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Regulation of intracellular calcium levels as a tool to control NK cell cytotoxicity

Regulace hladiny intracelulárního vápníku jako nástroj kontroly cytotoxicity NK buněk

Diploma thesis

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Prague, 2024

I hereby declare that my thesis represents my own original research work. Wherever the contribution of others is involved, every effort is made to indicate this clearly including reference to the literature. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

Prague, 29.4.2024 Bc. Vojtěch Graman

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// Abstract

NK cells, a subset of innate lymphoid cells, play a crucial role in recognizing and eliminating virally infected or cancerous cells, making them a promising cell-based immunotherapy for AML patients. However, NK cell-based immunotherapies face unforeseen efficacy problems. Intracellular Ca^{2+} signalling was shown to play a crucial part in NK cell cytotoxicity. Maintaining the intricate balance of intracellular Ca^{2+} signalling is vital for NK cell-mediated target cell killing. In the complex microenvironment of the patient's body, NK cells encounter various stimuli, which can potentially disrupt the balance of intracellular Ca^{2+} signalling. Stimulation of PRRs was shown to affect intracellular Ca^{2+}, further influencing overall NK cell cytotoxicity. This study investigated the impact of TLR stimulation on Ca2+ signalling and NK cell functions. The effect of TLR stimulation was assessed using Ca^{2+} influx measurement, functional cytotoxicity, and degranulation assay, as well as gene expression analysis. Exposure to TLR ligands resulted in elevation of intracellular Ca^{2+} levels, accompanied by a reduction of cytotoxic activity at low effector-to-target ratios. An increasing trend in degranulation was observed. Furthermore, gene expression analysis unveiled upregulation of NFAT and Orai1 in NK cells stimulated with TLR ligands. These findings suggest that chemotherapy-induced overexpression of DAMP molecules, may disrupt the Ca^{2+} homeostasis of adoptively transferred NK cells, impairing their cytotoxic activity, and contributing to the suboptimal outcomes of NK cell immunotherapy for AML patients.

Key words: AML, NK cell, Ca^{2+}, TLR, cytotoxicity, chemotherapy, immunotherapy

Abstrakt

NK buňky, podskupina přirozených lymfoidních buněk, hrají klíčovou roli při rozpoznávání a eliminaci virem infikovaných a rakovinných buