- (132) (133). AAs are divided into essential amino acids which are not produced by the body and can be obtained from the diet such as leucine, isoleucine, valine, phenylalanine, threonine, methionine, lysine, histidine and tryptophan, whereas the non-essential amino acids can be produced by the body such as alanine, arginine, glutamine, glycine, aspartic acid, serine, asparagine and cysteine (134).

The classification of AAs transporters is based on the gene sequences and there are 6 major families of transporters in the solute carrier (SLC) gene superfamily (SLC1, SLC6, SLC7, SLC36, SLC38 and SLC43 families) as well as an orphan transporter, SLC16, which is a monocarboxylate transporter that transports aromatic AAs (129). The transporter glycoproteins are composed of several transmembrane domains (TMDs) that are organized around a central pore region (10-12). SLC3 gene family is also an AA transporter, however their glycoproteins form a single TMD that acts as a regulatory subunit for SLC7 transporters (135, 136). The AA binding sites of AA transporters are specific and recognize AAs with similar structures. For example, large neutral AAs (LNAAs), small neutral AAs (SNAAs), cationic AAs (CAAs) or anionic AAs (AAAs) have similar structures within each group of AAs. The expression of AA transporters is tissue specific and many types of cells express many AA transporters with overlapping specificity. The activity and the substrate competition of AA transporters are important to regulate and maintain the flux of AAs through the cell membrane (136, 137). AA transporters have an important role upstream and downstream of mTORC1, which regulates and monitors the extracellular and intracellular AA availability (138) (139). AA transporters can activate signaling pathways directly either by causing a conformation change in the transporter leading to the initiation of signal transduction, such as

signaling through *SLC38A2* transporters (140); or by acting as a channel to deliver AA to activate the signaling pathway, which is the case for the leucine transporter, *SLC7A5* (141).

The increase of intracellular AA concentrations will allow the Rag-dependent translocation of mTORC1 from the cytosol to the surface of the lysosome. LNAAs which include leucine, isoleucine, valine, phenylalanine, and tryptophan are required to activate mTORC1 along with glutamine and arginine (142). There are many putative cytosolic AA sensors that can activate mTORC1 and some of them bind directly to AAs such as leucine. Some examples of those sensors include leucyl-tRNA synthetase (LRS), glutamate dehydrogenase (GDH), and unbranched chain AA receptors 1 and 2 (UBR1-2) (138, 143). Leucine binds to LRS, serving as a GAP for RagD and attaches the Rag protein to the lysosome membrane leading to the recruitment of mTORC1 (Fig. 1.6) (143). Thus, amino acids (AAs) are known as a direct activator of mTORC1, and the absence of one of the branched-chain amino acids (leucine, isoleucine, and valine) from the culture medium will result in the inhibition of protein synthesis in lymphocytes (144). Glutamine and LNAAs can activate mTORC1 and the deprivation of glutamine can decrease mTORC1 levels without affecting intracellular leucine concentrations (145). AA limitation can be involved in some infections, where pathogens compete with lymphocytes for the uptake of amino acids (146). This AA limitation also occurs in other diseases such as liver cirrhosis that is associated with low levels of branched-chain amino acids in the plasma (147). AA transporters are upregulated in activated T cells and the absence of these transporters will inhibit the response to antigens due to the loss of mTORC1 activation, which is involved in the metabolic reprogramming of T cells (148). In the tumor microenvironment, nutrient availability is poor and the loss of effector function of tumor-infiltrating-lymphocytes (TILs) might be due to the competition

for nutrients such as amino acids and glucose (149). During tumorigenesis, TILs showed low phosphorylation levels of SK6, which indicates a reduced mTORC1 activity, negatively affecting the metabolic reprogramming of TILs and anti-tumor response (150).

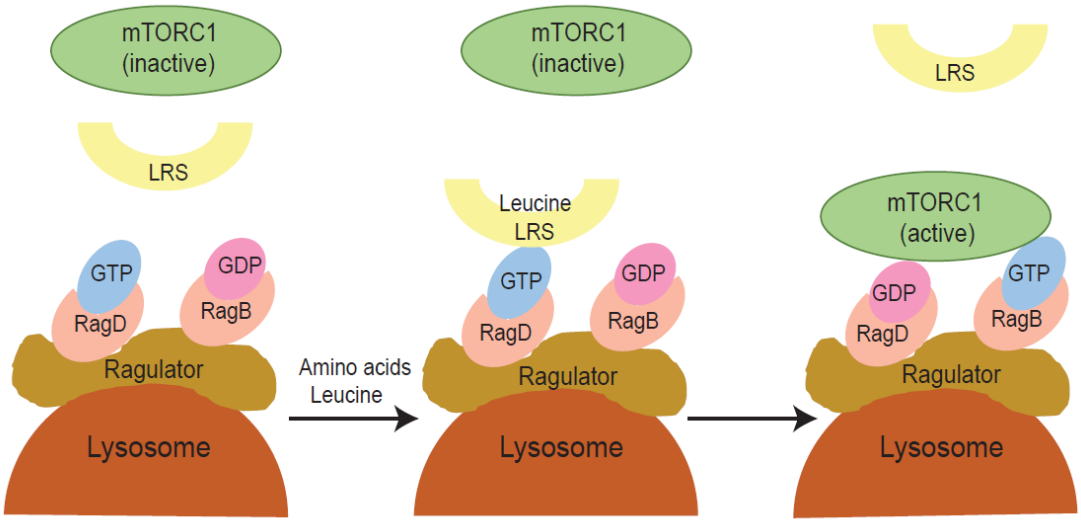

Figure 1.6. The role of leucyl-tRNA synthetase (LRS) in amino acid-induced mTORC1 activation. When leucine is present, LRS senses its concentration and binds to it. Then, LRS directly interacts with GTP-bound RagD, which is converted to the GDP-bound form, and then dissociates from the active Rag heterodimer to activate mTORC1.

The AA transporters at the plasma membrane of epithelial cells are different from those at the nonepithelial tissues (129). In epithelial cells, *SLC6A19* and *SLC6A14* can transport LNAAs and SNAAs directly into the cells and can activate signaling upstream of mTORC1 activation (151) (152). On the other hand, in nonepithelial cells, LNAAs are taken up through exchange mechanisms, whereas SNAAs transport into the cell is coupled with the movement of Na^+ ions down their concentration gradient. Some SNAAs such as glutamine and alanine accumulate in some tissues like skeletal muscles and serve as a source of nitrogen; whereas LNAAs do not accumulate and equilibrate between intracellular and extracellular fluids (153, 154). Any mutation or inactivation of these AA transporters inhibits the growth and proliferation of the cells in culture due to the reduction of mTORC1 signaling (140, 148). The function of *SLC7A5* gene expression is to serve as an initiating factor for the activation of mTORC1 by hypoxia-inducible factor 2-α (HIF2α) (155) and during T cell activation (148). Fibroblasts express high levels of *SLC7A5*, which is correlated with leucine-induced mTORC1 activation in these cells (156). The intracellular glutamine accumulation through *SLC1A5* acts as a limiting factor for the activation of mTORC1 because *SLC7A5* requires intracellular glutamine to transport leucine into the cell (141).

During rapid cell growth and proliferation, the AA supply becomes a limiting factor for protein synthesis. In lymphocytes, during an immune response, the availability of LNAAs such as leucine for protein synthesis and SNAAs such as glutamine for cell metabolism depends on the expression of AA transporters on the cell surface. Sustained growth of the lymphocytes requires upregulation of AA transporters (129). For T cell activation, the cells need to increase protein synthesis, which is associated with high induction of *SLC7A5* mRNA,

as well as the upregulation of *SLC1A5,* to increase the uptake of AA (148). Increased leucine uptake by *SLC7A5* in activated T cells is required for the activation of mTORC1, the upregulation of c-Myc mitogen, as well as increasing the energy to support the metabolic pathways (148). Indeed, *SLC7A5* inhibitors are potent immunosuppressants (157) (158). Compared to T cells treated with rapamycin, T cells deficient in *SLC7A5* show more severe defect in proliferation and growth, indicating that *SLC7A5* has a role in controlling cell division and proliferation independently of mTORC1 (148).

Reactive oxygen species (ROS)

ROS are highly reactive short-lived oxygen-containing molecules, which have unpaired electrons; hence these molecules are called radicals. There are many forms of ROS, such as super oxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), hypochlorous acid (HOCl), lipid peroxidise (ROOH), and singlet oxygen ($O^{\bullet}$) (159). The most well-known ROS are $O_2^{\bullet -}$ and H_2O_2, which are involved in the regulation of biological processes. When $O_2^{\bullet -}$ is produced, it will react directly with the surrounding molecules, or turn into H_2O_2 either spontaneously or in the presence of superoxide dismutase (SOD) (160). H_2O_2 can be converted to $OH^{\bullet}$, to HOCl by myeloperoxidase or to H_2O by catalase, peroxidase or peroxiredoxin (161). $OH^{\bullet}$ and HOCl are highly reactive and cause irreversible damage to the molecules. The mitochondria are one of the primary sources of ROS in the cells. They express the electron transport chain (ETC) complexes, through which the electrons are transferred, from NADH and succinate to be accepted by O_2. In order for O_2 to be reduced to H_2O, four electrons need to be transferred through the ETC complexes. Due to occasional electron leakage from the ETC, O_2 may undergo only one or two electron reductions in the presence of SOD forming $O_2^{\bullet -}$ or H_2O_2. More specifically, complexes I and III of the ETC are the major sources of the

mitochondrial $O_2^{\bullet -}$ (162, 163). There are many metabolic enzymes that produce ROS as intermediates or by-products of their reactions, and they are found in the mitochondria, endoplasmic reticulum, peroxisomes, and cytosol. Examples of such enzymes include ERO-1, cytochromes P-450 and b5, lipoxygenases, cyclooxygenases, α-ketoglutarate,glycerol phosphate dehydrogenases, as well as hydroxyacid, urate, xanthine, monoamine, diamine, polyamine, and amino acid oxidases (164). Also, there is an enzyme complex called NADPH oxidase that can produce ROS. In addition, ROS can be produced from exogenous sources such as smoking and air pollutants, ultraviolet and gamma radiation, as well as some drugs and chemicals (165).

ROS can damage proteins, lipids, and nucleic acids, and the cells use various mechanisms to neutralize the high level of ROS called the antioxidant system. The antioxidant system consists of several antioxidant enzymes including SODs, catalases, glutathione peroxidases (GPXs), peroxiredoxins (PRXs), thioredoxins (TRXs), glutaredoxins (GRXs), sulfiredoxins (SRXs), thioredoxin reductases, glutathione reductases, and methionine sulfoxide reductases (166), as well as non-enzymatic antioxidant molecules such as glutathione, ascorbate, pyruvate, α-ketoglutarate, and oxaloacetate (167). When the level of ROS is elevated and the antioxidant system is not able to neutralize it, the cells will undergo oxidative stress, which affects the cell's function and causes cell death or malignant transformation (168, 169).

During the last decade, there has been numerous lines of evidence demonstrating that ROS have an crucial role in cellular signaling (170). ROS mediated-signaling are involved in several cellular processes such as cell growth, stem cell renewal, tumorigenesis, cell death, cell migration and immune responses (171). H_2O_2 serves as the major signaling messenger

because it is stable, can cross the cell membrane and is able to react with cysteine residues (172). The level of ROS involved in signaling transduction is less than what is produced during oxidative stress. Because the antioxidants are abundant in the cells, ROS are not able to travel very far, and therefore the ROS mediated-signaling occurs locally, meaning that the sources and targets of ROS signaling will be located in the same site (173). ROS signalling can be regulated locally through specific antioxidants. For example, glutathione, a scavenger of many ROS, can react with HOCl but reacts slowly with H_2O_2, while PRXs can react with H_2O_2 (166, 171)

There are many signaling pathways that can be initiated by H_2O_2, such as the signaling of many receptors, which occur by oxidizing the receptors directly or through the activation of the intracellular protein tyrosine kinases (PTKs) that are important for the signaling of certain receptors. H_2O_2 can also inhibit protein tyrosine phosphatases (PTPs), which negatively regulate the signaling transduction of the receptors and activating PTKs (174) (175). In addition to acting as ROS scavengers, antioxidants can be involved in the signaling pathways. For example, thioredoxin (TRX) is involved in the antioxidant system in the cells by catalyzing the reduction of the oxidized proteins (176).

H_2O_2 is released into the extracellular space and it is stable in the oxidized environment. It can diffuse from activated cells to the cells nearby and initiate cellular signals (174). As a host defense mechanism, phagocytic cells produce high amount of H_2O_2 to destroy the pathogen and to activate dendritic cells, which initiate the antigen-specific immune response (170). On the other hand, during chronic inflammatory diseases, NK cell and T cell functions are impaired by the ROS, which are produced by the tumor-associated macrophages, making the ROS an immunomodulator and suppressor of immune response (177).

Mitochondrial ROS are involved in anti-bacterial and anti-viral signaling (178). ROS can help the anti-bacterial activity of macrophages. TLRs can enhance the production of ROS and the translocation of tumor necrosis factor receptor-associated factor 6 (TRAF6), which is the TLR signaling adaptor required for this response. TRAF6 deficient macrophages showed low levels of TLR-induced mitochondrial ROS and a decreased level of their ability to kill *S. typhimurium*. Also, similar results have been shown by the overexpression of mitochondrial catalase in macrophages (178). IFNγ produced by activated macrophages depends on the expression of mitochondrial respiratory chain nuclear genes and the production of mitochondrial ROS (179). The activation of the nuclear receptor, estrogen-related receptor α (ERRα), and coactivator, PPARγ-coactivator-1β (PGC-1β), are required for inducing the IFNγ gene and producing ROS. The absence of ERRα and PGC-1β in macrophages showed a decreased level of mitochondrial ROS and impaired ability to clear *L. monocytogenes* (180). For type I interferon and pro-inflammatory cytokine production, RIG-I-like receptor (RLR) family is required for viral infection response; and increasing ROS production will increase the RLR signals (181). Also, ROS are required for RLR-mediated interferon regulatory factor (IRF)-3 activation and downstream IFN-β expression (182).

During cancer, NK cells produce ROS that are required for NK cell-mediated cytolysis (183). In chronic granulomatous disease (CGD), although NK cells have a defect in NADPH oxidase function and decrease of $O_2^{\bullet -}$ production, NK cells have comparable killing ability because of their ability to produce $OH^{\bullet}$ that might be required for the secretion of cytotoxic granules. ROS from monocytes of healthy individuals or cancer patients can suppress NK cell function, activation and proliferation but the monocytes recovered from CGD do not affect the function of NK cell, indicating the involvement of NADPH oxidase in this process (184).

Stimulating NK cells by IL-12 will increase the level of ROS; however, the prolonged stimulation with IL-12 will increase the accumulation of ROS and cause apoptosis, demonstrating the role of ROS as a mechanism of IL-12 regulation (185). Human NK cells subsets have different levels of sensitivity to ROS. ROS produced from monocytes decrease the activating receptors NKG2D and NKp46 in $CD56^{dim}$ NK cells but not in $CD56^{bright}$ NK cells. $CD56^{bright}$ NK cells are more resistant to apoptosis and functional suppression induced by ROS than $CD56^{dim}$ subset (186). The reason of ROS sensitivity is due to the accumulation of antioxidants in $CD56^{bright}$ NK cells compared to the $CD56^{dim}$ population (187).

Glutaredoxin 2 (Glrx2)

Glutaredoxin belongs to the oxidoreductase family and has two isoforms, cytosolic glutaredoxin 1 (Grx1) and mitochondrial glutaredoxin 2 (Grx2) (188). Upon ROS-induced oxidative stress, there are oxidative repair systems in the cells, including the Grx and Trx systems. Grx is GSH-dependent, and is in involved in the dethiolation of either glutathionylated proteins or protein thiol mixed disulfide (PSSG) (189). There are several enzyme substrates for Grx1 dethiolase, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), protein tyrosine phosphatase 1B (190), HIV protease (191), p65 and caspase 3 (188). Grx2 is found in the nucleus and many tissues, such as in the brain, liver, heart, pancreas and the eye. Grx2 has been found in almost all ocular tissues, including the cornea, iris, lens, retina, and optic nerve (192). The molecular weight of Grx2 is 16 kDa and shares only 34% of the sequence identity with Grx1 (188). Grx2 has a high affinity to the glutathionylated proteins and does not become inactive through oxidation. Grx2 is the first oxidoreductase family member found to have iron-sulfur (Fe-S) containing protein. The Fe-S cluster connects two Grx2 molecules to make a stable and inactive dimer in the presence of glutathione (193).

During oxidative stress or in the presence of high levels of the oxidized glutathione (GSSG), the Grx2 dimer components will dissociate from the Fe-S cluster to make active monomers used as a redox sensor (193). Grx2 can serve as an antioxidant to protect the cells from oxidative stress by preserving the activity of complex I of the ETC through glutathionylation (194).

During oxidative stress, redox changes such as glutathionylation reactions, which are post-translation modifications, are required to control protein function (195). In order for these modifications to happen, there is formation of a disulfide bridge between GSH and the protein cysteine thiol. Depending on the cell's conditions, these reactions can occur nonenzymatically or enzymatically (196). The non-enzymatic glutathionylation reactions can occur non-specifically and cause hyper-glutathionylation of the proteins, changing their activity (197). The enzymatic glutathionylation reactions are controlled and very specific post-translation modifications that usually occur during oxidative stress (198).

The thiol group in the deglutathionylated proteins (PSH) is a sensitive target and undergoes irreversible and reversible redox modifications (199). PSSG can affect the function, activity, and structure of proteins or enzymes, as well as disrupt cell signaling. Grx proteins are the primary enzymes that catalyze these reactions and deglutathionylate PSSG (200). Grx1 in the cytosol and Grx2 in the mitochondria are required to deglutathionylate the target proteins (201). The deglutathionylation of proteins by Grx2 happens in two steps. The first involves Grx2 deglutathionylating PSSG through a thiol-disulfide exchange reaction, generating PSH and Grx2 glutathionylated protein (SSG) intermediate. The second step involves Grx2-SSG binding to GSH, regenerating Grx2 and producing GSSG (202). Moreover, Grx2 can deglutathionylate and glutathionylate proteins and the reversible nature

of Grx2 depends on the changes in 2GSH/GSSG ratio (Fig. 1.7). High 2GSH/GSSG causes protein deglutathionylation, whereas low 2GSH/GSSG activates Grx2 glutathionylase activity (202). The main target of Grx2 is complex I, which is affected by the increase and the decrease of 2GSH/GSSG. Complex I was shown to be a Grx2 target in lens epithelia and liver mitochondria (203). Also, uncoupling proteins UCP2 and UCP3 are controlled by Grx2-mediated glutathionylation reactions (204).

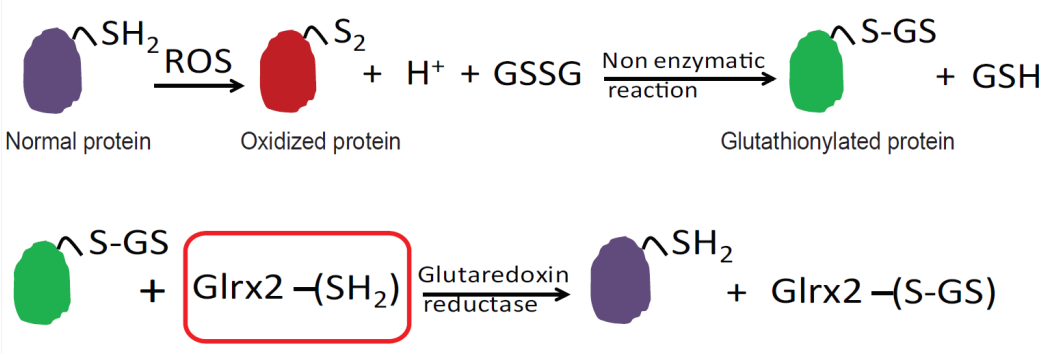

Figure 1.7. Model for the role of Glrx2 in redox system. Glutathione disulfide (GSSH) spontaneously interacts with the oxidized proteins and results in glutathionylation of these proteins and generates glutathione (GSH). The mitochondrial glutaredoxin Grx2 deglutathionylates the target protein by exchanging its thiol group with the S-GS group on the target protein. Glutathione interacts with the glutathionylated Grx2 and restores its thiol group, generating disulfide glutathione (GSSG). Disulfide glutathione is converted to glutathione by glutathione reductase and NADPH.

The importance of Grx2 in protecting proteins and enzymes from oxidative stress was shown in a cataract model. The functions of Grx2 were examined by using primary cells from Grx2-deficient and wild-type mice (WT). Although the deletion of Grx2 in mice does not show any changes in the phenotype of both morphological and biochemical characteristics compared to WT mice, the Grx2-deficient cells showed high sensitivity to oxidative stress (189). Grx2-deficient cells do not show any phenotypic changes because Grx2 forms an inactive complex with the Fe-S cluster in a normal and reduced environment, however it dissociates from the Fe-S cluster during oxidation, becoming an active monomer and activating the protective function of Grx2 (205). Also, the Grx2-deficient cells show reduced ability to detoxify oxidants in the presence of H_2O_2, which results in the reduction of ATP and GSH levels, affects the viability and the mitochondrial membrane integrity, and suppresses the activity of complex I (189). The treatment of Grx2-deficient cells with recombinant Grx2 protein was able to rectify all these changes, implying the critical role of Grx2 in protecting the function of the mitochondria. The overexpression of Grx2 in human lens epithelial cells protects the cells from H_2O_2-induced apoptosis, whereas the deletion of Grx2 results in the opposite effect (206).

Hypotheses and Objectives

IL-18 was identified as an IFNγ-inducing factor and was not known for its proliferative role. Later, studies have shown that IL-18 can support NK cell proliferation in the presence of IL-2 in vitro (207, 208) and during MCMV infection, IL-18 has a selective role in Ly49H+ NK cell expansion (19). Taking together, IL-18 is involved in NK cell proliferation, however, the mechanisms behind the role of IL-18 in inducing NK cell proliferation have not been identified yet. Here, I hypothesised that IL-18 is a potent cytokine that is involved in regulating NK cell metabolism through upregulating the nutrient transporters to support their proliferation. The main objective of the study in chapter 2 is investigating the role of IL-18 in inducing metabolic changes in NK cells.

Mitochondrial metabolism has a critical role in regulating T cell activation both in vivo and in vitro (209). In addition, studies showed that ROS are required for the induction of IL-2 and IL-4 cytokines in T cells (210). Treating the cells with H_2O_2 will induce IFNγ production from human NK and T cells, however, this study did not show if the secretion of IFNγ is specifically from NK cell (211). Antioxidant systems are required to balance the amount of ROS to avoid excessive oxidative stress (212). Glrx2 is an antioxidant enzyme and its absence is expected to increase ROS levels (189), however the role of Glrx2 has not been identified in NK cells. For this, the Glrx2-deficient mice would be an useful model to study the effect of ROS in immune cells. Thus, I hypothesized that ROS are essential to increase the effector functions of NK cells and the deletion of Glrx2 will enhance the function and the activation of immune cells. The objectives for the study in chapter 3 are: investigate whether ROS levels increase in cells upon stimulation, whether the total ROS and m-ROS are required for NK cell

effector functions, and investigate the role of Glrx2 in NK cell function upon in vitro stimulation or during MCMV infection in vivo.

Chapter 2: IL-18 upregulates amino acid transporters and facilitates amino acid-induced mTORC1 activation in natural killer cells

Preface

The following chapter consists of an original research project demonstrating that IL-18 enhances amino acid transporter expression on NK cells. This research was originally published in the Journal of Biological Chemistry

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The specific contributions of each author to this paper are listed below:

Saeedah Musaed Almutairi: Designed and performed most of the experiments and contributed to the writing/editing of the manuscript.

Alaa Kasim Ali: Performed all the injections, the radioactive experiments, sorting immune cells experiments, quantitative real-time PCR and contributed to the writing/editing of the manuscript.

William He and Doo-Seok Yang: Performed quantitative real-time PCR.

Peyman Ghorbani: Performed western blot experiments.

Lisheng Wang and Morgan D. Fullerton: Reviewed the manuscript.

Seung-Hwan Lee: Supervised experimental design and execution and contributed to the writing/editing of the manuscript.

Abstract

Upon inflammation, NK cells undergo metabolic changes to support their high energy demand for effector function and proliferation. The metabolic changes are usually accompanied by an increase in the expression of nutrient transporters leading to increased nutrient uptake. Among various cytokines inducing NK cell proliferation, the mechanisms underlying the effect of IL-18 in promoting NK cell proliferation is not completely understood. Here, we demonstrate that IL-18 is a potent cytokine that can enhance the expression of the nutrient transporter CD98/LAT1 for amino acids independently of mTORC1 pathway and thereby induce a dramatic metabolic change, associated with an increased proliferation of NK cells. Notably, the treatment of IL-18-stimulated NK cells with leucine activates the metabolic sensor mTORC1, indicating that the high expression of amino acid transporters induces amino acid-driven mTORC1 activation. Inhibition of the amino acid transporter CD98/LAT1 abrogated the leucine-driven mTORC1 activation and reduced NK cell effector function. Interestingly, during MCMV infection, in which several cytokines including IL-18 are produced, NK cells upregulated their nutrient transporters as they proliferated. However, signaling through IL-18 receptor was not required for the upregulating of nutrient transporters in vivo, suggesting that multiple independent pathways can modulate NK cell metabolism during infection and inflammation. Taken together, our study identified a novel role of IL-18 in upregulating nutrient transporters on NK cells and thereby inducing metabolic changes including the mTORC1 activation by amino acids.

Introduction

The inflammatory signals induce a metabolic change in immune cells as they shift from a quiescent state to an activated state, resulting in increased proliferation, cytokine production, and effector functions. Natural killer (NK) cells are innate lymphocytes that play a critical role in anti-viral immunity. NK cells are the first proliferating lymphocytes and serve as the principal cytotoxic cells during the early stages of infection (213-215). During virus infection, naive NK cells undergo rapid metabolic reprogramming to support their expansion and differentiation into potent effector NK cells (123, 216). IL-2 and IL-15 activate the mammalian target of rapamycin (mTOR) complex 1 (mTORC1), a key molecule required for glycolytic reprogramming that induces the upregulation of several glycolytic enzymes and GLUT1 in NK cells (106, 123, 124, 216-218). In immune cells including NK cells, the mTORC1 pathway is linked to the acquisition of effector functions, which is exemplified by defective effector functions in mTORC1-deficient or rapamycin-treated mice (123, 124, 216, 218).

Several cytokines including interleukin (IL)-2, 10, 12, 15, 18, 21, and type I IFNs are known to modulate NK cell proliferation and effector functions during infection and inflammation. Among them, IL-18 is a member of the IL-1 family of cytokines and was originally identified as IFN-γ inducing factor because NK cells and T cells secrete IFN-γ upon stimulation with IL-18 (219). It is mainly produced by macrophages and dendritic cells and is processed from an inactive precursor by caspase 1/11-mediated cleavage into the active form upon inflammasome activation in mice (220, 221). The cytosolic DNA sensor AIM2 inflammasome is known to induce systemic biologically active IL-18 during infection in vivo (222). So far, most studies on IL-18 have shown synergistic functions with IL-12 including

IFN-γ induction (220, 221). The critical role of IL-12 and IL-18 in IFN-γ production, which is important in directly or indirectly controlling virus replication, was previously demonstrated during MCMV infection (223, 224). In addition, we have identified that IL-12 and IL-18 can upregulate IL-2Rα chain that renders NK cells highly sensitive to IL-2 stimulation (225). This IL-12/18 pathway enhanced our understanding of NK cell proliferation and is currently being employed for the adoptive transfer of ex vivo expanded NK cells that can be sustained longer in vivo (226, 227). Another mechanism by which IL-18 exhibits its synergistic effect with IL-12 was described in previous work, where IL-18 was shown to prime NK cells in vivo to produce IFN-γ upon subsequent stimulation with IL-12 (228).

Interestingly, several reports presented the supportive role of IL-18 in NK cell proliferation during IL-2 stimulation in vitro (207, 208). Because IL-18 is not a proliferative cytokine that induces the STAT5 pathway, the effect of IL-18 in inducing proliferation might be indirect and influenced by other unidentified factors. We have shown that IL-18 induces the expression of CD25, the IL-2Rα chain, on NK cells (225), and thus the enhanced proliferation could be mediated by IL-18-induced CD25 upregulation on NK cells. However, a similar synergistic role between IL-18 and IL-15 was also demonstrated during NK cell proliferation (229), indicating that IL-18 utilizes an alternative pathway to promote NK cell proliferation. In addition, IL-18 was shown to support the selective expansion of the Ly49H+ NK cells during murine cytomegalovirus (MCMV) infection (230). Taken together, IL-18 is suggested to support the proliferation of NK cells; however, the mechanisms of IL-18 in promoting NK cell proliferation have not been clearly established.

In multicellular organisms, glucose and amino acids are plentiful in the extracellular milieu, but these molecules have to cross the cell membrane through transporters in order to be used as building blocks or for generating ATP (231). The nutrient transporters are comprised of the numerous solute carrier (SLC) groups of membrane transport proteins (>400 members) and show redundancy and promiscuity in their specificity (133, 232). For example, there are 11 SLC families dedicated to the transport of all 20 amino acids (233, 234). One well-studied amino acid transporter is CD98 that is encoded by *Slc3a2*. CD98 is not in itself an amino acid transporter, but forms disulfide-linked heterodimers with a variety of multiple membrane-spanning light chains that are responsible for the amino acid transport properties of the complex (233). One of the light chains is LAT1 (L-type amino acid transporter 1) that is encoded by *Slc7a5*. CD98 and LAT1 form a complex called the System L transporter, which preferentially imports large neutral amino acids (LNAA) such as leucine, isoleucine, and valine in exchange for the efflux of intracellular glutamine (141, 233). In addition to their roles as metabolites, accumulating evidence has demonstrated that essential amino acids (EAA) such as leucine, tryptophan, phenylalanine and arginine are known to activate mTORC1 in lysosomal compartments (235-238). A previous report demonstrated the ability of Rag GTPases to physically interact with mTORC1 and regulate its subcellular redistribution in response to leucine (239, 240).

In the present study, we characterized a mechanism by which IL-18 induces NK cell proliferation. In spite of the largely overlapping functions of IL-12 and IL-18, the signaling pathways of these two cytokines are different, suggesting a unique function of IL-18. We identified a novel IL-18-specific role on NK cells in which IL-18 can upregulate nutrient transporters and thereby modulate cellular metabolism. More importantly, the increased

expression of the System L amino acid transporter CD98/LAT1 enabled the activation of mTORC1 by enhanced uptake of leucine. Altogether, our findings demonstrated that IL-18 is the potential cytokine inducing metabolic changes in NK cells.

Results

Nutrient transporters are highly expressed on proliferating NK cells upon IL-18 stimulation

As previously demonstrated, enriched NK cells stimulated by IL-2/IL-18 showed increased proliferation upon increasing concentrations of IL-18 in a dose-dependent manner. Stimulation with 300 U/ml of IL-2 and 30 ng/ml of IL-18 for 3 days increased NK cells number up to 60 folds (Fig. 2.1A). Since we reasoned that the metabolic demands needed for the intense NK cell proliferation can be fulfilled by high expression of nutrient transporters, the expression of amino acid transporter CD98 on proliferating NK cells in regard to cell division was analyzed. Notably, higher expression of CD98 was observed in NK cells that were dividing fast, showing a strong correlation (Fig. 2.1B).

LPS treatment is known to induce IL-18 production through the inflammasome-dependent pathway (241). Notably, NK cells proliferate upon LPS treatment (242, 243) and the proliferation antigen Ki-67 as well as BrdU incorporation were highly increased in NK cells on day 2 post LPS treatment (Fig. 2.1C). To investigate whether the expression of nutrient transporters is also upregulated in LPS-induced inflammation, we analyzed the expression of nutrient transporters on NK and T cells from mice injected with LPS for 2 days. The LPS treatment in vivo resulted in the upregulation of CD98 and transferrin receptor (CD71) expression on NK cells (Fig. 2.1D). There was minor change in CD98 and CD71 expression on T cells. Thus, NK cell proliferation upon IL-18 stimulation is accompanied with a high expression of nutrient transporters, suggesting that their high expression is required to support the robust NK cell proliferation.

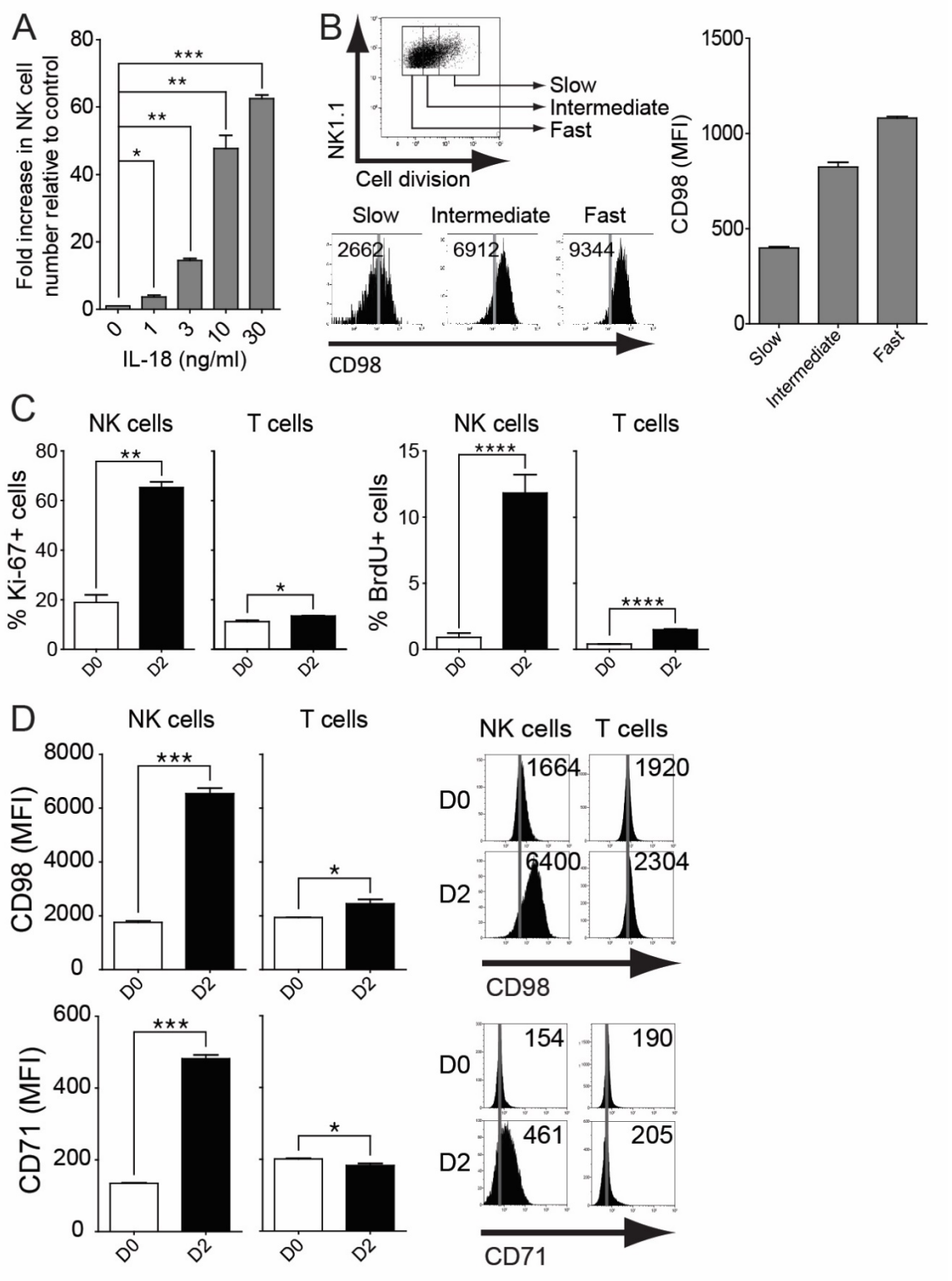
A
Fold increase in NK cell number relative to control
IL-18 (ng/ml)
B
NK1.1
Cell division
Slow
Intermediate
Fast
2662
6912
9344
CD98
CD98 (MFI)
C
NK cells
T cells
% Ki-67+ cells
% BrdU+ cells
D0
D2
D
CD98 (MFI)
CD71 (MFI)
1664
1920
6400
2304
154
190
461
205
CD71

Figure 2.1. Higher proliferation of NK cells is associated with enhanced expression of nutrient receptors upon IL-18 treatment in vitro and LPS treatment in vivo. (A) The number of expanded NK cells upon ex vivo stimulation with various concentrations of IL-18 relative to IL-2 alone was quantified. (B) NK cells from the spleen of naive C57BL/6 mice were ex vivo stimulated with IL-2 and IL-18. NK cells were prelabeled with cell proliferation dye and cultured with 300 U/ml of IL-2 and 3 ng/ml of IL-18 for 3 days. Dot plot depicts the dilution of cell proliferation dye on NK cells while the histograms depict the MFI of CD98 expression on NK cells gated in regard to cell proliferation. (C and D) C57BL/6 mice were either left untreated or challenged with 100 μg LPS intraperitoneally and sacrificed on the indicated days for further analysis. (C) NK cell proliferation upon treatment with LPS as measured by Ki-67 expression and BrdU incorporation. (D) Representative plots depict the MFI of CD98 and CD71 expression on NK cells and T cells in the spleens of naive (D0) or LPS-treated mice at day 2 post-injection (D2). For A and B, data are from one experiment representative of two independent experiments, with three replicates per group. For C and D, data are from one experiment representative of three independent experiments, with two to three mice per group. Data represent mean + SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

IL-18 can upregulate nutrient transporters on NK cells

To determine whether IL-18 can directly induce the upregulation of the nutrient transporters CD98 and CD71, we stimulated enriched NK cells for 18 hours with IL-18 or various other recombinant cytokines which are known to activate NK cells. The list of cytokines includes IL-4, -12, -15, -18, -21, IFN-α, -β, -γ, and TNF-α. 100 U/ml of IL-2 was included in order to maintain NK cell survival during stimulation. Even though IL-2 slightly increased the expression of CD98 and CD71, the surface expression of these nutrient transporters was exceedingly upregulated upon treatment with IL-18 (Fig. 2.2A). Since the adaptor protein MyD88 is known to transmit the signal downstream of IL-18 receptor, we investigated whether MyD88 signaling is required for the upregulation of CD98 and CD71. Notably, NK cells from *Myd88*-deficient mice stimulated with IL-18 failed to upregulate the nutrient transporters, as compared to NK cells from WT mice (Fig. 2.2B). In spite of the largely synergistic functions of IL-12 and IL-18, it is noteworthy that IL-18, but not IL-12, could induce high expression of CD98 and CD71 on NK cells upon stimulation with increasing concentrations of IL-12 or IL-18 (Fig. 2.2C), indicating a function of IL-18 that is unique from that of IL-12 in regard to the upregulation of nutrient transporters. Similarly, IL-18, but not IL-12, could drastically increase glucose uptake in NK cells (Fig. 2.2D), suggesting that IL-18 signaling induces the uptake of various nutrients.

Since IL-12 and IL-18 induce the expression of IL-2Rα chain that renders NK cells highly responsive to IL-2 (225), we investigated the effect of cytokines on enriched NK cells supplemented with IL-15 to exclude the possibility that the increased nutrient receptor expression was due to an increased sensitivity of NK cell to IL-2 by IL-18. Consistent with the results from IL-2/18-stimulated NK cells, whereas IL-15/18-stimulated NK cells greatly

upregulated the expression of nutrient transporters and showed increased glucose uptake, IL-15/12-stimulated NK cells failed to induce any significant change (Fig. 2.3).

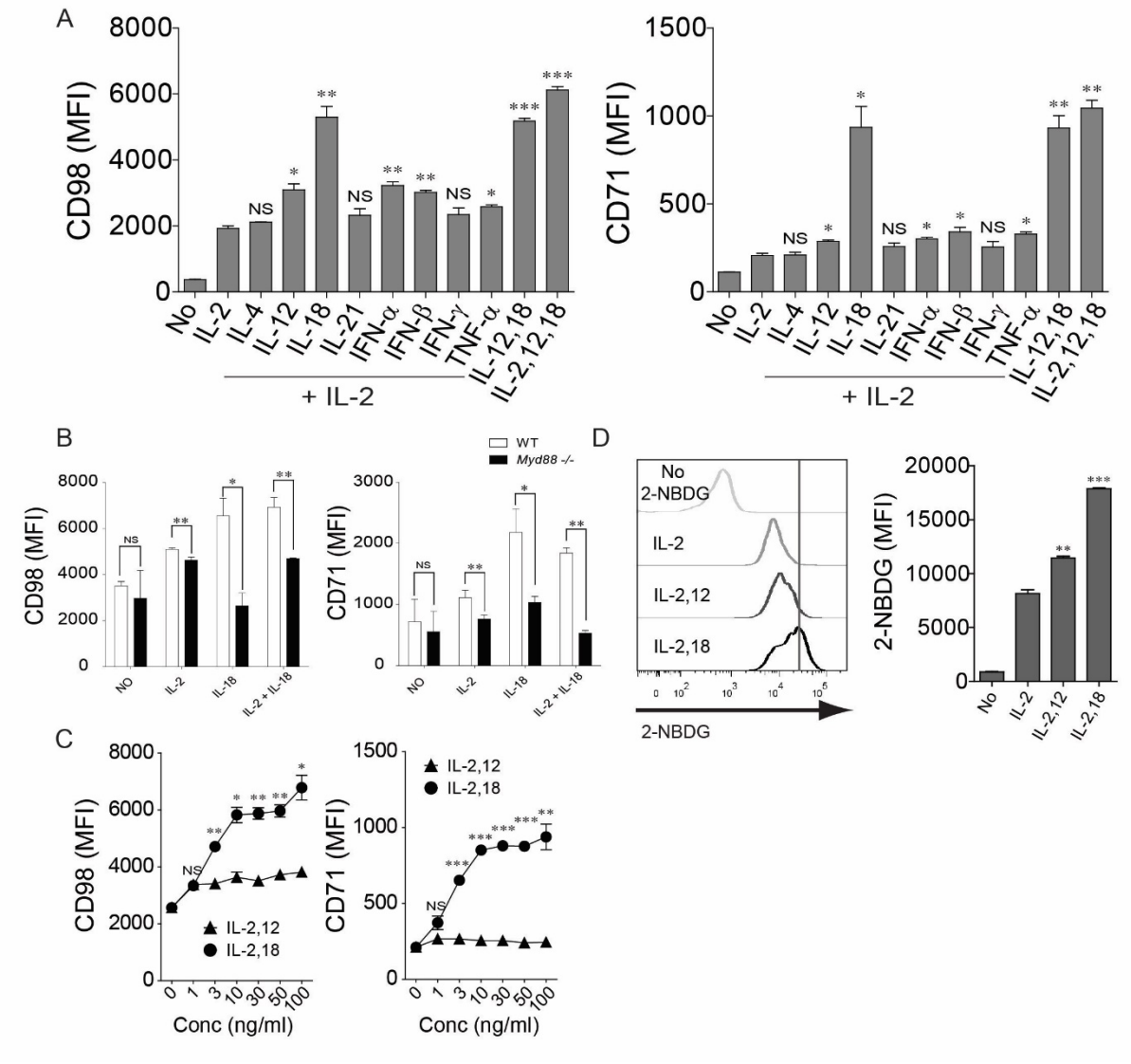

Figure 2.2. IL-18 can induce the upregulation of nutrient transporters on NK cells in vitro. NK cells were enriched from the spleens of naive C57BL/6 mice and stimulated with the indicated cytokines in vitro for 24 hours. 100 U/ml of rhIL-2 was added to maintain NK cell survival. (A) Representative plots depict the MFI of CD98 and CD71 expression on cytokine stimulated NK cells. Statistics are comparing samples to IL-2 stimulated NK cells. (B) Spleen cells of naive C57BL/6 and *Myd88-/-* mice were stimulated with the indicated cytokines for 24 hours. For the statistical analyses of the data, the p values were obtained by comparing the stimulated NK cells with the unstimulated NK cells (without IL12 or IL-18 treatment). (C) The expression of CD98 and CD71 on NK cells was measured upon stimulation with different concentrations of IL-12 and IL-18 . (D) Representative histograms of the glucose uptake by cytokine stimulated NK cells as measured by the MFI of 2-NBDG. Statistics are comparing samples to IL-2 stimulated NK cells. Data are from one experiment representative of four independent experiments, with two replicates per group. Data represent mean + SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

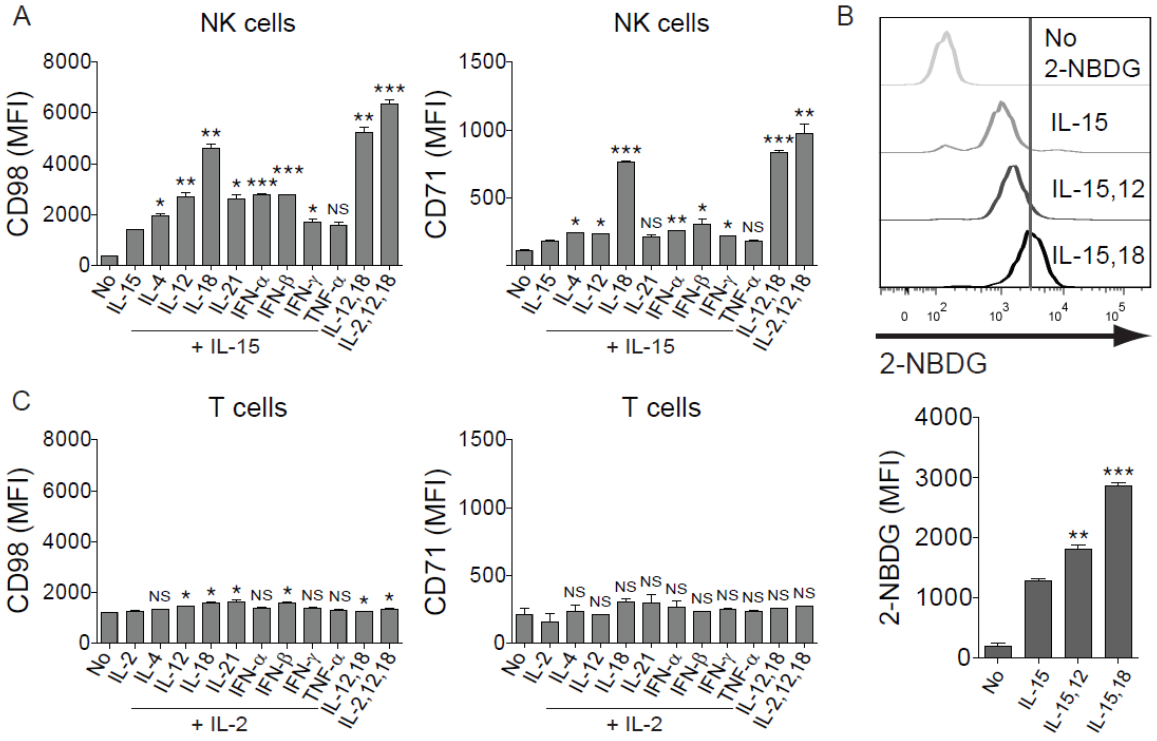

Figure 2.3. IL-18 induces the upregulation of nutrient transporters on NK cells in the presence of IL-15/IL-15Rα complex (3ng/ml). NK cells were enriched from the spleens of naive C57BL/6 mice and stimulated with the indicated cytokines in vitro for 24 hours. (A) Representative plots depict the MFI of CD98 and CD71 expression on cytokine stimulated NK cells. (B) Representative histograms and plots of the glucose uptake by cytokine stimulated NK cells as measured by the MFI of 2-NBDG. (C) Representative plots depict the MFI of CD98 and CD71 expression on T cells stimulated with the indicated cytokines. Data are from one experiment representative of four independent experiments, with two replicates per group. Data represent mean + SD. Statistics are comparing samples to IL-2 stimulated NK cells. ns: non significant; *p < 0.05; **p < 0.01; ***p < 0.001.

Interestingly, the upregulation of nutrient transporters by IL-18 stimulation predominately occurs on NK cells, and not on other immune cells such as T cells (Fig. 2.1D and Fig. 2.3C). To determine whether the selective effect of IL-18 on NK cells is determined by the differential expression level of its receptor, we analyzed IL-18Rα surface expression on several cell populations among naïve splenic leukocytes. Notably, NK cells are the only cell population expressing a basally high level of IL-18Rα among naïve splenic leukocytes (Fig. 2.4A). Real-time PCR analysis also indicated that genes encoding the IL-18 receptor α- and β-chains, *Il18r1* and *Il18rap*, are highly expressed on sorted NK cells compared to NK cells-depleted total leukocytes (non-NK cells) (Fig. 2.4B). Similar to IL-18, IL-1α and IL-1β are known to signal via a TIR motif in the cytoplasmic domain of their receptors. Treatment of IL-1α/β failed to upregulate nutrient transporters on NK cells (Fig. 2.4C), presumably due to the low expression level of genes for the IL-1 receptor, *Il1r1* and *Il1rap*, on NK cells (Fig. 2.4B). Taken together, IL-18 can upregulate nutrient transporters on NK cells and the basally high expression of IL-18 receptor presumably supports the immediate induction of nutrient transporters upon IL-18 stimulation.

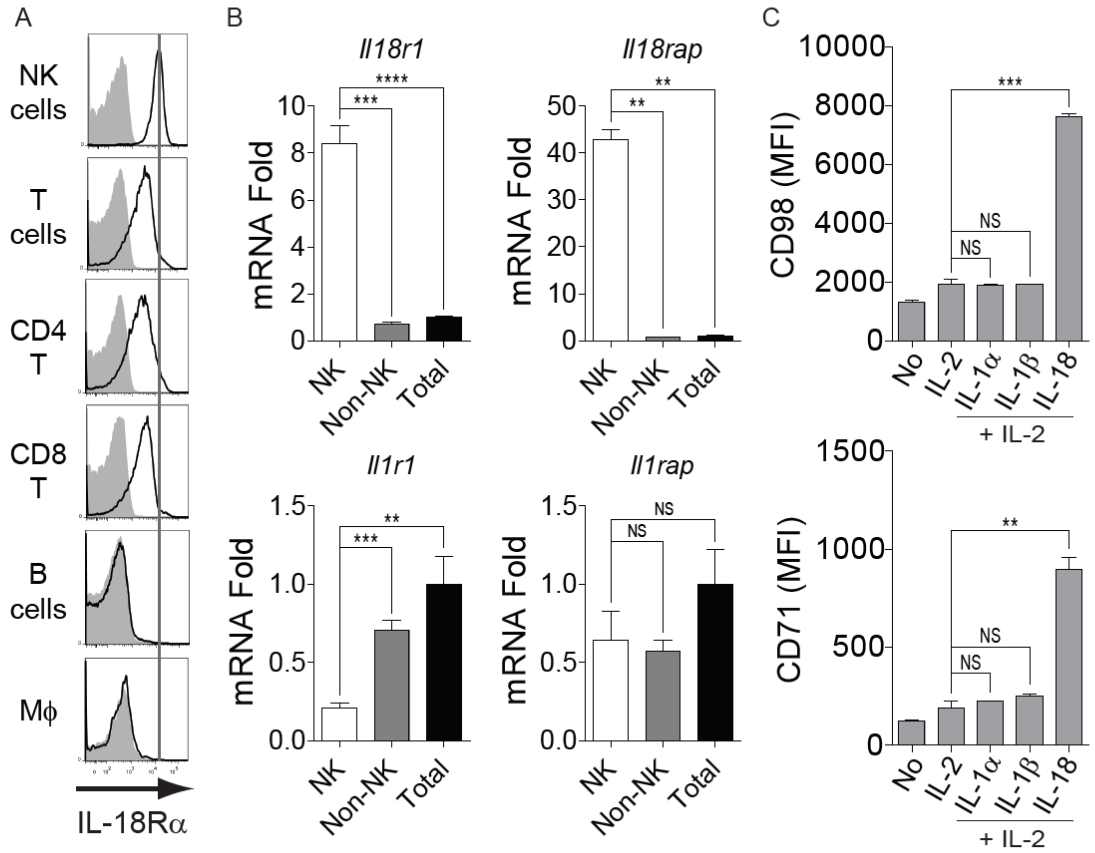

Figure 2.4. IL-18 and IL-1 receptors expression on NK cells. (A) Surface protein expression of the IL-18 receptor alpha subunit on various splenic leukocytes from C57BL/6 mice. (B) NK cells were isolated by flow sorting from the spleen of C57BL/6 mice and compared with non-NK cells (total splenic leukocytes except NK cells) and total splenic leukocytes. mRNA was extracted from these three populations and the transcript levels of IL-18 and IL-1 receptors were quantified by quantitative real-time PCR. (C) Representative plots depict the MFI of CD98 and CD71 expression on enriched NK cells from the spleen of naive C57BL/6 mice upon stimulation with different cytokines for 24 hours. Data are from one experiment representative of three independent experiments, with three replicates per group. Data represent mean + SD. ns: non significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

IL-18 upregulates nutrient transporters on NK cells independently of mTORC1 pathway

mTORC1 pathway is known to induce the upregulation of nutrient transporters on NK cells (124, 216). To determine whether IL-18 simulation is a non-redundant pathway distinct from mTORC1 pathway for inducing the expression of nutrient transporters, we stimulated NK cells with IL-18 in the presence of rapamycin, an mTORC1 inhibitor, and analyzed the expression of CD98 and CD71 on NK cells. In general, the rapamycin treatment reduced the expression of CD98 and CD71. However, it was noteworthy that IL-18, but not IL-12, can induce the upregulation of CD98 and CD71 expression by similar fold changes (Fig. 2.5, A and B), indicating that IL-18 can induce the upregulation of nutrient transporters independently of mTORC1 pathway. The abrogated S6 phosphorylation indicated the efficiency of mTORC1 inhibition by rapamycin (Fig. 2.5C). To exclude the possibility that IL-18 signaling activates mTORC1 pathway before the pathway is completely blocked by rapamycin, we measured the expression of CD98 and CD71 on NK cells preincubated with rapamycin before IL-18 stimulation. Consistently, we observed the upregulation of CD98 and CD71 by similar fold changes (Fig. 2.6). Therefore, our results indicated that IL-18 can induce the expression of nutrient receptors independently of mTORC1 pathway.

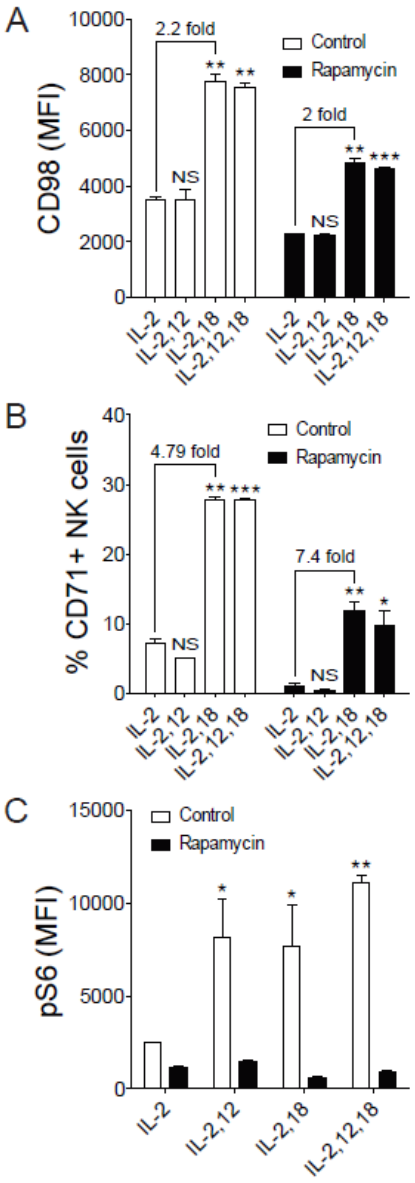

Figure 2.5. IL-18 induces the expression of nutrient receptors independently of mTORC1 pathway. NK cells from the spleen of naive C57BL/6 mice were stimulated with different cytokines for 24 hours in the presence or absence of rapamycin. (A) Representative plots depict the MFI of CD98 expression on NK cells. (B) Representative plots depict the percentage of CD71 expressing NK cells. (C) Representative plots depict the MFI of phosphorylated ribosomal protein S6 (pS6) in NK cells. Data are from one experiment representative of three independent experiments, with two replicates per group. Data represent mean + SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

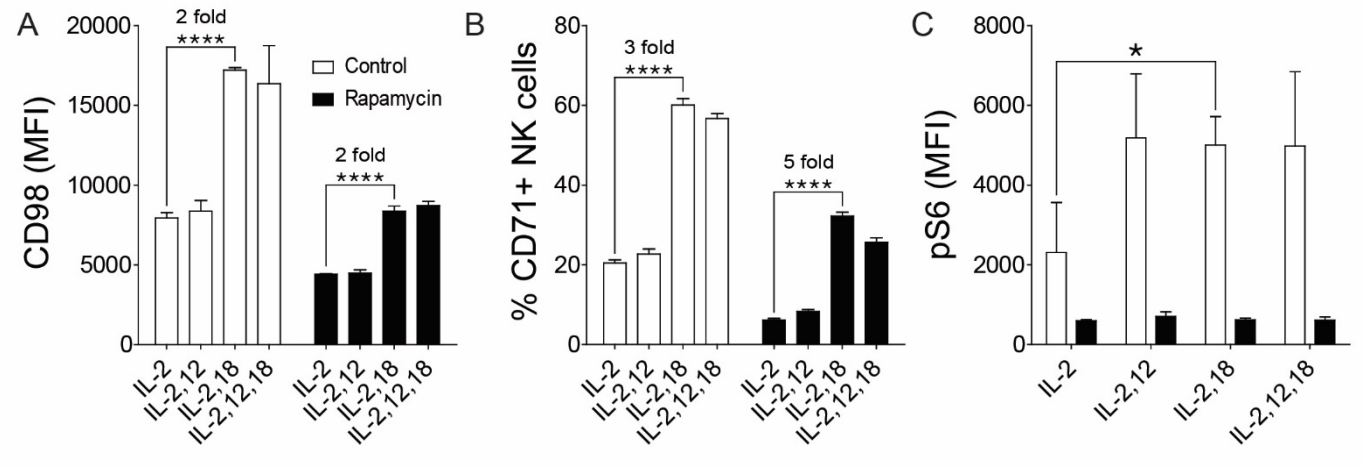

Figure 2.6. IL-18 induces the expression of nutrient receptors independently of mTORC1 pathway. NK cells from the spleen of naive C57BL/6 mice were pretreated with or without rapamycin for 30 min and stimulated with different cytokines for 24 hours. (A) Representative plots depict the MFI of CD98 expression on NK cells. (B) Representative plots depict the percentage of CD71 expressing NK cells. (C) Representative plots depict the MFI of phosphorylated ribosomal protein S6 (pS6) in NK cells. Data are from one experiment representative of two independent experiments, with two replicates per group. Data represent mean + SD. *$p < 0.05$; ****$p < 0.0001$.

IL-18 induces metabolic changes in NK cells

Heterodimers of CD98 (SLC3A2) and LAT1 (SLC7A5) form the bidirectional System L transporter that exchanges the simultaneous efflux of glutamine and influx of leucine (141, 233). For this exchange to occur, SLC1A5, a high affinity transporter for glutamine, is required to preload the cells with glutamine. To investigate whether the effect of IL-18 can induce the upregulation of all the necessary nutrient transporters for the exchange of glutamine for leucine, we purified splenic NK cells via fluorescence activated cell sorting, stimulated the NK cells with IL-2, IL-2/12 or IL-2/18 for 18 hours, and analyzed the transcripts of nutrient transporters by real-time PCR. The mRNA expression of *Slc3a2 (Cd98)*, *Tfrc (Cd71)*, and *Slc2a1 (Glut1)* was highly upregulated upon stimulation with IL-18, consistent with the flow cytometry data (Fig. 2.7A). Notably, expression of *Slc7a5* and *Slc1a5* were also upregulated by IL-18 (Fig. 2.7A), indicating that all nutrient transporters necessary for the bidirectional transport of leucine/glutamine via the System L transporter are highly induced by IL-18 stimulation.

To elucidate the metabolic change of NK cells by IL-18, we analyzed the metabolic profiles of glycolysis of expanded NK cells upon IL-2/18 stimulation by measuring the extracellular acidification rate (ECAR) using Seahorse technology. Clearly, NK cells treated with IL-18 showed enhanced glycolysis, suggesting the activation of mTORC1 (Fig. 2.7B). Indeed, NK cells stimulated with IL-2/18 elicited higher expression of phosphorylated S6 ribosomal protein (pS6), a substrate of mTORC1 activation, compared to those stimulated with IL-2 alone (Fig. 2.7C). Interestingly, IL-18 is able to enhance the rate glycolysis in the presence of rapamycin (Fig. 2.7D), similar to the mTORC1-independent upregulation of nutrient transporters on NK cells by IL-18 shown in Figure 4.

Next, to evaluate the importance of the System L transporter, consisting of the CD98/LAT1 heterodimer, for the induction of effector functions in NK cells upon stimulation with IL-2/18, we used 2-aminobicyclo-(2,2,1)-heptanecarboxylic acid (BCH), a competitive inhibitor of the System L transporter. Notably, NK cells stimulated with IL-2/18 in the presence of BCH showed reduced effector functions in regard to IFN-γ production, granzyme B expression, and mTORC1 activation (Fig. 2.7E). Altogether, these results demonstrated that IL-18 can upregulate the expression of a wide range of amino acid transporters and induce metabolic changes in NK cells. Moreover, CD98 expression on NK cells stimulated with IL-18 is required for the induction of effector functions.

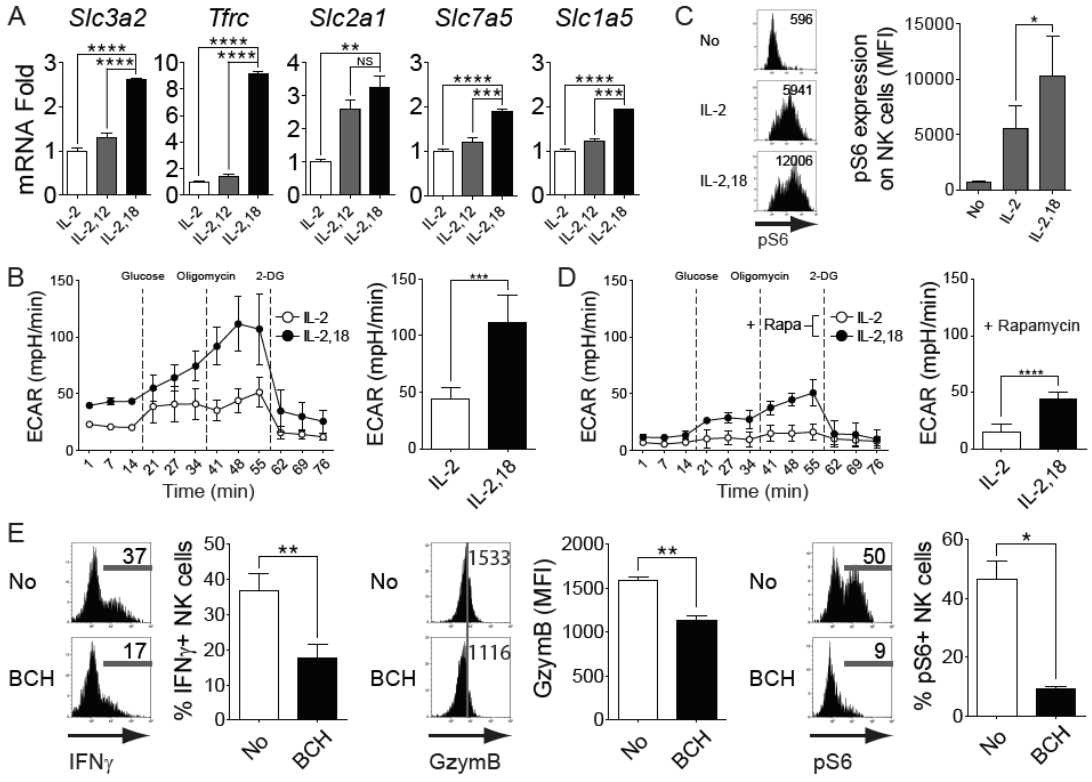

Figure 2.7. NK cells show increased glycolysis (ECAR) upon IL-18 stimulation. (A) NK cells isolated by flow sorting were stimulated with IL-2 and either IL-12 or IL-18 for 24 hours. Graph depicts the transcript levels of several nutrient receptors as quantified by quantitative real-time PCR. (B) The ECAR levels of NK cells stimulated with IL-2 or IL-2/18 are shown. (C) Representative histograms depict the MFI of phosphorylated ribosomal protein S6 (pS6) in NK cells upon stimulation with the indicated cytokines for 24 hours. (D) The ECAR levels of stimulated NK cells upon rapamycin treatment are shown. (E) NK cell functions are reduced upon inhibition of the amino acid transporter LAT1. NK cells from the spleen of naive C57BL/6 mice were stimulated with IL-2 and IL-18 cytokines for 18 hours in the presence or absence of BCH. Representative histograms depict the proportion of NK cells expressing IFNγ, granzyme B, and pS6. Data are from one experiment representative of three independent experiments, with three to five replicates per group. Data represent mean + SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

IL-18 can induce leucine-driven mTORC1 activation

Amino acids such as leucine can directly activate mTORC1 (235-238). Since NK cells stimulated with IL-2/18 showed increased transcript expression of the three necessary transporter components for the System L transporter, we investigated whether increased expression of these transporters allow leucine to activate mTORC1. NK cells expanded with IL-2 were further stimulated with either IL-2 or IL-2/18. The cells were then washed, and starved for 3 hours in amino acid-depleted media. mTOCR1 activity in NK cells diminished during amino acid starvation (Fig. 2.8A). The NK cells showing inert mTORC1 activity were preloaded with L-glutamine and were then subjected to stimulation with L-leucine. Remarkably, robust S6 phosphorylation was only observed in IL-2/18-stimulated NK cells by the addition of L-leucine in a dose-dependent manner (Fig. 2.8A) and was abrogated by rapamycin treatment (Fig. 2.8B). Signaling through the mTORC1 pathway leads to the activation of the 70kDa ribosomal protein S6 kinase (S6K), which then phosphorylates S6. L-leucine-induced mTORC1 activation in IL-2/18-stimulated NK cells was also confirmed by western blot, as these cells showed high phosphorylation of S6K upon leucine treatment (Fig. 2.8C). The enhanced S6 phosphorylation returned to the basal level within 60 min following stimulation with L-leucine (Fig. 2.8D).

The System L transporter allows the bidirectional exchange of L-glutamine for essential amino acids such as L-leucine (141, 233). To determine whether the L-leucine-driven mTORC1 activation is mediated by the bidirectional exchange of L-glutamine for L-leucine in IL-2/18-stimulated NK cells, we analyzed L-leucine influx and L-glutamine efflux upon L-leucine treatment using ^{3}H-labeled amino acids. Notably, increased L-leucine influx and L-

glutamine efflux were observed during L-leucine treatment in IL-2/18-stimulated cells compared to IL-2 stimulated cells (Fig. 2.8, E and F).

The cellular uptake of L-glutamine was shown to be the rate limiting step that activates mTORC1 (141, 233). To determine the importance of intracellular L-glutamine for the L-leucine-induced mTORC1 activation, we have performed an experiment in which IL-18 activated cells were treated with leucine without L-glutamine preloading. Notably, L-leucine could not induce mTORC1 activation without the intracellular L-glutamine (Fig. 2.8G).

To determine the requirement of components for the System L transporter consisting of the CD98/LAT1 heterodimer, BCH, an inhibitor of LAT1, was used. Treatment of BCH abrogated S6 phosphorylation in NK cells stimulated with IL-2/18 in a dose-dependent manner (Fig. 2.8H). Such changes were negligible in conditioned medium of NK cells stimulated with IL-2. α-(methylamino)isobutyric acid (MeAIB), an inhibitor of system A transporters, did not show any effect on leucine-induced mTORC1 activation (Fig. 8I). In addition, L-γ-Glutamyl-p-nitroanilide (GPNA), an inhibitor of SLC1A5-regulated transport (244), suppressed S6 phosphorylation in a dose-dependent manner (Fig. 2.8J), indicating that intracellular L-glutamine, transported into the cell via SLC1A5, is critical as an efflux substrate for the System L transporter in order to activate mTORC1 pathway. The leucine structural antagonist N-acetyl–leucine amide (NALA) exerted similar inhibitory effects on S6 phosphorylation (Fig. 2.8K). Treatments of IL-18 stimulated NK cells with the aforementioned inhibitors did not induce noticeable cell death on all tested concentrations of inhibitors (Fig. 2.9). Taken together, the data demonstrated that increased expression of the System L amino acid transporter by IL-18 stimulation can intensify mTORC1 activation by leucine.

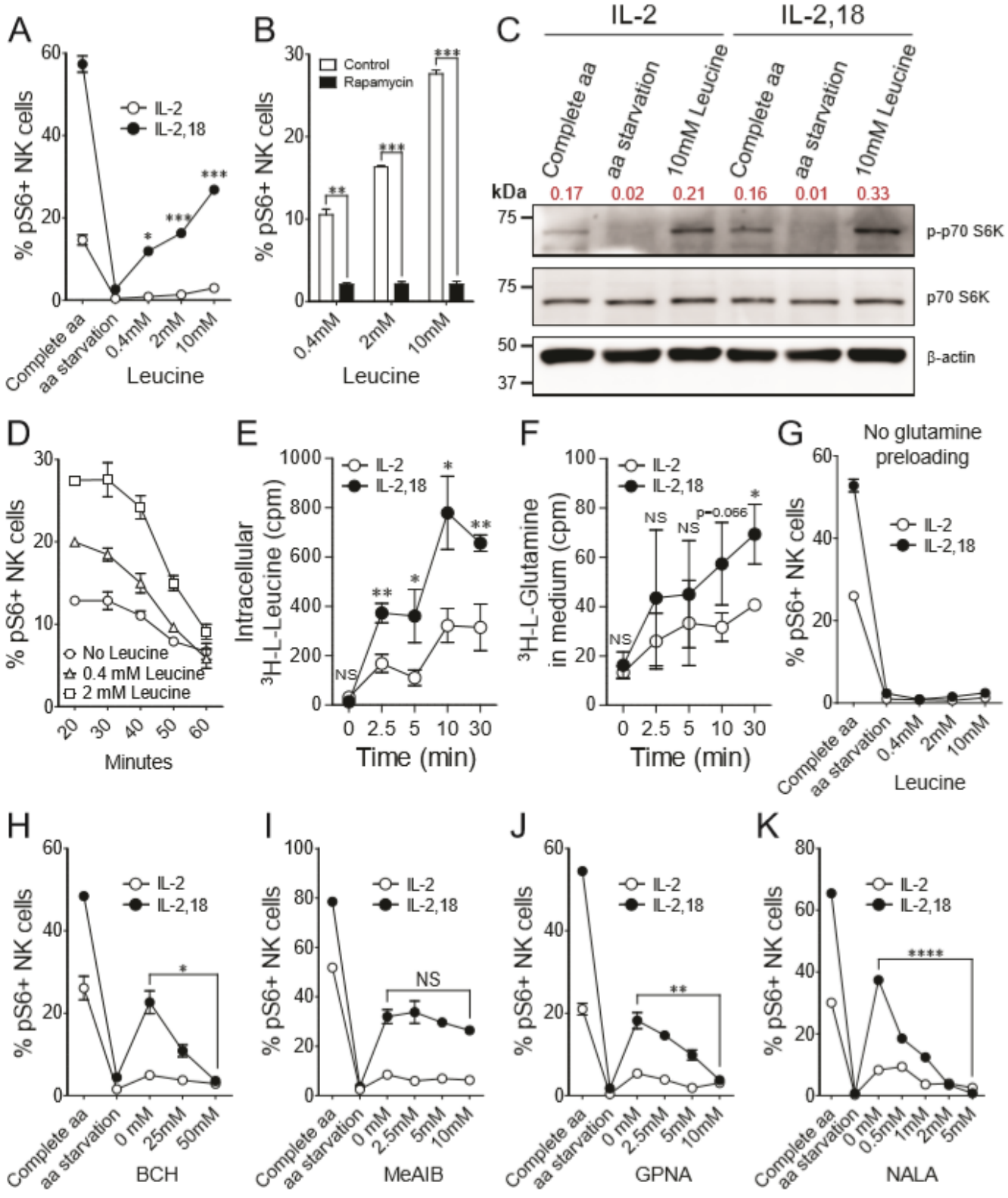
A
% pS6+ NK cells
IL-2
IL-2,18
Complete aa
aa starvation
0.4mM
2mM
10mM
Leucine
B
% pS6+ NK cells
Control
Rapamycin
0.4mM
2mM
10mM
Leucine
C
IL-2
IL-2,18
Complete aa
aa starvation
10mM Leucine
kDa
0.17
0.02
0.21
0.16
0.01
0.33
75
p-p70 S6K
75
p70 S6K
50
37
β-actin
D
% pS6+ NK cells
No Leucine
0.4 mM Leucine
2 mM Leucine
Minutes
E
Intracellular
3H-L-Leucine (cpm)
IL-2
IL-2,18
NS
Time (min)
F
3H-L-Glutamine
in medium (cpm)
IL-2
IL-2,18
NS
NS
p=0.066
Time (min)
G
No glutamine
preloading
% pS6+ NK cells
IL-2
IL-2,18
Complete aa
aa starvation
0.4mM
2mM
10mM
Leucine
H
% pS6+ NK cells
IL-2
IL-2,18
Complete aa
aa starvation
0 mM
25mM
50mM
BCH
I
% pS6+ NK cells
IL-2
IL-2,18
NS
Complete aa
aa starvation
0 mM
2.5mM
5mM
10mM
MeAIB
J
% pS6+ NK cells
IL-2
IL-2,18
Complete aa
aa starvation
0 mM
2.5mM
5mM
10mM
GPNA
K
% pS6+ NK cells
IL-2
IL-2,18
Complete aa
aa starvation
0 mM
0.5mM
1mM
2mM
5mM
NALA

Figure 2.8. IL-18 can induce mTORC1 activation by leucine treatment. Ex vivo expanded NK cells were stimulated with IL-2 or IL-2/IL-18 for 18 hours, starved for 3 hours in amino acid-depleted media, then replenished with L-glutamine for 1 hour before being treated with L-leucine for 30 minutes. (A) Phosphorylation of ribosomal protein S6 upon addition of leucine in NK cells was measured by flow cytometry. (B) NK cells were treated with leucine and the effect of rapamycin on mTORC1 activation was measured. (C) Phosphorylation of p70 S6 kinase upon addition of leucine was measured by western blot. Densitometry values indicate the ratio of phosphorylated p70 S6K to total p70 S6K protein expression. (D) IL-2/18-stimulated NK cells were treated with leucine for the indicated time before measuring pS6. (E) Starved NK cells were preloaded with unlabeled L-glutamine for 1 hour, and treated with 3Hlabeled L-leucine for the indicated time up to 30 minutes. The intracellular levels of 3H-labeled L-leucine are shown. (F) Starved NK cells were preloaded with unlabeled glutamine and 3H-labeled glutamine for 1 hour, and treated with unlabeled leucine for the indicated time up to 30 minutes. The levels of 3H-labeled glutamine in the culture medium are shown. (G) NK cells were treated with leucine without preloading with glutamine. (H-K) NK cells stimulated with IL-2 or IL-2/IL-18 were treated with leucine and the effects of BCH (H), MeAIB (I), GPNA (J), and NALA (K) on mTORC1 activation were measured. Data are from one experiment representative of three to four independent experiments, with two to three replicates per group. Data represent mean + SD. aa: amino acid; ns: non significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

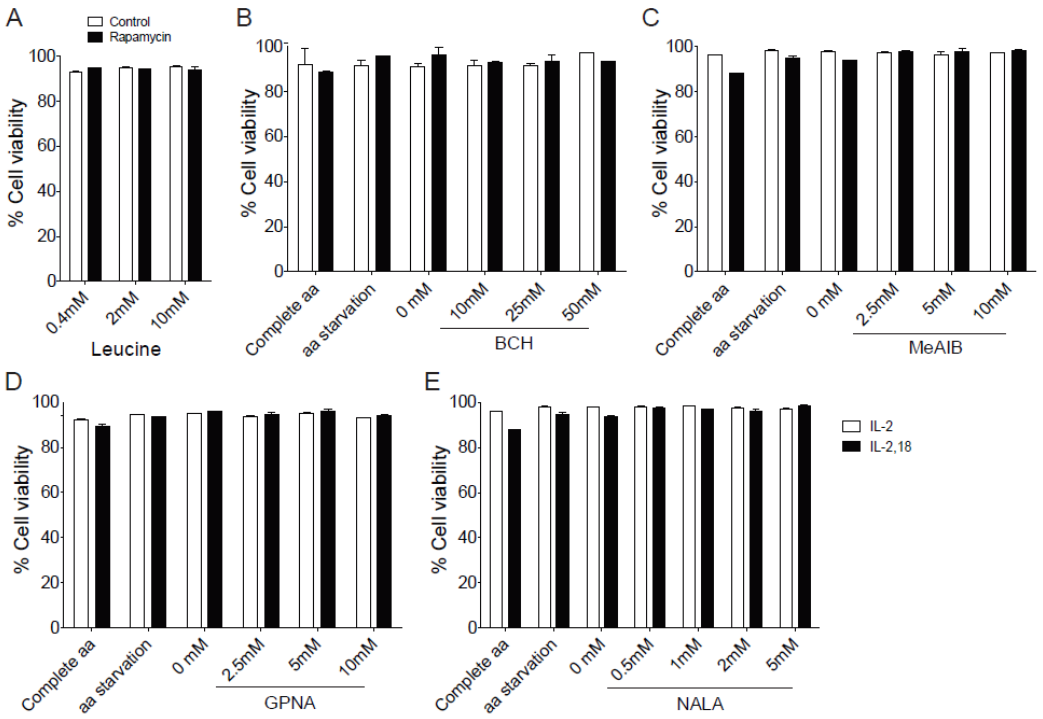

Figure 2.9. The viability of NK cell upon treatment with various inhibitors during. leucine driven mTORC1 activation was assessed. (A) NK cell viability was assessed upon treatment with rapamycin for 24 hours. (B-E) Ex vivo expanded NK cells were stimulated with IL-2 or IL-2/IL-18 for 18 hours, starved for 3 hours in amino acid-depleted media, and then replenished with glutamine for 1 hour before being treated with leucine for 30 minutes. NK cell viability was assessed upon treatment with BCH for 30 min (B), MeAIB for 1 hour (C), GPNA for 1 hour (D), or NALA for 30 min (E). Data are from one experiment representative of three independent experiments, with two replicates per group. Data represent mean + SD.

IL-18 is dispensable in the expression of nutrient transporters and proliferation of NK cells during MCMV infection

NK cells are known to proliferate during MCMV infection (48, 49). BrdU incorporation assay on splenic leukocytes from immunocompetent C57BL/6 mice infected with MCMV for 1.5, 3, and 5 days indicated that the proliferative response of NK cells during MCMV infection peaks on day 3 post infection (p.i) (Fig. 2.10A). We reasoned that since the metabolic demands needed for the intense NK cell proliferation can be fulfilled by high expression of nutrient transporters, the expression of amino acid transporter CD98 and transferrin receptor CD71 on splenic leukocytes were analyzed. The peak surface expression of CD98 and CD71 were observed on NK cells at day 3 p.i. (Fig. 2.10B). The expression on T cells showed a marginal increase on day 3 and gradually augmented up to day 5 during MCMV infection. Increased glucose uptake was also observed on NK cells at day 3 p.i. (Fig. 2.10C). Therefore, the upregulation of nutrient transporters on NK cells coincides with the peak of proliferation during MCMV infection.

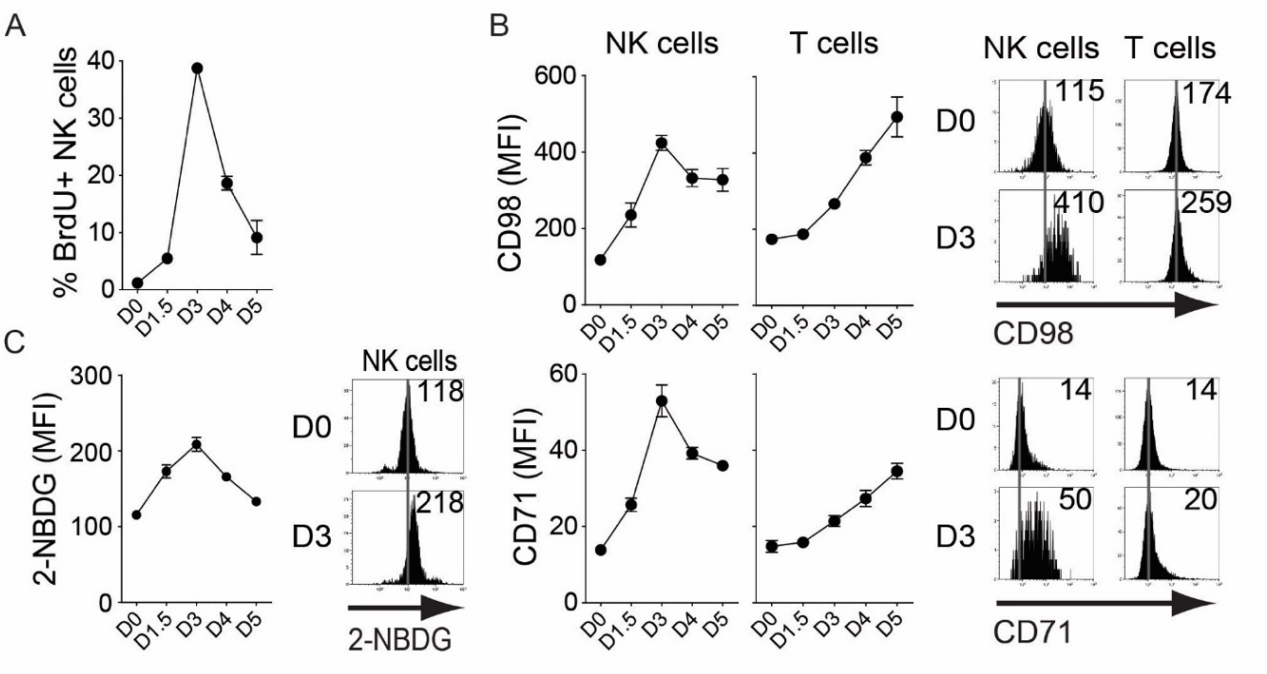

Figure 2.10. Increased proliferative rate of NK cells correlates with higher expression of nutrient receptors during MCMV infection in vivo. C57BL/6 mice were either left untreated or infected with 3,000 PFU MCMV intraperitoneally, and analyzed on the indicated days. (A) Kinetics of NK cell proliferation during MCMV infection as measured by BrdU incorporation. (B) Representative plots depict the mean fluorescence intensity (MFI) of CD98 and CD71 expression on NK cells and T cells in the spleens of naive (D0) or MCMV-infected mice at day 3 post-infection (D3). (C) Representative plots of the glucose uptake by NK cells during MCMV infection as measured by the MFI of 2-NBDG. Data are from one experiment representative of two independent experiments, with three to five mice per group.

To further assess NK cell activation in vivo, we evaluated the requirement of IL-18 pathway by using caspase-1-deficient mice, which are known to have reduced levels of active IL-18 (245). Caspase-1-deficient mice were infected with 5,000 PFU MCMV via the intraperitoneal route. The proliferation antigen Ki-67 on NK cells was measured, and it was comparable between the WT and caspase-1-deficient mice (Fig 2.11A). Next, I evaluated the expression of nutrient transporters on NK cells from MCMV-infected caspase-1-deficient mice. Unexpectedly, NK cells in caspase-1-deficient mice could upregulate CD98 and CD71 during MCMV infection (Fig 2.11B). Since the active form of IL-18 can be generated by non-caspase proteases (69), I decided to assess the importance of IL-18 using *Il18r1*-deficient mice during MCMV infection. B6 mice and *Il18r1*-deficient mice were infected with MCMV. On day 3 p.i., the viral loads in the organs of infected *Il18r1*-deficient mice were comparable to those of control mice, suggesting an efficient clearance of MCMV by NK cells in the absence of IL-18 signaling (Fig 2.11C). Surprisingly, the levels of CD98 and CD71 were similar in both groups, even though they were slightly lower in *Il18r1*-deficient mice (Fig 2.11D). In addition, comparable glucose uptake was observed on NK cells. NK cell proliferation as measured by BrdU incorporation was also similar between wild type and *Il18r1*-deficient mice during MCMV infection (Fig 2.11E), consistent with previous reports (246, 247).

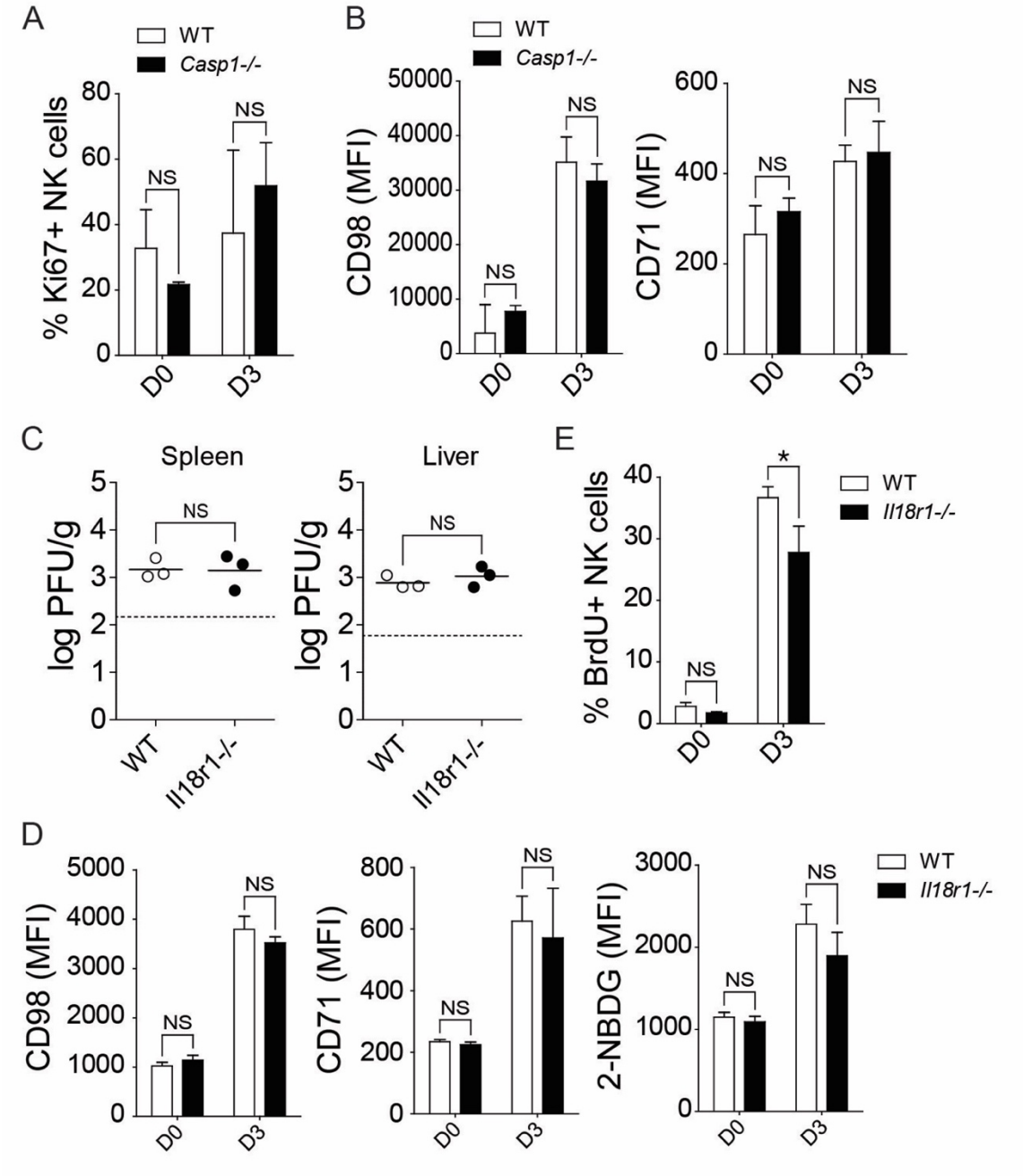
A
WT
Casp1-/-
% Ki67+ NK cells
NS
D0
D3
B
CD98 (MFI)
CD71 (MFI)
C
Spleen
Liver
log PFU/g
WT
Il18r1-/-
E
% BrdU+ NK cells
*
Il18r1-/-
D
2-NBDG (MFI)

Figure 2.11. The requirement of IL-18 signaling during MCMV infection. C57BL/6 and caspase-1-/- mice were either left untreated or infected with 3,000 PFU MCMV via the intraperitoneal route and analyzed at day 3 post-infection (D3). (A) NK cell proliferation as measured by Ki-67 expression. (B) The mean fluorescence intensity (MFI) of CD98 and CD71 expression on NK cells in the spleens of naive or MCMV-infected mice at day 3 post-infection. C57BL/6 and *Il18r1 -/-* mice were infected with 3,000 PFU MCMV. (C) The viral titers in the spleens and livers of infected C57BL/6 and *Il18r1-/-* mice. (D) The mean fluorescence intensity (MFI) of CD98, CD71 expression and glucose uptake as measured by 2-NBDG on NK cells and T cells in the spleens of naive or MCMV-infected mice at day 3 post-infection. (E) The percentage of BrdU incorporation of NK cells in the spleens of naive (D0) or MCMV-infected mice at day 3 post-infection. Data are from one experiment representative of two independent experiments, with three to four mice per group. Data represent mean + SD. ; ns: non significant; *$p < 0.05$.

Since the results indicated that IL-18 signaling is not required for upregulating nutrient transporters on NK cells during MCMV *in vivo*, I hypothesized that there are different pathways involved in the upregulation of the nutrient transporters. On day 3 following MCMV infection, Ly49H+ NK cells preferentially proliferate while Ly49H- NK cells start to diminish (57). I reasoned that the preferential proliferation of Ly49H+ NK cells is due to the differential expression of nutrient transporters on NK cells in regard to Ly49H expression. B6 mice were infected with MCMV and the levels of CD98 expression were analyzed on Ly49H+ NK cells at different time points. The data showed that a higher expression of CD98 is prominent on Ly49H+ NK cells starting from 3 days p.i. and onwards (Fig 2.12A), which suggests that the activating signal through the m157-Ly49H axis drives the heightened CD98 expression. Indeed, the stimulation of NK cells with m157-expressing BAF3 cells induced expression of CD98 (Fig 2.12B), demonstrating that signaling through the activating receptor Ly49H also can induce high expression of CD98. Since I have identified two pathways that can upregulate CD98 expression, I investigated the contribution of IL-18 in the absence of Ly49H signaling on CD98 expression by using *Ly49h*- deficient and *Ly49h*$^{-/-}$*Il18r1*$^{-/-}$ mice. These mice were infected with MCMV and peripheral blood was collected at different time points to analyze the expression of CD98 and CD71. Strikingly, the level of the nutrient transporters was comparable in both groups of mice (Fig 2.12C). On day 10, the spleens were harvested and the expression of CD98 and CD71 was the same in both groups (Fig 2.12D), suggesting that IL-18 signaling is dispensible for the upregulation of nutrient transporters during MCMV *in vivo*.

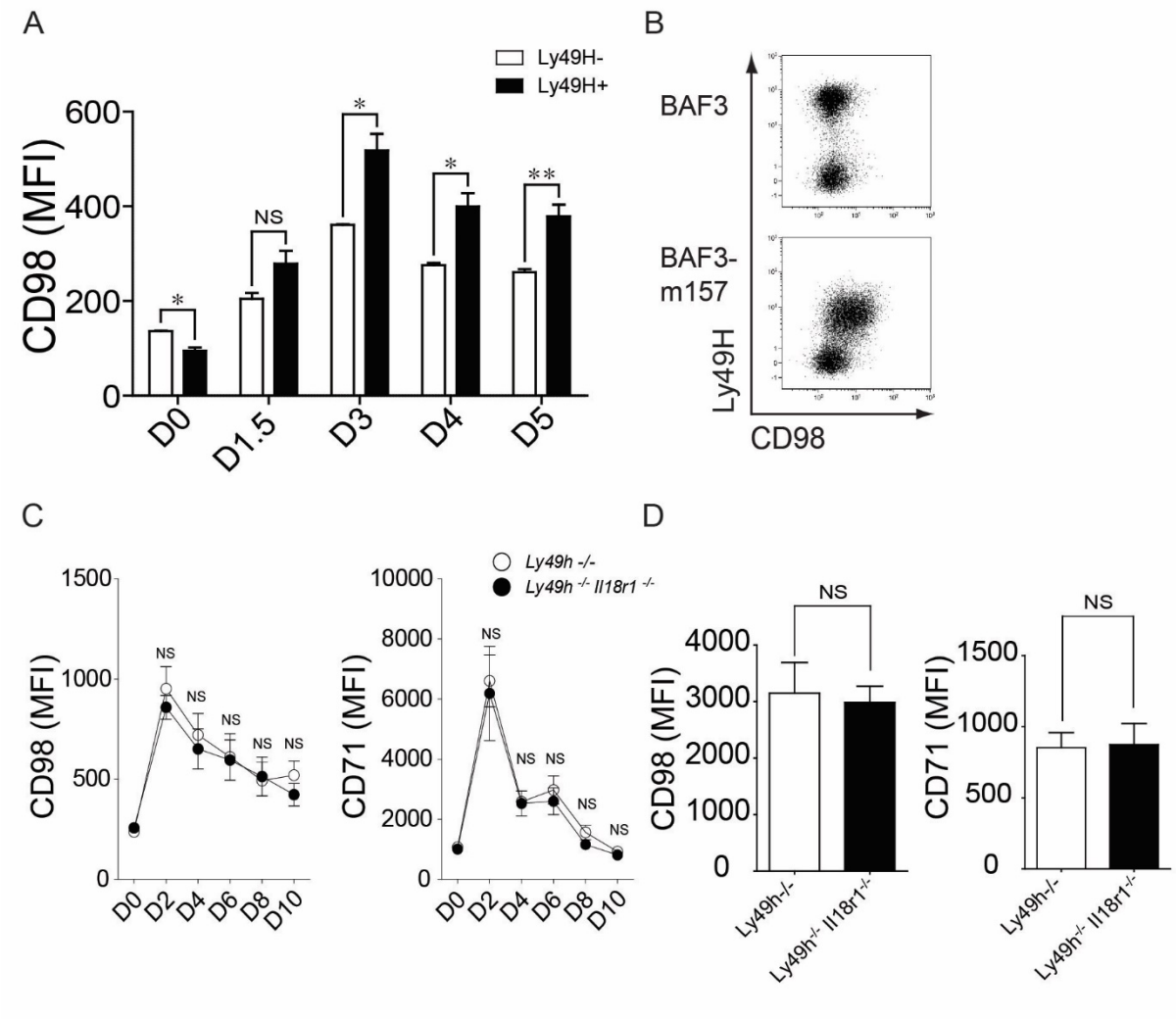

Figure 2.12. Ly49H+ NK cells show enhanced CD98 upregulation upon MCMV infection. (A) CD98 expression on Ly49H- and Ly49H+ NK cells during the course of MCMV infection. (B) Splenic NK cells were stimulated with either BAF3 or BAF3-m157 cells in vitro. Ly49h$^{-/-}$ and Ly49h$^{-/-}$Il18r1$^{-/-}$ mice infected with 5,000 PFU MCMV intraperitoneally, and analyzed on the indicated days. (C) The mean fluorescence intensity (MFI) of CD98 and CD71 expression on NK cells in the blood of naive (D0) or MCMV-infected mice at different time points post-infection. (D) The mean fluorescence intensity (MFI) of CD98 and CD71 expression on NK cells in the spleen of MCMV-infected mice at day 10 post-infection (D10). Data represent mean + SD. **p < 0.01; ***p < 0.001; ****p < 0.0001.

Discussion

The inflammasome pathway induces inflammation in response to infectious microbes and molecules derived from host proteins by the activation of caspase-1 (248-250). Due to the fact that the signaling pathway emanated from IL-18 receptor is distinct from that of IL-12 receptor, the inflammasome-induced IL-18 was thought to play non-redundant roles on immune cells. Here, we demonstrated a previously unappreciated role of IL-18 in increasing nutrient accessibility by upregulating nutrient transporters including the amino acid transporters and the transferrin receptor on NK cells in a MyD88-dependent manner.

In this report, we demonstrated that the nutrient transporters upregulation by IL-18 stimulation occurs predominately on NK cells. Notably, the increased expression of CD98/LAT1, the System L amino acid transporter, granted dramatic activation of mTORC1 in NK cells upon the addition of leucine, one of the substrates for the System L transporter. To our knowledge, this is the first time that the activation of mTORC1 by leucine has been observed in primary immune cells (Fig. 2.13), highlighting the effect of IL-18 on immunometabolism during inflammation. Therefore, our data demonstrated that IL-18 is a potent cytokine for inducing high expression of nutrient transporters on NK cells and thereby intensifying mTORC1 activation through the transport of leucine.

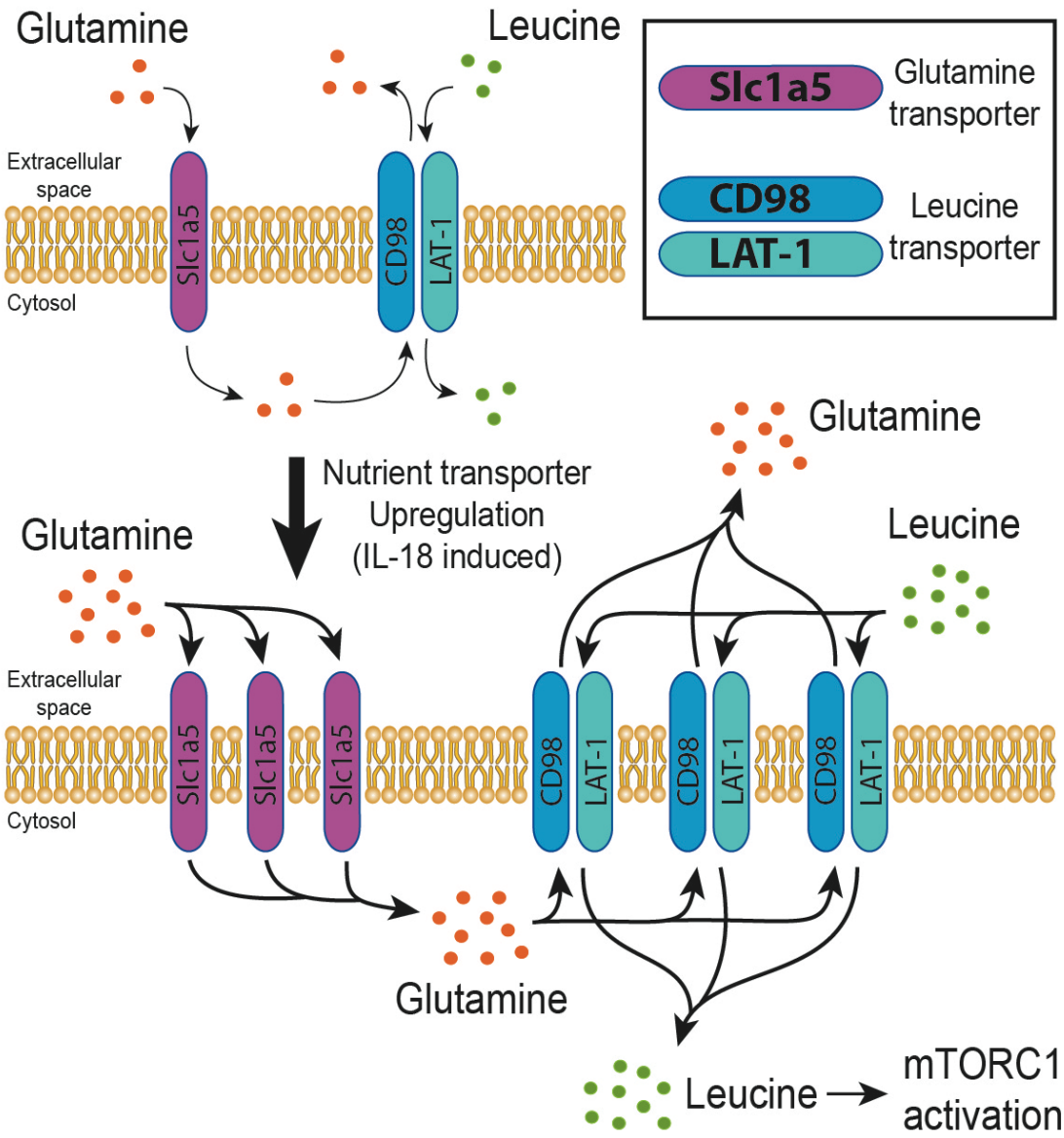

Figure 2.13. Enhanced expression of CD98/LAT1 amino acid transporter by IL-18 induces leucine-driven mTORC1 activation. The System L transporter is a bidirectional transporter importing leucine into the cell in exchange for the export of glutamine, requiring intracellular glutamine for the efficient transport of leucine into the cell. IL-18 upregulates glutamine transporter (SLC1A5) and leucine transport (CD98/LAT1) which will increase the uptake of leucine and the efflux of glutamine resulting in mTORC1 activation.

The mechanism by which CD98 promotes the proliferation of immune cells is an emerging area of recent research. It is conceivable that CD98 allows the robust proliferation of immune cells by boosting the transport of amino acids because the rapidly proliferating immune cells have intense metabolic demands. However, several lines of evidence have recently indicated that amino acids can have a predominant role in metabolic processes in addition to their role as protein building blocks (240). Notably, the CD98-mediated exchange of glutamine for leucine has been identified as a rate-limiting step in the activation of mTORC1 (141), suggesting the existence of a critical pathway through which CD98 could stimulate the proliferation of immune cells by sustaining mTORC1 activity.

To our surprise, I have observed that NK cells in caspase-1-deficient mice could upregulate their nutrient transporters during MCMV infection. In caspase-1-deficient mice, the ability to produce the biologically active form of IL-18 is impaired (245). However, there are some conditions where caspase-1 is not required for IL-18 processing. For example, FasL stimulation in macrophages from caspase-1-deficient mice is able to release active IL-18 because Fas signaling activates caspase-8, which cleaves the precursor form of IL-18 to produce the active form (251, 252). This might explain why the expression of nutrient transporters is high during MCMV infection in caspase-1-deficient mice. In addition, *Il18r1*-deficient mice were able to upregulate CD98 and CD71 in vivo during MCMV infection (Fig. 2.11). These data might suggest that other pathways can compensate for the need of IL-18 in the upregulation of nutrient transporters during inflammation and infection. Indeed, Ly49H signaling can upregulate CD98 on NK cells, but it is not required for inducing the expression of the nutrient transporters, as seen in *Ly49h*$^{-/-}$ *and Ly49h*$^{-/-}$*Il18r1*$^{-/-}$ mice. It is noteworthy to mention that the concentrations of IL-18 used in our *in vitro* studies were higher than those

found *in vivo* during MCMV infection. We usually detected 200-600 pg/ml of IL-18 in the serum of MCMV-infected mice, whereas in our *in vitro* experiments, I used 30ng/ml of IL-18. Alternatively, the discrepancies observed between *in vitro* and *in vivo* conditions in regard to NK cell phenotype in the context of the IL-18 signaling could be explained by the microbiome found in *Il18r1*-deficient mice. The microbiota is known to have broader effects that contribute to innate and adaptive immunity at multiple levels, including metabolic regulation (59, 60). Recently, several reports showed that IL-18 produced by intestinal epithelial cells plays a critical role in supporting the intestinal barrier, while abrogation of IL-18 signaling leads to an altered microbiota as compared with that in wild-type mice (61-64). NK cell-specific deletion of *Il18r1* in mice might be useful to determine the role of IL-18-induced metabolic changes in NK cells in conditions with equivalent microbiomes.

Several discrete pathways have been described to induce CD98 expression. In addition to our results showing that IL-18 can induce high expression of CD98 on IL-18 receptor-expressing cells such as NK cells, early studies showed that conventional stimulation such as phytohemagglutinin (PHA), phorbol 12-myristate-13-acetate (PMA) and ionomycin, or α-CD3 resulted in a dramatic increase of CD98 mRNA level in resting human T cells (253, 254). The induction by α-CD3 stimulation in T cells might be comparable to that induced by the α-Ly49H stimulation in NK cells (Fig. 2.12). In naïve NK cells, the proliferative cytokines IL-2 and IL-15 also induce a marginal expression of CD98 via the PI3K-mTORC1 pathway (123, 124, 216, 255). Our data showing the potent effect of IL-18 on the upregulation of CD98 in the presence of rapamycin supported the conclusion that IL-18 modulates the nutrient uptake by NK cells through a mechanism that is non-redundant of the mTORC1 pathway. The presence of multiple pathways capable of inducing CD98 might guarantee that immune cells

are able to upregulate nutrient transporters during inflammation. Interestingly, the seahorse data showed that IL-18 is able to enhance the rate of glycolysis in the presence of rapamycin (Fig. 5D). Even though we demonstrated in this report that IL-18 can induce metabolic changes via leucine-driven mTORC1 pathway, the seahorse data suggests that IL-18 can increase glycolysis independently of mTORC1. Presumably, the upregulation of glucose transporters by IL-18 contributes to the increased glycolysis.

To understand the sustained mTORC1 activity during inflammation, we propose a new model with two distinct mTORC1 activation pathways; 1) IL-2/15 initiates the metabolic reprogramming and induces mTORC1 activity, and 2) CD98/LAT1 sustains mTORC1 activity by amino acid-driven mTORC1 activation. For the first time, our model indicated that IL-18 is a potent cytokine for inducing high expression of nutrient transporters on metabolically active immune cells and thereby sustains mTORC1 activation by amino acid transport. Our model is supported by a recent report demonstrating that amino acid transport through CD98 is required to sustain levels of cMyc, a central regulator of metabolism (256).

This specific role of IL-18 for upregulating nutrient transporters on naïve NK cells might be due to the basally high level of both chains of the heterodimeric complex of IL-18 receptor as shown here and elsewhere (229, 257). The IL-18 receptor expression on T cells has been known to be induced in effector T cells and maintained in memory T cells (258-260). Therefore our model is also informative to understand the sustained mTORC1 activation during T cell proliferation (257-260). Presumably, the high expression of IL-18 receptor supports the immediate induction of the System L amino acid transporter on NK and memory T cells upon IL-18 stimulation during infections. This single transporter system seems responsible for mediating the uptake of LNAA such as leucine in immunologically activated

NK and T cells and is thus the dominant LNAA transporter in such cells without redundancy with other System L1 or System y+L transporters. For example, the roles of CD98/LAT1 in T cells have been extensively studied in the context of infection and autoimmune models. Anti-CD98 mAb completely prevented the onset of cyclophosphamide-induced diabetes in NOD mice, coincident with decreased proliferation of pathogenic CD4+ T cells (261). T cell-specific deletion of LAT1 resulted in dramatically reduced T cell clonal expansion in an autoimmune model (262). Moreover, LAT1-deficient T cells were unable to reprogram their metabolism in response to antigens and did not undergo clonal expansion or effector differentiation. Interestingly, naïve mice deficient in CD98 or LAT1 in T cells do not show any developmental defects, suggesting that the transporter is exclusively required as a critical metabolic checkpoint for immune cell proliferation during inflammation (148, 262).

Pre-activation of NK cells with a combination of IL-12/15/18 was shown to generate so-called cytokine-induced memory-like NK cells which can maintain sustained proliferation and effector function after adoptive transfer and reject established tumors in mice (226). Similarly, the first human clinical trial with NK cells pre-activated ex vivo with IL-12/15/18 demonstrated that adoptively transferred memory-like NK cells proliferated and expanded in AML patients and generated robust responses against leukemia targets (263, 264). For the mechanism underlying the sustained proliferation in vivo, we have shown that both IL-12 and IL-18 can induce expression of CD25, the IL-2Rα chain, on NK cells (225). Even though the enhanced survival and proliferation of adoptively transferred NK cells is mediated by their increased sensitivity to endogenous levels of low IL-2 due to the high affinity IL-2 receptor on NK cells, the unique role of IL-18 in the cytokine combination has not been demonstrated. Notably, IL-18 was able to act synergistically with IL-15 in stimulating in vitro NK cell

proliferation (229), however the mechanism has not been characterized. Our results demonstrated that IL-18 can induce the metabolic changes of NK cells via the modulation of nutrient transporters expression to support NK cell proliferation and provided an insight for therapeutic applications in infection, cancer, and autoimmune diseases by modulating the metabolism and function of immune cells via targeting the IL-18-mediated pathways.

Materials and methods

Mice, MCMV infection and LPS injection

Wild type C57BL/6 mice were purchased from Charles River. *Il18r1*-deficient mice, caspase-1-deficient mice and *Myd88*-deficient mice on the C57BL/6 background were purchased from the Jackson Laboratory. All mice were housed in a specific pathogen-free environment. In order to obtain *Ly49h*$^{-/-}$ *Il18r1*$^{-/-}$ mice, we crossed *Ly49h*-deficient mice with *Il18r1*-deficient mice. All mice used for experiments were aged between 6 to 12 weeks old. All procedures were approved by and conducted in accordance with the institution's animal guidelines of the University of Ottawa. Smith strain MCMV stocks were generated in our laboratory from the salivary glands of infected BALB/c mice. C57BL/6, *Il18r*1-deficient mice, caspase-1-deficient mice and *Ly49h*$^{-/-}$ *Il18r1*$^{-/-}$ mice were infected with 5,000 PFUs MCMV intraperitoneally. C57BL/6 mice were injected intraperitoneally with 100 μg of LPS in PBS. To measure BrdU incorporation in vivo, the mice were injected intraperitoneally with 2 mg BrdU two hours prior to sacrifice.

Cell isolation, NK cell isolation and in vitro stimulation

Spleens were harvested, and a single-cell suspension was generated following red blood cells lysis and filtration through a 70-μm filter. NK cells were enriched from the spleen by negative selection using the MagniSort Mouse NK cell Enrichment Kit (eBioscience). Purities of enriched NK cells (NK1.1+TCRβ-) were >80%. Isolated NK cells were cultured for 24 hours in the presence of 100 U/ml of recombinant human (rh) IL-2, with different cytokines at the indicated concentrations; IL-1α and β (100 ng/ml), IL-4 (100 ng/ml), IL-12 (10 ng/ml), IL-15/IL-15Rα complex (3 ng/ml), IL-18 (50 ng/ml), IL-21 (100 ng/ml), IFN-α (1,000 U/ml), IFN-β (1,000 U/ml), IFN-γ (100 ng/ml), and TNF-α (100 ng/ml). For expanding

NK cells, NK cells were cultured in RP-10 media (RPMI-1640 medium containing 10% FBS, 1× penicillin/streptomycin, 2 mM L-glutamine, 10 mmol HEPES, 50 μmol 2-mercaptoethanol) for 7-10 days in the presence of 1,000 U/ml of recombinant human (rh) IL-2 (obtained from NCI Preclinical Repository). For NK cell proliferation assay, NK cells were enriched from the spleen as mentioned above. Enriched NK cells were labeled with Cell Proliferation Dye eFluor 450 (eBioscience). Cells were then cultured in RP-10 media with 300 U/ml of rhIL-2 and various concentrations of IL-18 for 3 days.

Flow cytometric analysis

The following mAbs were used: anti-CD3 (17A2 and 145-2C11), anti-TCRβ (H57-597), anti-CD8 (53-6.7), anti-CD49b (DX5), anti-granzyme B (NGZB), anti-IL-18Rα (P3TUNYA) and anti-phospho-S6(S235/S236) (CUPK43K) from eBioscience; anti-CD19 (1D3), anti-CD4 (RM4-5), anti-F4/80 (T45-2342), anti-NK1.1 (PK136), anti-CD107a (1D4B), anti-IFNγ (XMG1.2), anti-Ki-67 (B56), and anti-BrdU (3D4) from BD Biosciences; anti-CD71 (RT7217), anti-CD98 (RL388) from Biolegend; and Live/Dead Fixable Yellow Dead Cell Stain from Invitrogen. Intracellular staining of Ki-67 was carried out using a Foxp3 staining kit (eBioscience). Cells were acquired using BD LSRFortessa or BD FACSCelesta and analyzed using Kaluza 1.3 Analysis software (Beckman Coulter) or FlowJo (Tree Star). The intracellular staining of anti-phospho-S6 (S235/S236) was performed using BD Cytofix/Cytoperm protocols (BD Biosciences).

Quantitative real-time PCR

For the quantification of transcripts of IL-1 and IL-18 receptors, NK cells from C57BL/6 mice were isolated by cell sorting. Purities of sorted NK cells were >98%. Total RNA was extracted from the sorted NK cells using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. For the quantification of transcripts of nutrient receptors genes, NK cells from C57BL/6 mice were isolated by cell sorting as mentioned above. NK cells were labeled with NK1.1, TCRb, and dead, the labeled cells were then flow sorted by using MoFlo XDP-sorter from Beckman Coulter (Stem Core laboratories, OHRI, Ottawa) to obtain NK cells and non NK cells. The sorted NK cells were then stimulated with IL-12 (30 ng/mL) and/or IL-18 (30 ng/mL) for 18 hours and the total RNA was extracted as mentioned above. cDNA was reverse transcribed in a 20 µl reaction using the iScript Reverse Transcription Supermix (Bio-Rad). For quantification of target genes by real-time PCR, cDNA was added to a 20 µl reaction of FastStart Universal SYBR Green Master (Rox) (Roche) and amplified using Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific). Expression of target genes was normalized to β-actin levels. The primer sequences for the target genes are shown in Table 1

Table 1: List of oligo sequences for qPCR used in the study

Name	Sequence	References
Actb-F	CAGCCTGGATGGCTACGTAC	Newly generated sequence.
Actb-R	GTTACCAACTGGGACGACATG	Newly generated sequence.
Il18r1-F	ACTTTTGCTGTGGAGACGTTAC	(265)
Il18r1-R	CCGGCTTTTCTCTATCAGTGAAT	(265)
Il18rap-F	CAGTACTGGCTCCATTCATTGTC	Newly generated sequence.
Il18rap-R	AGCTCGGACTGTCCAGGA	Newly generated sequence.
Il1r1-F	GTGCTACTGGGGCTCATTTGT	(266)
Il1r1-R	GGAGTAAGAGGACACTTGCGAAT	(266)
Il1rap-F	GGAACTGGTTATTCCCTGCAA	https://pga.mgh.harvard.edu/primerbank/
Il1rap-R	GCTCGGTGCATCCATTACCTT	https://pga.mgh.harvard.edu/primerbank/
Slc1a5-F	TACCGCAATCCTGTATCCAGC	https://pga.mgh.harvard.edu/primerbank/
Slc1a5-R	CACCAAAGACGATAGCGAAGAC	https://pga.mgh.harvard.edu/primerbank/
Slc2a1-F	CAGTTCGGCTATAACACTGGTG	(267)
Slc2a1-R	GCCCCCGACAGAGAAGATG	(267)
Slc7a5-F	CTGGTCTTCGCCACCTACTT	(268)
Slc7a5-R	GCCTTTACGCTGTAGCAGTTC	(268)
Tfrc-F	AGCCAGATCAGCATTCTCTAACT	(269)
Tfrc-R	GCCTTCATGTTATTGTCGGCAT	(270)
Slc3a2-F	CTTGGTGATATTCTGCCACTCAG	Newly generated sequence.
Slc3a2-R	CCTGCTCAGGCTGACATTG	Newly generated sequence.

ECAR measurement by Seahorse technology

Isolated NK cells were cultured for 7 to 10 days in the presence of 1,000 U/ml of recombinant human IL-2 and treated with or without 30 ng/ml of IL-18 for 24 hours in the presence or absence of 1.6 μM of rapamycin. XF 24-well microplates (Seahorse Bioscience) were pre-coated with CellTaq (BD Biosciences) for 2 hours before seeding the NK cells on the plate for real-time analysis of the extracellular acidification rate (ECAR). 10^6 NK cells were cultured per well and various inhibitors were added (Agilent Seahorse XF Cell glycolysis Stress Test) at the following concentrations: oligomycin (2 μM), 2-deoxyglucose (2-DG) (30 mM) and glucose (5 mM) which allow the accurate calculation of glycolysis (ECAR).

Glucose uptake assay

5×10^5 to 10^6 spleen cells/ml were washed with PBS and incubated for 15 minute in RPMI-1640 without glucose (Corning) supplemented with 10% of dialyzed serum (Thermo Fisher Scientific), 2 mM L-glutamine, 1 mM HEPES, 1% penicillin/streptomycin and 50 μmol 2-mercaptoethanol at 37°C. Cells were incubated for 1 hour in the glucose free medium with 50 μM of 2-NBDG (Life Technologies) at 37°C. Cells were washed twice with PBS and stained for NK1.1, TCRβ, and Fixable Yellow Live/Dead (Invitrogen) on ice for 25 minutes, before being analyzed using flow cytometry.

Leucine-driven mTORC1 activation

Expanded NK cells were stimulated with either 1,000 U/ml of recombinant human IL-2 alone or with 3 0ng/ml of recombinant mouse IL-18 for 18 hours. Cells were washed with PBS 3 times to remove the remaining amino acids and FBS. Cells were cultured in RPMI-1640 medium without L-glutamine and amino acids (MyBioSource) for starvation for 3 hours.

Cells were pretreated with 15 mM of L-glutamine (Sigma) for 1 hour and washed 3 times. Cells were cultured in starved medium supplemented with different concentrations of leucine (0.4 mM, 2 mM, and 10 mM) for different time points (20min, 30min, 40min, 50min, and 60min). mTOR inhibitor rapamycin (1.6 μM) and inhibitor of protein synthesis cycloheximide (10 μM) were purchased from Calbiochem. The leucine antagonist N-acetyl-leucine amide (NALA) was used for 30 minutes and was purchased from Bachem. The following inhibitors were purchased from Sigma and used for the indicated periods; 2-Amino-2-norbornanecarboxylic acid amino acid transports inhibitor (BCH) for 30 minutes, glutamine uptake inhibitor L-γ-Glutamyl-p-nitroanilide hydrochloride (GPNA) for 1 hour, and alpha-(methylamino)isobutyric acida amino acid transport system A inhibitor (MeAIB) for 1 hour. Cells were stained for NK1.1, TCRβ, pS6, and Live/Dead Fixable Yellow Dead Cell Stain and analyzed using flow cytometry. To study the bidirectional exchange of glutamine for leucine, ^{3}H-labeled L-glutamine (NET551250UC) and ^{3}H-labeled L-leucine (NET460250UC) were purchased from PerkinElmer. Cells were treated as mentioned above with the addition of ^{3}H-labeled amino acids. To measure leucine influx, starved NK cells were preloaded with unlabeled L-glutamine and treated with 0.05 μCi/mL ^{3}H-labeled L-leucine. The cells were then washed twice with ice cold PBS, resuspended in 50 uL PBS, lysed with 50 uL 2% SDS and transferred into liquid scintillation vials. To measure glutamine efflux into the culture medium, starved NK cells were preloaded with both 15 mM unlabeled L-glutamine and 0.5 μCi/mL ^{3}H-labeled L-glutamine and treated with unlabeled L-leucine. The cells were then centrifuged and 50 uL of conditioned media was transferred to liquid scintillation vials. 1 mL of EcoLite(+)^{TM} liquid scintillation cocktail (MP Biomedicals) was added to each vial and the

^{3}H-labeled amino acids were measured using the Tri-Carb 2910TR liquid scintillation analyzer (PerkinElmer).

Western blot

Samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed for total p70 S6K (Cat# 9202), phospho-p70 S6K (T389, clone 108D2), and beta-actin (clone 13E5) rabbit antibodies, all from Cell Signaling Technology, in 5% BSA in Tris-buffered saline/0.05% Tween-80 (TBST). Membranes were then incubated with HRP-linked anti-rabbit IgG (Cell Signaling Technology), developed with Clarity enhanced chemiluminescent substrate (Bio-Rad), and chemiluminescent signals visualized with the ImageQuant LAS 4010 system (GE Healthcare). Quantification was performed by using the ImageJ software (NIH).

Statistical analysis

The mean values in the experiment were tested by ANOVA. If the ANOVA rejected the null hypothesis of the same means among the conditions ($p < 0.01$), multiple comparisons were performed between selected pairs of means by two-tailed unpaired t test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$) using Prism Version 5 (GraphPad Software).

Chapter 3: Reactive oxygen species as signaling molecules in immunity

Abstract

Cellular processes are regulated by the redox state, which influences cell growth, metabolism, and death. Even though reactive oxygen species (ROS) have been regarded as undesirable by-products of redox reactions that can damage DNA, lipids, and proteins, recent reports have demonstrated that ROS can serve as signaling molecules in the immune response. During infection and tumorigenesis, the stimulation of immune cells subjects them to cellular stresses. However, in-depth knowledge of the role of ROS in NK cell proliferation and cytotoxicity is still not completely understood. We showed that ROS levels in immune cells increase upon stimulation along with NK cell production of IFNγ and cytotoxicity. Notably, treating NK cells with H_2O_2 increased their effector functions. *Glrx2* belongs to the oxidoreductase family, which protects the cells from oxidative stress. In fact, Glrx2-deficient mice have been shown to be sensitive to ROS, we investigated the role of Glrx2 in NK cell function, activation, and proliferation. To our knowledge, the role of Glrx2 in immune cell function has never been shown. Our initial findings demonstrated that NK cells and T cells deficient in Glrx2 showed enhanced IFNγ production, cytotoxic potential, and increased proliferation, upon a variety of stimulations. To exclude any effects from genetic variations caused by genes other than Glrx2, we decided to confirm our data by crossing Glrx2-deficient mice with wild type B6 mice and use the progeny to generate *Glrx2wt/wt, Glrx2wt/ko,* and *Glrx2ko/ko* littermate mice. Unexpectedly, NK cell and T cell IFNγ production, cytotoxic potential, and proliferation were comparable among the littermate groups. Due to the sensitivity of Glrx2-deficient mice to ROS, MCMV has been used as a model to study viral latency, which will be inducing oxidative stress that leads to the generation of ROS. During MCMV infection NK cells from *Glrx2wt/wt, Glrx2wt/ko,* and *Glrx2ko/ko* mice showed similar

levels of IFNγ production, LAMP-1 expression, proliferation, as well as Ly49H+ NK cells expansion. Also, there was no difference in NK cell effector functions during persistent infection with MCMV. Taken together, Glrx2 does not play critical role in NK cell effector function during acute and persistent MCMV infection.

Introduction

NK cells have an essential role in anti-viral and anti-tumor responses. Although NK cells are critical for the early immune responses, they can regulate the adaptive immune response by releasing cytokines such as IFNγ and TNFα as well as modulating the number of DCs (35). During MCMV infection, NK cells undergo clonal expansion and increase their effector function to control the infection. As the immune cells including NK cells become activated and proliferate, they change their cellular metabolism to accommodate their bioenergetic needs (124). Quiescent immune cells need to maintain a basal level of metabolism to support their housekeeping functions and trafficking, whereas proliferating immune cells need to produce more ATP to support their increased cytotoxicity and cytokine production, and to produce intermediates required for biosynthesis and cell division (209). Upon activation, the most dramatic change in NK cells (124) and T cells metabolism is the increase of glucose metabolism, which is regulated by the PI3K and Akt pathway and the transcription factors Myc and ERRa (271). In addition, T cells increase their glutamine metabolism, which is required for their proliferation and activation (272). Glucose and glutamine are two main nutrient sources, which can be derived into metabolites that can feed into the TCA cycle in the mitochondria. In the glycolysis pathway, glucose is metabolised into pyruvate, which can be converted to acetyl-CoA, a substrate for the TCA cycle. Glutamine can be converted to α-ketoglutarate and feed into the TCA cycle via a process called glutaminolysis (209). Since proliferating cells may rely on the TCA cycle as a source of energy for biosynthetic (99), the mitochondrial metabolism has an essential role in immune cell activation (209).

In addition to the role of mitochondria in supporting biosynthetic reactions, the mitochondria are the primary source of ROS. During cellular oxidation, NADH and $FADH_2$ generated in different mitochondrial pathways, such as fatty acid oxidation (FAO) and TCA cycle, provide electrons that pass through the electron transport chain (ETC) to be accepted by O_2. Leakage of these electrons at complexes I and III of the mitochondrial ETC can generate superoxide which is then converted to H_2O_2 (153). Although ROS are involved in oxidative stress which results in many human diseases; such as Parkinson's disease, Alzheimer's disease, cancer and heart disease, studies have shown that mitochondrial (mROS) can serve as signaling intermediates (273); (274); (275); (276). Interestingly, treating T cells with antioxidants reduced T cell expansion, (277). Notably, mROS are involved in T cell activation, and the mROS from complex III are required for CD4+ T cell activation as well as the antigen-specific CD4+ T cell expansion in vivo (209). Moreover, the oxidative signals from the mitochondria are important for nuclear factor of activated T cells (NFAT) activation, IL-2 induction (278) and regulation of IL-2 and IL-4 expression in T cells (210). Here, I report that stimulation of NK cells leads to increased levels of ROS. ROS signaling are required for NK cells effector function and activation. Notably, mROS are critical for NK cells to induce IFNγ production and LAMP-1 expression. Treating NK cells with the antioxidant N-acetyl cysteine (NAC) resulted in a reduction of IFNγ production as well as cytotoxicity.

Cells have an antioxidant system to control the level of ROS and protect proteins from being oxidized when the level of ROS are elevated. Glrx2 belongs to the oxidoreductase family and is involved in the antioxidant system. Upon oxidative stress in the mitochondria, the level of ROS increases as well as the level of proteins being oxidized. GSSH interacts with

the oxidized proteins and results in the glutathionylation of these proteins and generates glutathione (GSH). Glrx2 deglutathionylates the target protein by exchanging its thiol group with the S-GS group on the target protein (202). GSH interacts with the glutathionylated Glrx2 and restores its thiol group, generating GSSG. GSSG is converted to glutathione by glutathione reductase and NADPH (279). Deletion of Glrx2 was previously shown to increase cellular sensitivity to ROS, while the mitochondria showed have normal activity and morphology. When *Glrx2*-deficient cells were exposed to oxidative stress, they showed a decrease in ATP production, complex I activity and elevated level of ROS (189). Therefore, Glrx2-deficient mice are a useful model to study the effect of ROS on immune cell function, proliferation, and activation in naive state or acute and persistence infection. Thus, to further investigate the role of ROS in immune cells in vivo, I examined *Glrx2*-deficient mice during MCMV infection. Unexpectedly, the absence of Glrx2 did not show any defect in the function or the activation of NK and T cells during MCMV infection.

Results

Intracellular levels of ROS are increased upon different stimulations

To test the production of ROS by immune cells, spleen cells were first treated with ROS, in the form of H_2O_2. Treating the cells with H_2O_2 as a positive control showed an increase in the generation of intracellular levels of ROS (Fig. 3.1A), as measured by the oxidation of H2DCFDA (DCF in short). Treatment of spleen cells with the antioxidant NAC for 30 minutes after stimulation with H_2O_2 blocked ROS production (Fig. 3.1B).

Stimulating T cells with PMA and Ionomycin (PMA/Iono) activates protein kinase C (PKC) and Ca^{2+} signaling pathways, upregulates IL-2 gene expression, DNA synthesis and induces cell proliferation. PKC is also involved in ROS generation (280). To determine whether stimulation of immune cells with cytokines or PMA/Iono can generate ROS production, splenic cells were stimulated with IL-2/12 or PMA/Iono and the ROS level measured by the DCF oxidation. As expected, ROS were strongly induced upon PMA/Iono stimulation and slightly increased with IL-2/12 stimulation (Fig. 3.1C). Blocking ROS production with NAC treatment reduced ROS levels (Fig. 3.1D). Interestingly, when I measured the level of ROS in the naïve state, our data showed that NK cells produce more ROS levels as compared to T cells (Fig. 3.1E). To measure the level of ROS produced by NK cells upon stimulation, NK cells were stimulated with IL-2/12 or PMA/Iono and consistent with the results obtained with the spleen, ROS levels increased upon PMA/Iono treatment (Fig. 3.1F) and with IL2/12 stimulation (Fig. 3.1G), while NAC treatment was able to inhibit ROS production. Taken together, ROS are produced from spleen cells and NK cells upon stimulation with PMA/Iono, and NAC treatment neutralizes the ROS.

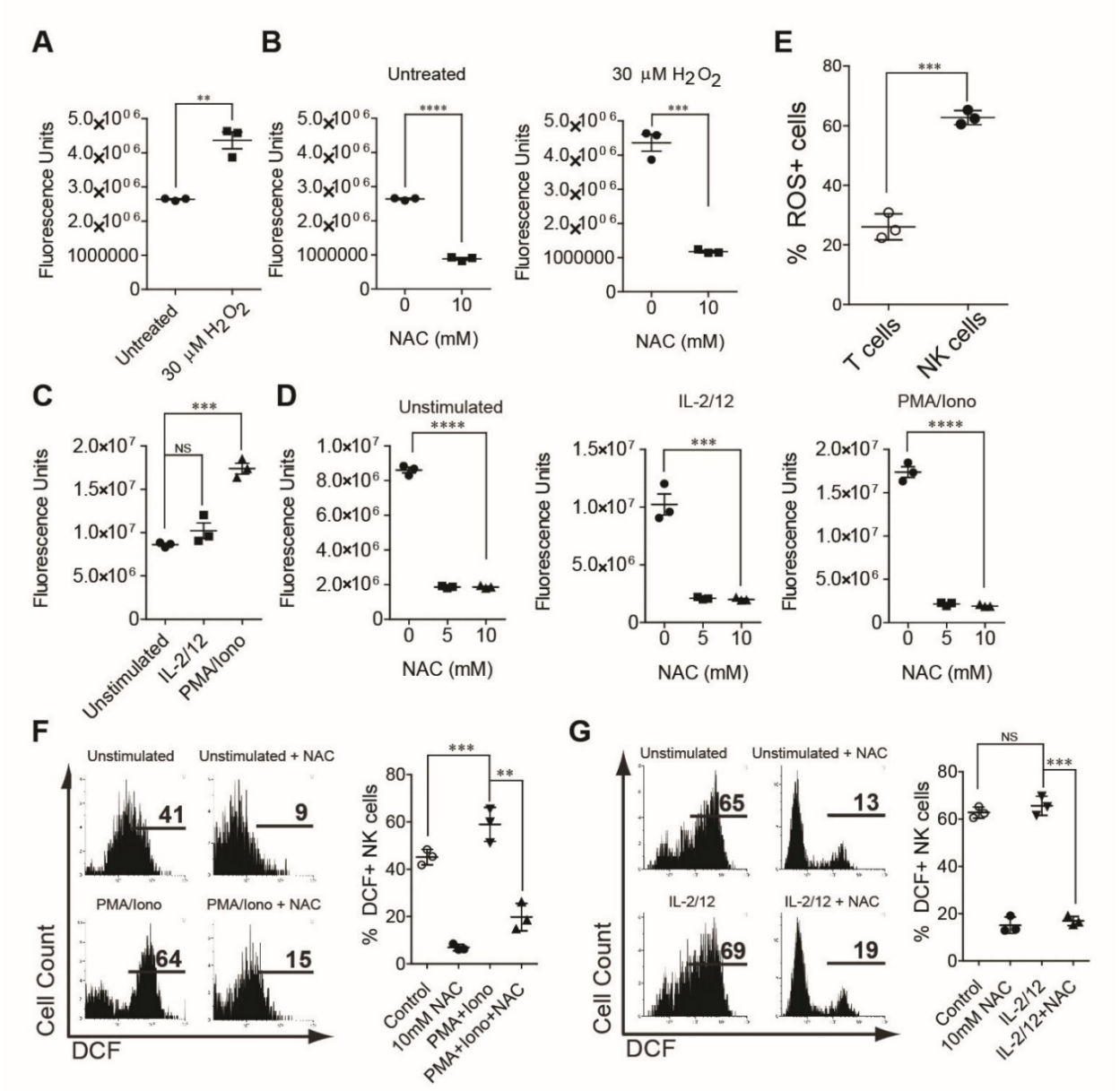
A
B
C
D
E
F
G
Fluorescence Units
Untreated
30 μM H2O2
NAC (mM)
Unstimulated
IL-2/12
PMA/Iono
% ROS+ cells
T cells
NK cells
NS
Unstimulated + NAC
PMA/Iono + NAC
IL-2/12 + NAC
Cell Count
DCF
% DCF+ NK cells
Control
10mM NAC
PMA+Iono
PMA+Iono+NAC
IL-2/12+NAC

Figure 3.1. Increased intracellular levels of ROS upon different stimulations. (A) Spleen cells were treated with 30 μM H2O2 for 4 hours and ROS levels were measured by spectrophotometry and stained with the free radical-sensitive probe carboxy-H2DCFDA (DCF) for 10 minutes. (B) Cells treated with NAC for 30 minutes. (C) The levels of ROS in splenic cells were measured upon stimulation with IL-2/12 or PMA/Iono for 4 hours. (D) After stimulation with IL-2/12 and PMA/Iono, the cells were treated with the indicated concentrations of NAC. (E) The induction of ROS in NK cells and T cells was analyzed in the naive state. (F and G) ROS levels were measured in NK cells upon PMA/Iono treatment or IL-2/12 stimulation folloId by NAC treatment. Data are from one experiment representative of three independent experiments, with two to three replicates per group. Data represent mean + SD. **p < 0.01; ***p < 0.001; ****p < 0.0001.

The total ROS and the mitochondrial ROS (mROS) are required for NK cell effector functions

To investigate whether ROS can induce NK cell effector functions, NK cells were treated with H_2O_2 and the production of IFNγ was analyzed. IFNγ was strongly induced upon H_2O_2 treatment (Fig. 3.2A), indicating that ROS can activate NK cells. I confirmed that the concentrations of H_2O_2 used in this study did not induce cell death (Fig. 3.2A). Since the stimulation of NK cells with PMA/Iono treatment showed increased levels of ROS, I investigated whether blocking ROS production can reduce NK cell function. Therefore, NK cells were stimulated with IL-2/12, PMA/Iono or through the activating receptor NK1.1 in the presence of the antioxidant NAC. IFNγ production (Fig. 3.2B) as well as LAMP-1 expression (Fig. 3.2C) in NK cells were induced upon different stimulations. Indeed, NAC treatment led to a significant inhibition of IFNγ and LAMP-1 in a dose-dependent manner, suggesting that the generation of ROS are involved in enhancing NK cell effector functions and using NAC would decrease the level of ROS, resulting in the reduction of NK cell effector functions. The treatment of NK cells with the concentrations of NAC used in this assay did not affect the viability of the cells (Fig. 3.2D).

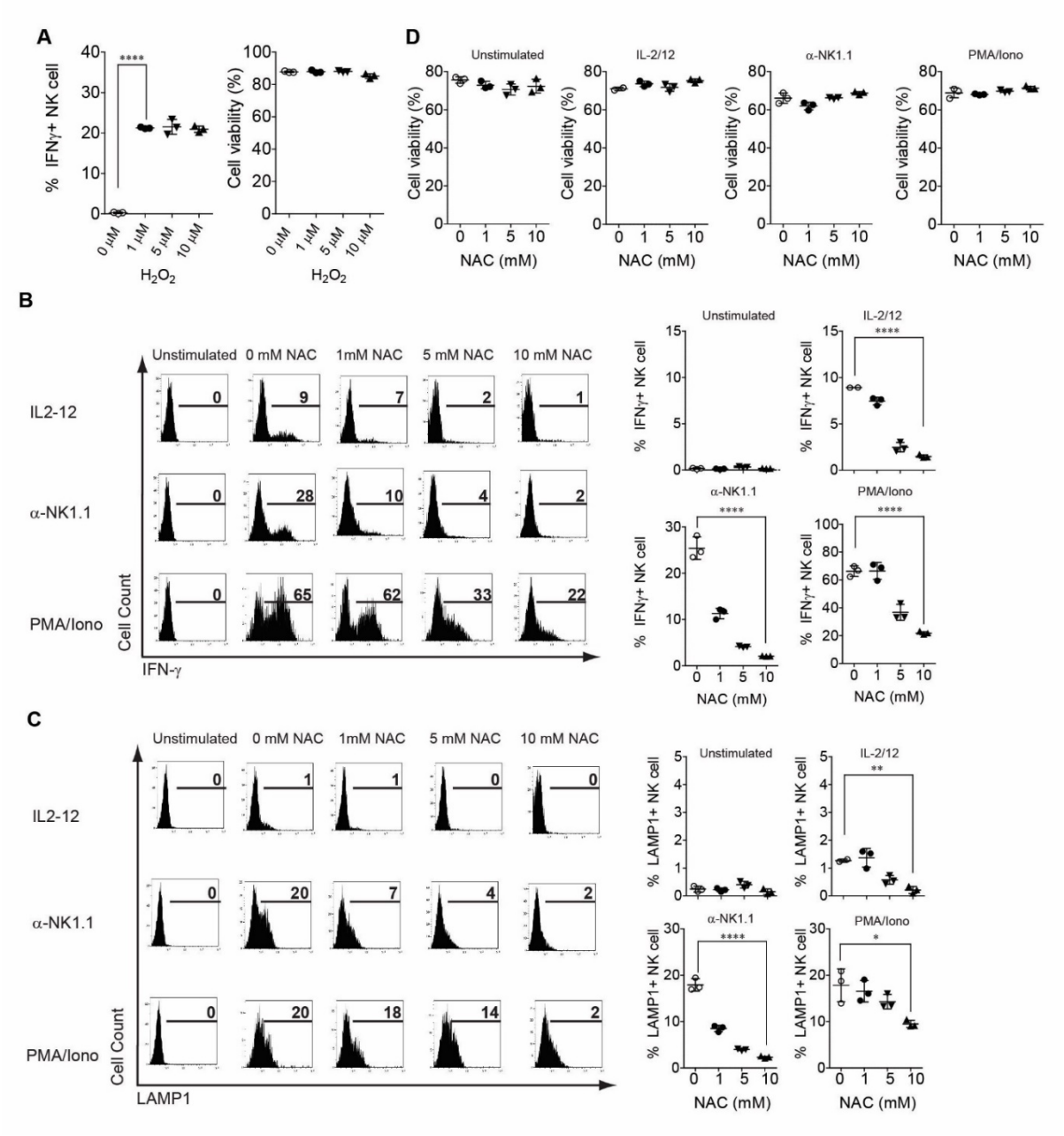

Figure 3.2. ROS are required for inducing effector function in NK cells. (A) NK cells were treated with different concentrations of H_2O_2 and the intracellular IFNγ production was analyzed. The cell viability of NK cells upon treatment with the indicated concentrations of H_2O_2 was measured. (B and C) Representative histograms of intracellular IFNγ and LAMP-1 expression in NK cells upon different stimulations IL-2/12, α-NK1.1 or PMA/Iono followed by treatment with different concentrations of NAC. (D) The cell viability of NK cells upon treatment with the indicated concentrations of NAC are shown. Data are from one experiment representative of two independent experiments, with three replicates per group. Data represent mean + SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

mROS have been previously shown to control T cell activation (210). To analyze whether the mROS derived from complexes I and III of the ETC are critical for NK cell effector function, NK cells were treated with rotenone and antimycin A which block complex I and complex III, respectively. These two complexes are known to be the major sources of ROS in the mitochondria. NK cells stimulated with IL-2/12 showed increased levels of IFNγ and LAMP-1 expression, but blocking mROS using rotenone (Fig. 3.3A) and antimycin A (Fig. 3.3B) at different concentrations resulted in the reduction of IFNγ production and LAMP-1 expression in a dose-dependent manner. Consistently, treating the cells with PMA/Iono in the presence of rotenone and antimycin A exhibited similar results (Fig. 3.3C). Altogether, these results indicated the importance of both complex I and complex III in NK cell effector functions. To elucidate whether rotenone and antimycin A treatment can in fact inhibit mROS levels, the cells were treated with both inhibitors for 4 hours and the mROS levels were measured by DCF staining. The level of mROS were decreased upon treatment with the ETC inhibitors in both purified NK cells and total spleen (Fig. 3.3D), showing that blocking the complexes from transferring electrons results in inhibiting ROS production. Treating the cells with the above inhibitors did not induce cell death on all tested concentrations (Fig. 3.3E).

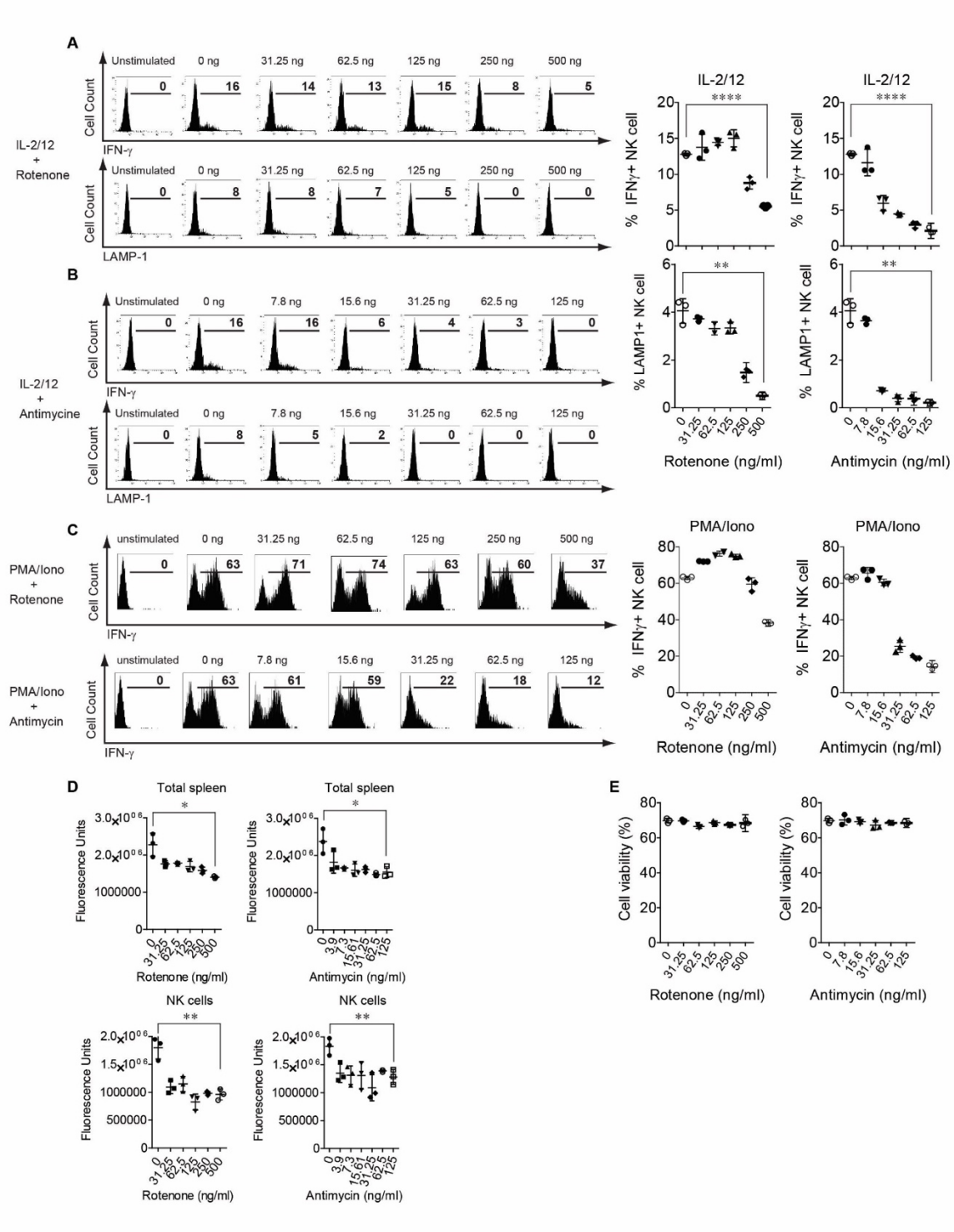
A
Unstimulated
0 ng
31.25 ng
62.5 ng
125 ng
250 ng
500 ng
IL-2/12 + Rotenone
Cell Count
IFN-γ
LAMP-1
IL-2/12
% IFNγ+ NK cell
% LAMP1+ NK cell
Rotenone (ng/ml)
B
7.8 ng
15.6 ng
IL-2/12 + Antimycine
Antimycin (ng/ml)
C
PMA/Iono + Rotenone
PMA/Iono + Antimycin
PMA/Iono
D
Total spleen
Fluorescence Units
NK cells
E
Cell viability (%)

Figure 3.3. Mitochondrial ROS (mROS) are required for degranulation and IFNγ production by NK cells. NK cells were treated with different concentrations of rotenone or antimycin A inhibitors. (A and B) Representative histograms of intracellular IFNγ and LAMP-1 expression in NK cells upon IL2/12 stimulation. (C) Representative histograms of intracellular IFNγ in NK cells upon PMA/Iono. (D) Spleen cells or purified NK cells were treated with the indicated concentrations of rotenone or antimycin A for 4 hours and the ROS levels were measured by spectrophotometry following staining with DCF for 10 minutes. (E) The cell viability of NK cells upon treatment with the inhibitors. Data are from one experiment representative of two independent experiments, with two to three replicates per group. Data represent mean + SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Analysis of general immune populations in Glrx2-deficient mice

The physiological role of Glrx2 and the effects of Glrx2 deletion on cellular function have been previously demonstrated (189). Mice deficient in Glrx2 develop cataracts early during aging, which was associated with a defective electron transport system (189). Moreover, *Glrx2*-deficient mice developed heart hypertrophy and became hypertensive (279). However, the phenotype of immune cells in Glrx2-deficient mice has never been investigated. Therefore, the phenotypic characteristics of immune cells from wild type and *Glrx2*-deficient mice were examined, and the proportions of various immune populations (NK, T, B, CD8+ T, and CD4+ T cells) were quantified. The proportions of NK, B, and CD8+ T cells were similar in both wild type and *Glrx2*-deficient mice, indicating that the deletion of Glrx2 does not affect the development of these cells. However, the percentage of total T cells and in particular CD4+ T cells were lower in *Glrx2*-deficient mice (Fig. 3.4A).

NK cells in Glrx2-deficient mice showed increased effector functions

To determine the role of Glrx2 on NK cell effector functions in regards to IFNγ production, LAMP-1 expression and proliferation, NK cells from wild type and *Glrx2*-deficient mice were stimulated using IL-2/12 and IL-2/12/18 or through NK cell activating receptors. Cytokines stimulation as well as cross-linking of the activating receptors NK1.1 or Ly49H resulted in significantly higher IFNγ production in NK cells from *Glrx2*-deficient mice, as compared to wild type mice (Fig. 3.4B). In addition, NK cells from *Glrx2*-deficient mice showed increased expression of LAMP-1 upon stimulation through NK cell activating receptors (Fig. 3.4C). Stimulation with IL-2 allows NK cells and T cells to proliferate and the proliferation is crucial to control the infection. To respond to low levels of physiological IL-2 concentrations in vivo, these immune cells need to bind to IL-2 with high affinity. NK and

T cells need to express IL-2Ra (CD25) to complete the hetero-trimeric high-affinity IL-2R composed of IL-2Rα, β, and the common γ-chain (281). Thus, I sought to investigate whether the defect in Glrx2 will affect the proliferation of NK and CD8+ T cells as well as the expression of CD25. Notably, NK and CD8+ T cells from *Glrx2*-deficient mice showed enhanced proliferation upon stimulation with IL-2 (Fig. 3.4D). CD8+ T cells from *Glrx2*-deficient mice expressed higher levels of CD25 than CD8+ T cells from WT mice (Fig. 3.4E).

Glrx2-deficient cells have been known to exhibit normal functions unless the cells undergo oxidative stress such as treatment with H_2O_2, in which case they will become highly sensitive (189). To confirm it, the basal ROS levels were analyzed in NK cells, and as expected, NK and T cells from exhibited higher levels of ROS, as compared to NK cells from wild type mice (Fig. 3.4F). Taking together, our data demonstrate that immune cells from *Glrx2*-deficient mice elicit elevated effector functions accompanied by high levels of ROS. Our data suggest that the deletion of Glrx2 increase the generation of ROS, which serve as signaling molecules to support the activation and the proliferation of NK cells and T cells.

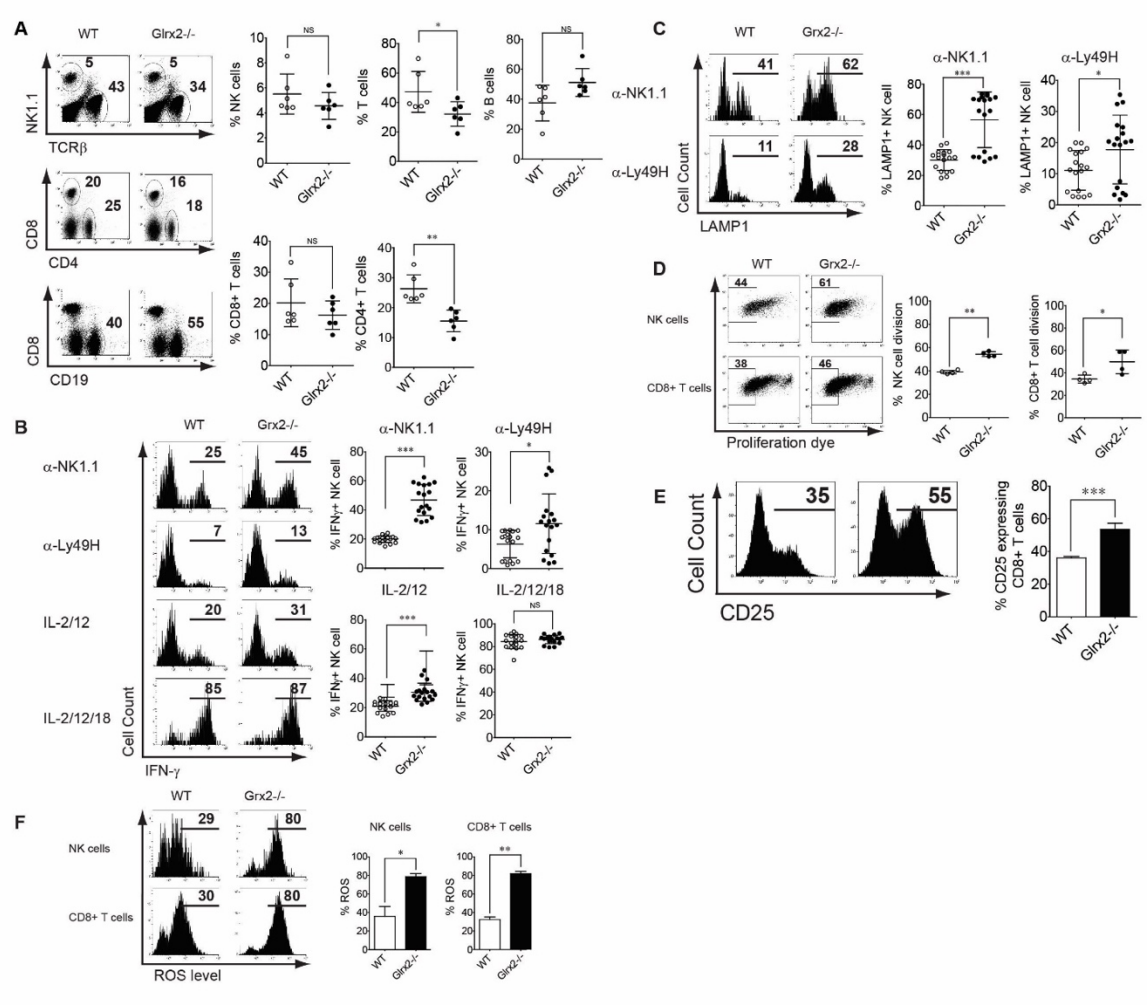

Figure 3.4. Analysis of general immune populations and the effector functions of immune cells in *Glrx2*-deficient mice. (A) Representative dot plots of NK, T, CD8+, CD4+ and B cell frequencies and percentage of NK, T, CD8+, CD4+ and B cells. (B and C) Representative histograms of intracellular IFN-γ and LAMP-1 expression in NK cells upon α-NK1.1, α-Ly49H, IL2/12 or IL-2/12/18 stimulation. Spleen cells were labeled with a cell division dye and stimulated with 1000 U/ml of IL-2. (D) Representative dot plot depict the proliferation of NK and CD8+ T cells following 5 days of culture with IL-2. (E) Representative histogram of CD25 expression in CD8+ T cells. (F) Representative histogram of ROS level produced from NK and CD8+ T cells. Data are from three experiments combined with three to four mice per group. Data represent mean + SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Generating littermate controls to study the effect of Glrx2 deletion in immune cell function

To confirm that our data showing enhanced effector function in NK cells from *Glrx2*-deficient mice are solely due to the deletion of Glrx2 and to avoid any effect from genetic variations other than *Glrx2*, I decided to generate littermate controls. To do so, wild type B6 mice were crossed with *Glrx2*-deficient mice, and the progeny (*Glrx2wt/ko*) was further crossed to obtain *Glrx2wt/wt, Glrx2wt/ko,* and *Glrx2ko/ko* littermates (Fig. 3.5A and B).

To investigate whether the development and function of immune cells is influenced by the Glrx2-mediated antioxidant system, NK, T, B, CD8+ T, and CD4+ T cell proportions in the spleen were examined. Consistent with the previous data, the proportions of these different immune cell populations were comparable between all the genotypes (*Glrx2wt/wt, Glrx2wt/ko, Glrx2ko/ko*) (Fig. 3.5C). Since *Glrx2*-deficient cells are known to be highly sensitive to oxidative stress (189), I tested the NK cell effector functions such as IFNγ production, LAMP-1 expression, and proliferation in all littermates. Spleen cells from mice of different genotypes were stimulated through NK cells activating receptors NK1.1 or Ly49H. Unexpectedly, the IFNγ production in NK cells was equivalent between *Glrx2wt/wt*, *Glrx2wt/ko*, and *Glrx2ko/ko* mice (Fig. 3.5D). The cytotoxic potential (LAMP-1 expression) of NK cells remained the same among the different genotypes. In addition, the proliferation of NK and T cells (Fig. 3.5E) and the activation of T cells were comparable in these mice (Fig. 3.5F). Taken together, these results indicate that the absence of Glrx2 does not affect the effector functions of NK cells and T cells at the steady state, and suggest that the differences in immune cells function seen in the previous data (Fig. 3.4) might be due to the genetic variations from genes other than *Glrx2*. The *Glrx2-deficient* mice were originally generated on a 129SV and B6 hybrid background and were then backcrossed into B6 mice to generate

homozygous *Glrx2-deficient* mice. Thus, it is possible that the control B6 mice used in previous experiments and *Glrx2-deficient* mice show genetic variations.

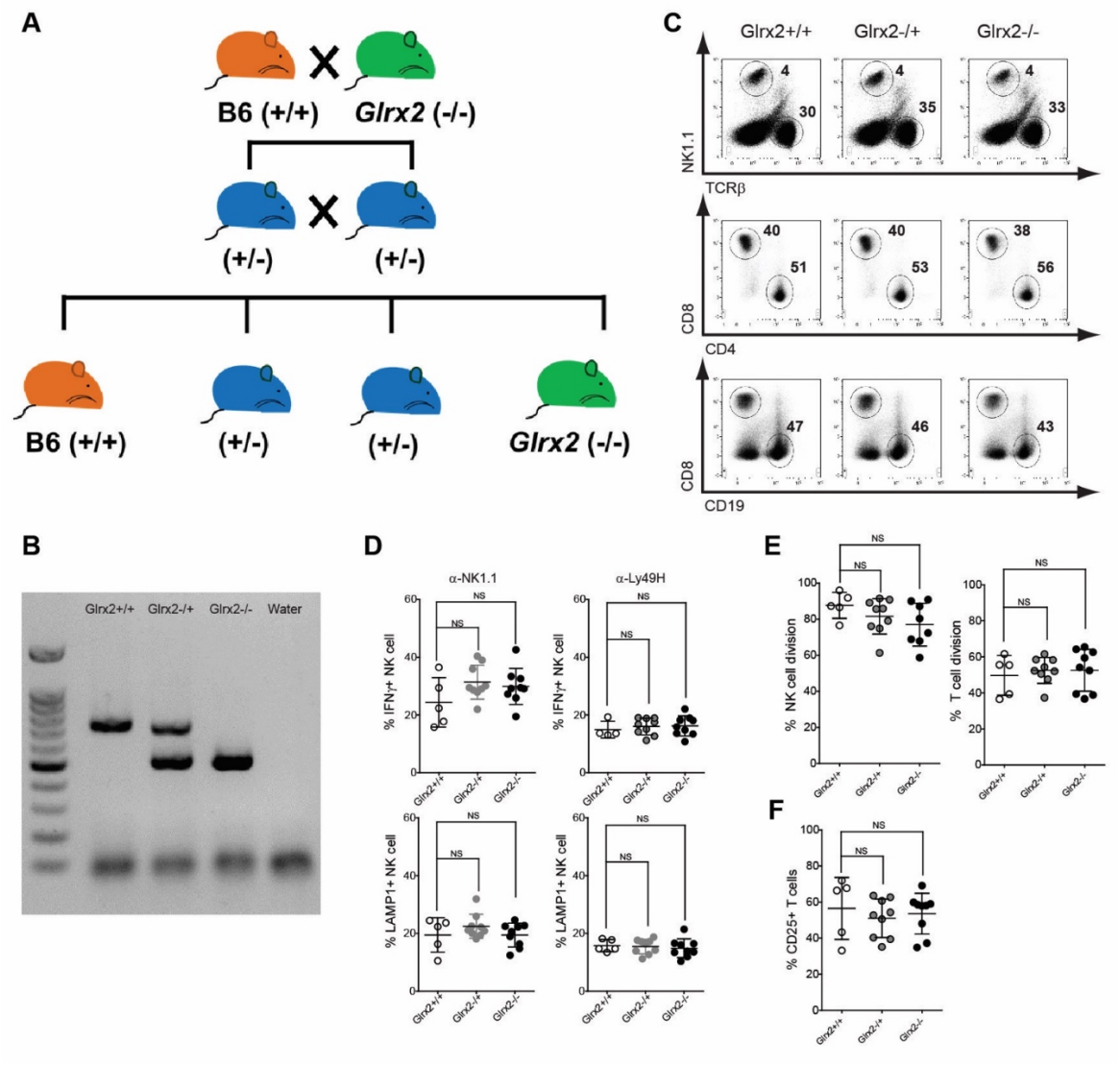

Figure 3.5.Glrx2 deletion does not affect immune cells development or effector functions. (A) Breading scheme. (B) Agarose gel picture depicts the PCR results for testing the presence or deletion of Glrx2 gene in *Glrx2wt/wt, Glrx2wt/ko,* and *Glrx2ko/ko* mice. (C) Representative dot plots of the frequencies of NK, T, CD8+, CD4+ and B cells. (D) Intracellular IFN-γ and LAMP-1 expression in NK cells upon α-NK1.1 or α-Ly49H stimulation. (E) Spleen cells were labeled with a cell division dye and stimulated with 1000 U/ml of IL-2. The proliferation of NK and CD8+ T cells following 4 days of culture with IL-2 is shown. (F) Expression of IL-2Rα (CD25) in T cells. Data are from one experiment representative of two independent experiments with five to eight mice per group. Data represent mean + SD.

Glrx2 is not required for NK cell effector function during MCMV infection

During a viral infection such as MCMV, oxidative stress occurs which can lead to an increased cellular content of ROS (282). *Glrx2*-deficient mice are sensitive to the oxidative stress since they show a defect in detoxifying ROS (189). In the previous results, I demonstrated that Glrx2 has no effect on IFNγ production, LAMP-1 expression, as well as the proliferation of both NK and T cells from naive F2 mice from the B6 and *Glrx2*-/- breeding upon different in vitro stimulations. It was a striking observation since enhanced effector functions was clearly observed in NK cells from Glrx2-deficient mice compared to those from wildtype control mice To dissect the discrepancy, I decided to study the role of Glrx2 during MCMV infection in vivo, which would impose oxidative stress on immune cells. To do so, *Glrx2wt/wt*, *Glrx2wt/ko*, and *Glrx2ko/ko* mice were infected with 10,000 PFU MCMV via the intraperitoneal route. During MCMV infection, the proportion of proliferating NK cells reaches its peak at day 3 post-infection in order to control the infection (283, 284). On day 3 post-infection, mice were sacrificed, and the proportions of immune cells were analyzed. The proportions of NK, T, B, CD8+ T, and CD4+ T cells were comparable among the different genotypes (Fig. 3.6A). In addition, the viral loads in the organs of infected mice were comparable in all three genotypes, suggesting an efficient clearance of MCMV by NK cells in the absence of Glrx2 (Fig 3.6B). Moreover, the proliferation antigen Ki-67 in NK and T cells was similar between *Glrx2wt/wt*, *Glrx2wt/ko*, and *Glrx2ko/ko* mice (Fig. 3.6C). The activation marker CD25 was also comparable in both NK and T cells (Fig. 3.6D). Thus, the data indicated that Glrx2 is not required for the proliferation of NK and T cells during MCMV infection. In addition, the level of ROS in NK cells was similar in all the mice (Fig. 3.6E). The resistance of the host against MCMV is achieved by the activating receptor Ly49H on NK

cells (285).The mechanism of action of Ly49H receptor is through its recognition of the viral protein m157 on the surface of MCMV infected cells, which will induce the killing of the infected cells by NK cells, along with an increase in the secretion of many cytokines, followed by the expansion of Ly49H+ NK cells (57).

To evaluate whether Glrx2 has a role in Ly49H expansion, *Glrx2wt/wt*, *Glrx2wt/ko*, and *Glrx2ko/ko* mice were infected with 10,000 PFU MCMV for 5 to 6 days. The expansion of Ly49H+ NK cells in the blood was normal in all the genotypes at day 5 post infection (Fig. 3.6F). By day 6 post infection, it is known that 80% of the splenic NK cells in infected mice express the Ly49H receptor (286). Notably, Ly49H+ NK cells were able expand to reach up to 80% of the total NK cell pool at day 6 post infection in *Glrx2wt/wt*, *Glrx2wt/ko*, and *Glrx2ko/ko* mice, suggesting that Glrx2 is not involved in the expansion of Ly49H+ NK cells during MCMV infection. Since the mice from all three genotypes showed comparable response during the acute stage of MCMV infection, I decided to investigate whether Glrx2 can play a role during MCMV persistent infection. Mice were infected with 5,000 PFU MCMV and left for 3 months to allow a persistent infection. After 3 months, the mice were sacrificed, and the spleen cells were stimulated with IL-2,12 or through the activating receptors NK1.1 and Ly49H to induce IFNγ and LAMP-1. The levels of IFNγ and LAMP-1 expression were equivalent between all three groups of mice (Fig. 3.6G). In addition, the proliferation of NK and T cells were comparable for all genotypes (Fig. 3.6H). Altogether, these data indicate that Glrx2 does not play a critical role in NK and T cell effector function during acute or persistent MCMV infection.

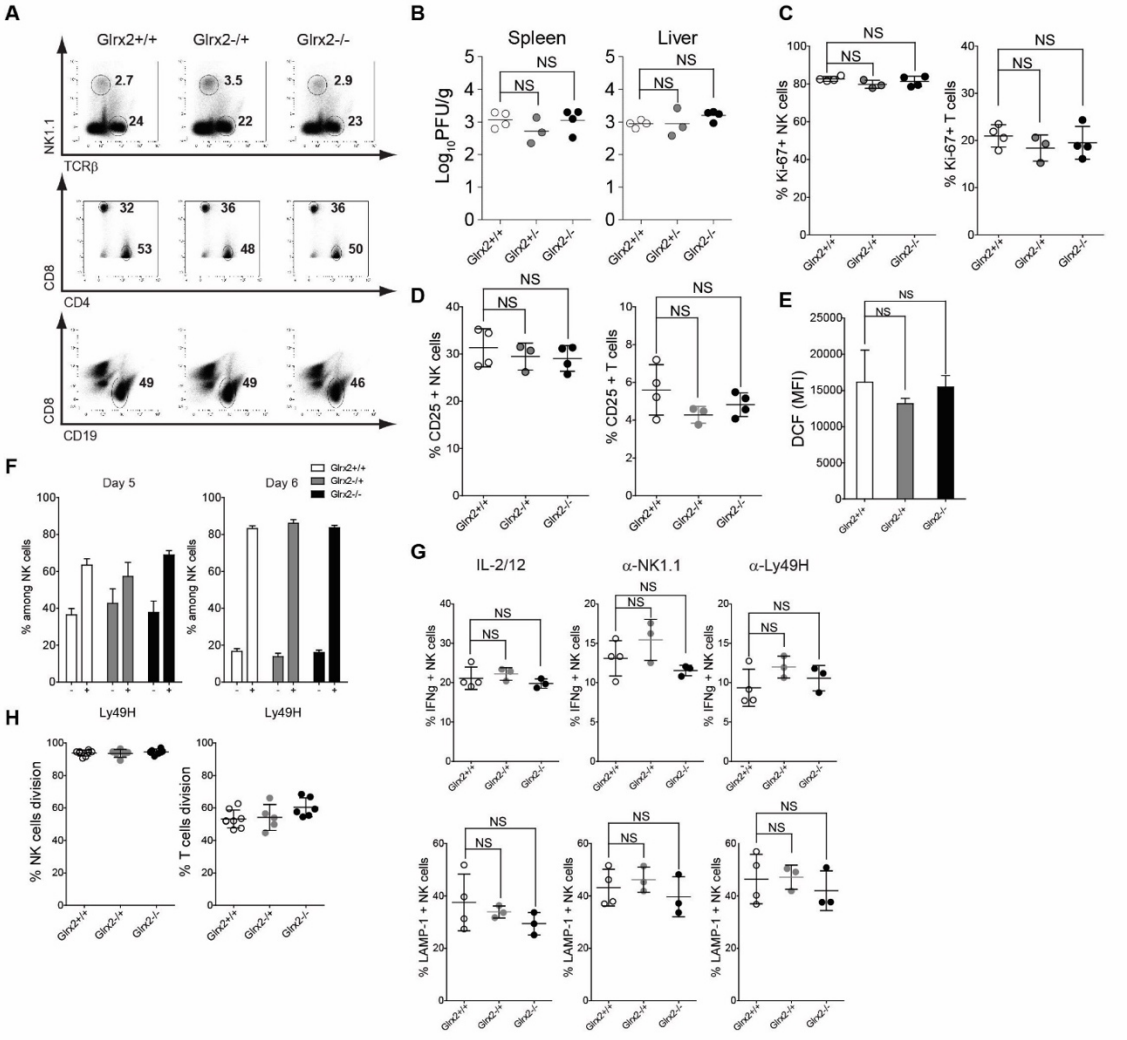
A
Glrx2+/+
Glrx2-/+
Glrx2-/-
NK1.1
TCRβ
CD8
CD4
CD19
B
Spleen
Liver
Log10PFU/g
C
% Ki-67+ NK cells
% Ki-67+ T cells
D
% CD25 + NK cells
% CD25 + T cells
E
DCF (MFI)
F
Day 5
Day 6
% among NK cells
G
IL-2/12
α-NK1.1
α-Ly49H
% IFNg + NK cells
% LAMP-1 + NK cells
H
Ly49H
% NK cells division
% T cells division
NS

Figure 3.6. Glrx2 deletion does not affect the function of NK and T cells during acute or persistence MCMV infection. Glrx2wt/wt, Glrx2wt/ko, and Glrx2ko/ko mice were infected with 10,000 PFU MCMV intraperitoneally, and analyzed at day 3 post infection. (A) Representative dot plots of the frequencies of NK, T, CD8+, CD4+ and B cells. (B) The viral titers in the spleens and livers of infected Glrx2wt/wt, Glrx2wt/ko, and Glrx2ko/ko mice. (C) NK and T cell proliferation upon MCMV infection is shown as measured by Ki-67 expression. (D) Expression of CD25 in NK and T cells. (E) The MFI of ROS levels on NK cells in the spleens of infected mice is shown. *Glrx2wt/wt*, *Glrx2wt/ko*, and *Glrx2ko/ko* mice were infected with 10,000 PFU MCMV intraperitoneally, and the blood samples were collected at day 5, before the mice were sacrificed at day 6 post infection. (F) The expansion of Ly49H-expressing NK cells in *Glrx2wt/wt*, *Glrx2wt/ko*, and *Glrx2ko/ko* mice. *Glrx2wt/wt*, *Glrx2wt/ko*, and *Glrx2ko/ko* mice were infected with 5,000 PFU MCMV intraperitoneally for 3 months. (G) Intracellular IFN-γ and LAMP-1 expression in NK cells upon IL-2/12, α-NK1.1 or α-Ly49H stimulation. Spleen cells were labeled with a cell division dye and stimulated with 1000 U/ml of IL-2. (H) Proliferation of NK and T cells following 4 days of culture with IL-2. Data are from one experiment with three mice per group. Data represent mean + SD.

Discussion

ROS, which have long been known to damage the cells and induce cell death. However, many studies have shown that ROS also act as intracellular signaling molecules both in steady-state and upon antigen recognition (287). Notably, ROS have been shown to regulate T cell function and proliferation (209, 210, 278). For example, low concentrations of ROS in T cells are required for cell survival, whereas high levels of ROS can lead to apoptosis (288). ROS are known to positively regulate IL-2 expression on T cells (210). However, whether cytokine stimulation of lymphocytes can induce ROS production is unknown. Here, I demonstrated the role of cellular ROS and mROS in modulating NK cell effector functions. In addition, treating the cells with H_2O_2 led to increased levels of intracellular ROS. Previous studies showed that ROS production is required for T cell mitogenesis (289, 290). Engagement of the TCR induces the generation of both H_2O_2 and ($O_2\bullet^-$) (291). The unique properties of H_2O_2 such as the reversible oxidation of cysteines, its membrane permeability, and the long half-life, favor it over other ROS as a possible second messenger (292). Activation of T cells with PMA/Iono is known to activate PKC and Ca^{2+} signaling pathway and results in a rapid flux of ROS (293). Consistently, our data showed that treating NK cells with PMA/Iono increased the level of intracellular ROS.

Glutathione (GSH) helps reduce oxidative stress and acts as an electron donor to glutaredoxin (292). NAC is a GSH precursor and known to enhance T cell proliferation, production of IL-2 and up-regulation of the IL-2 receptor (294). Our data demonstrated that treatment of NK cells with the antioxidant NAC blocked the PMA/Iono and IL-2/12-induced oxidative signal, suggesting that NAC enters the cells, is converted into GSH, which in turn stabilizes the intracellular antioxidant system by neutralizing ROS. There was a slight increase

of ROS level in NK cells stimulated with the cytokines IL-2/12, suggesting that the signal through IL-2/12 can increase the production of ROS.

During MCMV infection, various cytokines, such as IL-2, 15, 12, 18, and type I and type II IFNs, are produced to control the infection. In addition, NK cells can recognize viral proteins through their activating receptors such as NK1.1 and Ly49H (35). One potent effector function of NK cells is the production of IFNγ, which is critical to provide the host resistance against numerous intracellular pathogens (295). IL-12 stimulates NK cells through STAT4 to produce IFNγ and *STAT4*-deficient mice failed to induce IFNγ (296). IL-18 is also known to stimulate NK cells to produce IFNγ through activation of the MAPK pathway, which stabilizes IFNγ mRNA and enhances IFNγ secretion (297). The induction of IFNγ upon ROS such as H_2O_2 has been investigated more than three decades ago (211) when it was shown that IFNγ levels are increased upon H_2O_2 treatment in human peripheral mononuclear cells including NK cells and T cells, but not in B cells. To demonstrate that ROS can serve as a possible second messenger, which can induce the production of IFNγ, NK cells were stimulated with H_2O_2 for 4 hours and consistent with the previously published data, H_2O_2 was able to induce IFNγ as well as increase the expression of LAMP-1 in NK cells. These results suggest that ROS might be responsible for inducing the effector functions in immune cells.

Notably, stimulating NK cells with PMA/Iono and IL-2/12 increases the ROS levels. Based on these results, I hypothesized that ROS are required for NK cell functions. In fact, upon stimulation with IL-2/12, PMA/Iono or through the activating receptors NK1.1 and Ly49H, NAC treatment of NK cells led to reduced IFNγ production in a dose-dependent manner.

mROS from mitochondria complex I function as a generator of the activation-induced oxidative signal in preactivated human T cells (210). Rotenone, which inhibits complex I of the ETC, was able to block the activation-induced IL-2 and IL-4 expression and ROS production in resting T cells. In addition, IL-2 and IL-4 secretion which is induced by CD3/CD28 or PMA/Iono stimulations in resting T cells was inhibited by rotenone treatment (210). Therefore, I determined whether mitochondrial complex I and complex III-generated ROS influence IFNγ production and LAMP-1 expression in NK cells. Our data indicated that pre-treatment with sub-toxic doses of rotenone and antimycin A efficiently blocked the production of IFNγ as well as LAMP-1 expression, suggesting that the activity of mitochondrial complex I and complex III are critical to induce oxidative signals necessary for the production of IFNγ and cytotoxicity in NK cells.

Cells have developed a system consisting of antioxidant proteins which provide a reducing environment in the cells. Among these antioxidant proteins, the thioredoxin-1 (Trx1) and glutaredoxin (Glrx) enzymes are known to regulate multiple cellular processes, such as gene expression, antioxidant response, apoptosis, and proliferation (212). The Trx1 system has been studied in regard to the expansion of T cells. Notably, the deletion of thioredoxin reductase 1 (*Txnrd1*) prevented the expansion of the activated T cell population during viral and parasitic infections (298). However, the role of these antioxidant systems in regulating immune cell function in vivo is currently poorly understood. Here, I investigated the role of Glrx2 in NK cell effector functions. Glrx2 provides the first line of defense against protein oxidation or misfolded protein aggregation. In addition, the deletion of Glrx2 increases cellular ROS levels, making the cells more sensitive to oxidative stress (189). Therefore, as a model to study the role of oxidative stress in regulating immune cell function and activation,

I investigated the immune cell function in *Glrx2*-deficient mice. *Glrx2*-deficient mice have never been subjected to an investigation for immune responses. In this report, I determined that the proportions of immune cells including NK, T and B cells are comparable between *Glrx2*-deficient mice and control WT mice (C57BL/6N), which were kindly provided by Dr. Mary-Ellen Harper at the University of Ottawa. Interestingly, the function of NK cells including IFNγ production, LAMP-1 expression and proliferation, as well as the activation and proliferation of T cells upon stimulation with various cytokines or through activating receptors were significantly increased in NK cells from *Glrx2*-deficient mice. To determine whether ROS signaling is involved in mediating the outcome of immune cell response, ROS levels in immune cells were measured and were highly induced in *Glrx2*–deficient mice, suggesting that the function and activation of NK and T cells are controlled by the increased levels of ROS in *Glrx2*-deficient mice.

To exclude the effect of genetic variations other than the *Glrx2* gene, I generated F2 littermate mice (*Glrx2wt/wt, Glrx2wt/ko, Glrx2ko/ko*) by breeding a WT mouse with a *Glrx2*-deficient mouse, and mating the *Glrx2wt/ko* progeny together to generate all three genotypes. Unexpectedly, when I investigated the function and activation of NK and T cells via stimulation with different cytokines, no difference was observed among the three genotypes. Since Glrx2-deficient cells are sensitive to oxidative stress, I decided to induce intensive stress by infecting *Glrx2wt/wt, Glrx2wt/ko, Glrx2ko/ko* mice with MCMV. Consistent with in vitro data from various stimulations, the immune cell function of NK cells during both acute and persistent MCMV infection was similar between control mice and mice deficient in *Glrx2*.

One possible explanation for this discrepancy is that there are well known metabolic differences between C57BL/6N (B6N) or C57BL/6J (B6J) mice that have been identified (299). B6J mice carry a mutation in the nicotinamide (NAD) nucleotide transhydrogenase (*Nnt*) gene, that is not found in other B6 backgrounds. NNT is a mitochondrial protein that catalyzes the reduction of NADP+ to NADPH. NADPH is a reducing agent which detoxifies ROS in the mitochondria to maintain efficient ATP synthesis (300). The *Nnt* mutation was identified when researchers found that the insulin secretion and glucose tolerance in response to glucose challenge was different between B6J and C3H/HeH mice (299). Although our colony of WT mice originally came from Charles River and were B6N, we also received WT mice from Jackson which are B6J. It is unknown whether the WT parent mouse used in this breeding scheme is under a B6N or B6J background. Unfortunately, the breeding colony has been terminated, making it impossible for me to track down the substrain of B6 mice. The *Glrx2*-deficient mice were under the B6N background, but it is possible that the WT mice used for breeding were under the B6J background. It has been shown that *Nnt* mutation results in mitochondrial redox abnormalities, as exemplified by increased ROS levels and decreased amounts of NADP and glutathione in their reduced form (301). As a result, the basal oxidative stress in B6J mice is relatively high as compared with that of B6N mice. Therefore, the contribution of Glrx2 deletion in generating increased ROS levels may be masked by the increased ROS caused by the *Nnt* mutation. In other words, whether or not the mice express the *Glxr2* gene, the difference in oxidative stress might be outweighed by the *Nnt* mutation. These results suggest that breeding the mice using WT mice under the B6N background is required to determine whether the *Nnt* mutation can be responsible for nullifying the effect of

Glxr2 deletion. Thus, further study is warranted to determine the interaction of Glxr2 and Nnt in the ROS regulation.

Alternatively, it is possible that the Trx system can compensate for the absence of Glrx2. Interestingly, the proliferation and development of embryonic fibroblasts, cardiomyocytes, and hepatocytes is unaffected in the absence of Txnrd1 (302), and that is due to the redundancy provided by the Glrx2 system (303). Altogether, our findings demonstrated that ROS can be induced upon different stimulations and its generation is required for IFNγ production and LAMP-1 expression, indicating a link between the levels of ROS and NK cell effector functions (Fig. 3.7).

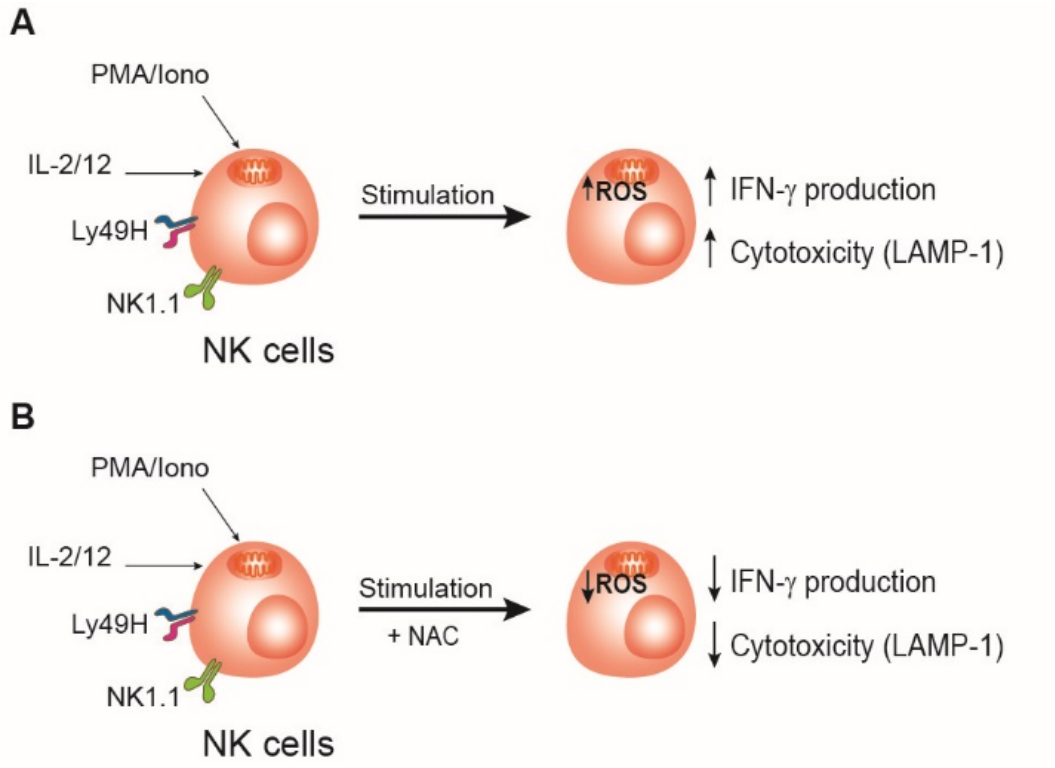

Figure 3.7. The role of ROS in NK cell effector functions upon different stimulations. (A) ROS levels are increased in NK cells upon different stimulations (PMA/Iono, IL-12/12 and through NK1.1 and Ly49H receptors) resulting in increased IFNγ production and LAMP-1 expression. (B) ROS levels are decreased in NK cells upon NAC treatment resulting in decreased IFNγ and LAMP-1 levels.

Materials and methods

Mice and MCMV infection

C57BL/6N and *Glrx2*-deficient mice were kindly provided by Dr. Mary-Ellen Harper (University of Ottawa). C57BL/6 and *Glrx2*-deficient mice were breed to obtain *Glrx2wt/wt, Glrx2wt/ko, Glrx2ko/ko* littermate mice. All mice were housed in a specific pathogen-free environment. The Glrx2-sufficient and Glrx2-deficient alleles were genotyped using the following primers: Forward primer (located in intron 2): 5'-GACCTAGCCTACCAGACTTGGCTGAAATTTATTC-3'; Reverse primer: 5'-CAT AGACACTCTTCACTTTCAAGCCCACCCTC-3'; Neo primer: 5'-CCTACATTTTGAATG GAAGGATTGGAGCTACGG G-3'. The PCR cycling conditions were as follows: 5 min at 94°C , then 30 cycles of 30 sec at 94°C, 1 min at 63°C , 1 min at 72°C, and 7 min at 72°C. All mice used for experiments were aged between 6 to 12 weeks old. All procedures were approved by and conducted in accordance with the institution's animal guidelines of the University of Ottawa. Smith strain MCMV stocks were generated in our laboratory from the salivary glands of infected BALB/c mice. C57BL/6 and Grx2-deficient mice were infected with 10,000 PFUs MCMV intraperitoneally for 3 and 6 days to induce an acute infection and with 5,000 PFU for 3 months to induce a persistent infection.

Free radical-sensitive probe carboxy-H2DCFDA (DCF) staining

Splenocytes were transferred into a 96 well v-bottom plate, washed with warm PBS and centrifuged at 1200rpm for 5min at room temperature. The cells were resuspended in RPMI no phenol red (Sigma-Aldrich) with 10μM of H2DCFDA (Sigma-Aldrich) and incubated for 10 min at 37°C. The cells were stimulated with the specific stimuli for 30 min and analyzed by spectrophotometry using excitation wavelength at 488 nm and recording

emission at 525 nm. For flow cytometric analysis, 0.25µl/Ill of Dead Fixable Blue Dead Cell Stain was added and the plate was placed on ice for 25 min, before proceeding immediately to acquisition by flow cytometry.

Measuring ROS production in lymphocytes and NK cells

Spleens were harvested, and a single-cell suspension was generated following red blood cells lysis and filtration through a 70-µm filter. Cells were labeled with DCF for 10 minutes in RPMI no phenol red medium at 37°C in the presence of recombinant human (rh)IL-2 (1000 U/ml; National In-stitutes of Health/National Cancer Institute at Frederick Biological Re-sources Branch Preclinical Repository), followed by treatment with different concentrations of N-acetyl cysteine (NAC) (Sigma-Aldrich) (1 mM, 5 mM, and 10 mM) in RPMI no +phenol red medium for 30 min at 37°C. Cells were then stimulated for 4 hours with hydrogen peroxide (H_2O_2) solution (Sigma-Aldrich), recombinant murine (rm)IL-12 (50ng/ml; eBioscience) or phorbol 12-myristate 13-acetate (100ng/ml) and ionomycin (1µg/ml) (PMA/Iono) and then analyzed by spectrophotometry. To measure ROS induction upon stimulation of NK cells, spleen cells were labeled with H2DCFDA for 10 minutes followed by treatment with NAC for 30 min. Cells were then stimulated for 1 hour with IL2/12 or PMA/Iono. Cells were washed once with RPMI no phenol red medium and stained with α-NK1.1 and α-TCRβ and incubated for 25 min 37°C. Cells were washed once with RPMI no phenol red medium and labeled with DCF for 10 min. Cells were analyzed by flow cytometry.

IFN-γ production and LAMP1 expression upon in vitro stimulation and NAC treatment

Spleens were harvested and treated with or without NAC for 30 minutes and cultured in RP-10 medium on Ab-coated plates (α-NK1.1 and α-Ly49H) or stimulated with rhIL-2 (1000 U/ml), mIL-12 (50ng/ml), rmIL-18 (50ng/ml; PeproTech) and PMA/Ionomycin. After 1 h of stimulation, brefeldin A and LAMP-1 was added to a final concentration of 5mg/ml, and cells were incubated for 4 h followed by staining for intracellular IFN-γ. To determine the mitochondrial ROS, cells were treated with different concentrations of rotenone (0, 31.25ng, 62.5ng, 125ng, 250ng and 500ng), an inhibitor of complex I or different concentrations of antimycin A (0, 7.8ng, 15.6ng, 31.25ng, 62.5ng and 125ng), an inhibitor of complex III following the stimulation.

In vitro assay for NK cell and T cell proliferation

Splenic leukocytes were resuspended at 2×10^7 cells/ml in PBS and mixed with an equal volume of 20 μM of Cell Proliferation Dye eFluor® 450 in PBS while vortexing the cells. Cells were incubated for 20 min at room temperature in the dark. Labeling was stopped by adding 4-5 volumes of cold complete medium (containing 10% serum) followed by incubation on ice for 5 minutes. Cells were washed 3 times with complete medium, and counted. Cells were then cultured in 96-well plates in triplicates in RP-10 medium with rhIL-2 (1000 U/ml) for 4 days.

Flow cytometric analysis

The following mAbs were used: anti-CD3 (145-2C11), anti-TCRβ (H57-597), anti-CD8 (53-6.7), and anti-CD49b (DX5) from eBioscience, anti-CD19 (1D3), anti-CD4 (RM4-5), anti-F4/80 (T45-2342), anti-NK1.1 (PK136), anti-CD107a (1D4B), anti-CD25 (PC61),

anti-IFNγ (XMG1.2), and anti-Ki-67 (B56) from BD Biosciences, and Live/Dead Fixable Blue Dead Cell Stain from Invitrogen. Intracellular staining of Ki-67 was carried out using a Foxp3 staining kit (eBioscience). The intracellular staining of IFNγ was performed using BD Cytofix/Cytoperm protocols (BD Biosciences). Cells were acquired using BD LSRFortessa or BD FACSCelesta and analyzed using Kaluza 1.3 Analysis software (Beckman Coulter) or FlowJo (Tree Star).

Statistical analysis

The mean values in the experiment were tested by ANOVA. If the ANOVA rejected the null hypothesis of the same means among the conditions ($p < 0.01$), multiple comparisons were performed between selected pairs of means by two-tailed unpaired t-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$) using Prism Version 5 (GraphPad Software).

Chapter 4: General Conclusion

Conclusion

The immune system provides a defence mechanism against pathogens to control infections. To achieve these functions, immune cells will increase their bioenergetics processes which result in the generation of ROS as signalling molecules and control the cellular metabolic pathways. This work contributes to better understand the link between metabolism and immune cell responses during inflammation and infection in chapter 2 as well as the role of ROS in enhancing immune cell effector functions in chapter 3.

In chapter 2, I have shown that IL-18 is involved in upregulating amino acid transporters and transferrin receptor on NK cells to increase the nutrient accessibility. The ability of amino acid to regulate mTOR signaling is critical for mTORC1 to integrate growth factor signals (304). The function of amino acid transporters upstream of mTORC1 allow the cells to sense amino acid availability which is involved in the anabolic responses such as increasing growth. The role of IL-18 in inducing amino acid transporters in NK cells to support their effector functions have not been identified previously. The upregulation of amino acid transporters occurs mainly on NK cells in response to IL-18 due to the high expression of IL-18R. Our data showed that the system L amino acid transporter CD98/LAT1 is increased upon IL-18 stimulation and treating the cells with leucine results in the activation of mTORC1 in NK cells.

mTORC1 pathway has been known to induce upregulation of amino acid transporters. Notably, I demonstrated that the upregulation of CD98 upon IL-18 treatment in the presence of rapamycin is a non-redundant pathway which is independant of mTORC1 pathway, suggesting that there are multiple pathways involving in CD98 upregulation on immune cells during infection or inflammation.

During immune cell proliferation, extensive metabolic regulations must be in place in order for the cells to uptake sufficient nutrients such as amino acids, which are necessary to support cell growth. CD98 supports immune cell activation by allowing the transport amino acids inside the cells (141). CD98 regulates an increase in the intracellular concentration of L-glutamine which is used as an efflux substrate to enable the uptake of extracellular L-leucine which leads to activation of mTORC1 (141). Here, I showed that treating NK cells with IL-18 enhanced the glutamine efflux and leucine uptake and blocking one of the system L transporter components failed to activate mTORC1. In addition, IL-18 stimulation upregulates CD98 which might increase NK cell proliferation by sustaining mTORC1 activity. The proliferative cytokines IL-2/15 signaling will induce moderate increase in CD98 expression and mTORC1 activity in NK cells. However, IL-18 signaling strongly increases the expression of CD98/LAT1, allowing enhanced uptake of amino acids, leading to sustained activation of mTORC1. Therefore, our finding demonstrated that IL-18 is a critical cytokine which induces elevated level of nutrient transporters on activated NK cells which sustain mTORC1 activity.

Surprisingly, during MCMV infection, IL-18 signaling was not required to upregulate the nutrient transporters on NK cells. Using different mouse models such as *caspase-1*-deficient mice and *Il18r1*-deficient mice to evaluate the importance of IL-18 during infection, I showed that the levels of CD98, CD71, glucose uptake and proliferation in NK cells from these mice were comparable with those in wild type mice during MCMV infection. Taken together, this data suggests that there are multiple redundant pathways involved in the upregulation of nutrient transporters. In fact, signaling through the activating receptor Ly49H that allows NK cells to directly recognize MCMV-infected cells can also upregulate the

nutrient transporters. However, by using *Ly49h*$^{-/-}$ *and Ly49h*$^{-/-}$*Il18r1*$^{-/-}$ mice, I determined that both Ly49H and IL-18 signaling are dispensable for the induction of nutrient transporters in NK cells during MCMV infection.

The deletion of CD98 or LAT1 in T cells resulted in developmental defects and reduced clonal expansion, suggesting the importance of the nutrient transporters for immune cells during inflammation (148, 262). In this study, I showed that blocking CD98/LAT1 with BCH inhibitor blocked the activity of mTORC1 as well as the production of IFNγ and granzyme B in NK cells. The preactivation of NK cells with IL-18 has been shown to maintain their effector function and proliferation after adoptive transfer in vivo (226), and the mechanism for sustaining the proliferation in vivo has been shown (305). Our study showed the unique function of IL-18 in changing NK cell metabolism by increasing the nutrient transporters and induction of leucine-driven mTORC1 activity.

ROS are known to cause a wide variety of human diseases (273), but can be essential for biological functions, including cell survival, cell growth, proliferation, differentiation, and immune responses. Any disruption between the oxidation and the antioxidant system will lead to oxidative stress. In chapter 3, I showed that ROS production is required for immune cell effector functions. Treating the cells with H_2O_2 will induce the production of IFNγ in NK cells. Activating NK cells will increase the production of ROS and blocking the level of ROS by using the antioxidant NAC will result in the inhibition of IFNγ as well as the expression of LAMP-1. mROS have been shown to play a critical role in regulating the induction of IL-2 and IL-4 in T cells as well as the proliferation (209, 210). Here, I showed that blocking mROS from both ETC complexes leads to the inhibition of IFNγ and LAMP-1 expression.

Studies have shown discordant effects of the antioxidant system on immune cells function. Trx1 system is critical for T cell expansion (298) and Glrx1 is not required for the development of B cells (306), suggesting that the role of antioxidant enzymes is cell type-dependent. Glrx2-deficient mice are known to be sensitive to oxidative stress and were used to study the role of the antioxidant system in the cells (189). Here, I showed that Glrx2 is not necessary for IFNγ production, cytotoxicity, and proliferation in NK and T cells in the steady-state. During acute and persistent infection with MCMV, the absent of Glrx2 did not affect the function and development of immune cells, suggesting that Glrx2 is dispensable for NK cell effector function.

Concluding remark

The metabolic reprogramming of cancer cells is associated with disease progression and is considered as the key mechanism of cancer's resistance to treatment. Cancer progression can be more aggressive when the function and metabolic activity of immune cells are compromised. Therefore, understanding the metabolic profiles of cancer cells and immune cells is important for identifying mechanisms that can favor the metabolic activity of immune cells over cancer cells.

The activation of immune cells by different signals such as cytokines or ROS results in dramatic reprogramming of their cellular metabolism. Understanding these processes will enable us to identify metabolic pathways that are shared between the target cells and immune cells and will allow the selection of metabolism-targeting drugs to control the infection and to be used as immunotherapy.

The major goal of immunotherapy is generating tumor-specific immune cells with enhanced function. As metabolic changes are known to drive immune cell differentiation and

alter the phenotype and the function of these cells, combining metabolism-targeting drugs with checkpoint inhibitors will result in enhancing the differentiation of tumor-specific immune cells to generate better immune effector cells (307).

The activation of immune cells, through the activating receptors in NK cells or through the TCR in T cells, stimulates mitochondria and generates ROS. The level of activation-induced ROS can affect the downstream functions of immune cells, including proliferation, differentiation, and survival. Therefore, controlling the level of ROS through cellular antioxidant pathways is important to maintain proper immune cells mediated immunity (308). Understanding these pathways may lead to novel treatments for many diseases.

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Curriculum Vitae

Saeedah Musaed Almutairi

PhD Candidate

Department of Biochemistry, Microbiology, and Immunology
Faculty of Medicine, University of Ottawa

Language skills

English and Arabic (spoken and written)

Education

2014 – Present **PhD student**, Program in Microbiology and Immunology
University of Ottawa, Ottawa, Ontario, Canada

2010 – 2013 **MSc student**, Master of Science, program in Medical Microbiology
University of Manitoba, Winnipeg, Manitoba, Canada
Overall GPA of 3.5/4

2003 – 2007 **BSc student**, Program in Botany and microbiology
King Saud *University, Riyadh, Saudi Arabia*
Overall GPA of 4.29/5 with the second class honors degree

Publications

1. Alaa Kassim Ali, Amandeep Kaur Komal, **Saeedah Musaed Almutairi**, and Seung-Hwan Lee. (2019) Natural killer cell-derived IL-10 regulates T cell response to prevent liver damage during sustained murine cytomegalovirus infection. *Front Immunol.* 10: 2688.

2. **Saeedah Musaed Almutairi***, Alaa Kassim Ali*, William He, Doo-Seok Yang, Peyman Ghorbani, Lisheng Wang, Morgan D Fullerton and Seung-Hwan Lee. (2019) Interleukin-18 up-regulates amino acid transporters and facilitates amino acid-induced mTORC1 activation in natural killer cells. *J Biol Chem.* **294**: 4644-4655. *Equal contribution (Article was selected to appear in a special virtual issue on "Immunometabolism" at *JBC* as part of the exciting advances in immunometabolism in recent years)

Oral presentations

- **Saeedah Musaed Almutairi**, Alaa Kassim Ali, William He, Doo-Seok Yang, Peyman Ghorbani, Lisheng Wang, Morgan D. Fullerton, and Seung-Hwan Lee. IL-18 upregulates amino acid transporters and facilitates amino acid-induced mTORC1 activation in natural killer cells. At the CIACCO, May 2019.

- **Saeedah Musaed Almutairi**, Alaa Kassim Ali, William He, Doo-Seok Yang and Seung-Hwan Lee. IL-18 upregulates nutrient transporters and facilitates amino acid-induced mTORC1 activation in natural killer cells. At 31th Annual spring Meeting of the Canadian Society for Immunology, June 2018.

- **Saeedah Musaed Almutairi**, Alaa Kassim Ali, William He, Doo-Seok Yang and Seung-Hwan Lee. IL-18 upregulates nutrient transporters and facilitates amino acid-induced mTORC1 activation in natural killer cells. At The CIACCO, May 2018.

- **Saeedah Musaed Almutairi**, Alaa Kassim Ali, and Seung-Hwan Lee. IL-18 increases nutrient transporter on natural killer that is essential for proliferation during infection. At The BMI Seminar Day, February 2018. **Won 3rd place**

- **Saeedah Musaed Almutairi**, Alaa Kassim Ali, Mary-Ellen Harper, and Seung-Hwan Lee. The Role of Reactive Oxygen Species in Immune Cell Function. At The CIACCO, May 2017.

- **Saeedah Musaed Almutairi**, Alaa Kassim Ali, Mary-Ellen Harper, and Seung-Hwan Lee. Reactive Oxygen Species as Signaling Molecules in Immunity. At 30th Annual spring Meeting of the Canadian Society for Immunology, April 2017.

- **Saeedah Musaed Almutairi**, Alaa Kassim Ali, Mary-Ellen Harper, and Seung-Hwan Lee. Reactive Oxygen Species as Signalling Molecules in Immunity. At The BMI Seminar Day, May 2014.

Poster presentations

- **Saeedah Musaed Almutairi**, Alaa Kassim Ali, William He, Doo-Seok Yang, Peyman Ghorbani, Lisheng Wang, Morgan D. Fullerton, and Seung-Hwan Lee. IL-18 upregulates amino acid transporters and facilitates amino acid-induced mTORC1 activation in natural killer cells. At 32th Annual spring Meeting of the Canadian Society for Immunology, April 2019.

- **Saeedah Musaed Almutairi**, Alaa Kassim Ali, William He, Doo-Seok Yang and Seung-Hwan Lee. IL-18 upregulates nutrient transporters and facilitates amino acid-induced mTORC1 activation in natural killer cells. At 31th Annual spring Meeting of the Canadian Society for Immunology, June 2018.

- **Saeedah Musaed Almutairi**, Alaa Kassim Ali, William He, Doo-Seok Yang and Seung-Hwan Lee. IL-18 upregulates nutrient transporters and facilitates amino acid-induced mTORC1 activation in natural killer cells. At The BMI symposium day, May 2018.

- **Saeedah Musaed Almutairi**, Alaa Kassim Ali, and Seung-Hwan Lee. IL-18: A Key Cytokine Linking Metabolism to Immunity in Natural Killer Cells. At The BMI Seminar Day, May 2017. **Won 2nd place**

- **Saeedah Musaed Almutairi**, Alaa Kassim Ali, Mary-Ellen Harper, and Seung-Hwan Lee. Reactive Oxygen Species as Signaling Molecules in Immunity. At 30th Annual spring Meeting of the Canadian Society for Immunology, April 2017.

- **Saeedah Musaed Almutairi**, Alaa Kassim Ali, Mary-Ellen Harper, and Seung-Hwan Lee. Reactive Reactive oxygen species as signaling molecules in immunity. At The McGill University Research Center on Complex Traits (MRCCT), November 2016.

- **Saeedah Musaed Almutairi**, Minjun Kim, Alaa Kassim Ali, and Seung-Hwan Lee. Metabolic reprogramming of IL-2 expanded NK cells by glutamine deprivation for cancer immunotherapy. At The CIACCO, May 2016. **Won 3rd place**

- **Saeedah Musaed Almutairi**, Minjun Kim, Alaa Kassim Ali, and Seung-Hwan Lee. Metabolic reprogramming of IL-2 expanded NK cells by glutamine deprivation for cancer immunotherapy. At 29th Annual spring Meeting of the Canadian Society for Immunology, April 2016.

- **Saeedah Musaed Almutairi**, Alaa Kassim Ali, Mary-Ellen Harper, and Seung-Hwan Lee. Reactive Reactive oxygen species as signaling molecules in immunity. At The BMI Poster Day, May 2015.

Trainings

May 2007 – July 2007

Summer training at National Guards Hospital, *Riyadh, Saudi Arabia*

- Office Skills:
- Entry and retrieval of patients' information in the computer database
- Maintaining patient relations through information exchange
- Laboratory Skills:
- Withdrawal of blood and analysis of blood samples

June 2006 – August 2006

Summer training at King Saud Chest Diseases hospital, *Riyadh, Saudi Arabia*

- Streaked samples (Urine and Ear, Throat, and Vaginal swabs)
- Prepared bacterial media (Blood and Chocolate agar)

- Gram stained slides
- Performed Antibiotics sensitivity tests
- Used Biochemical tests to identify bacterial strains

Honours and awards

2018 – 2019 Received International Doctoral Scholarship from the University of Ottawa
2018-02 Won Third place as a presenter at the BMI seminar Day 2017 at uOttawa
2017-05 Won second place as a poster presenter at the BMI seminar Day 2017 at uOttawa
2016-05 Won third place as a poster presenter at on Cytokines in Inflammation, Ageing, Cancer and Obesity (CIACCO-2016)
2008 – 2013 King Abdullah Scholarship Program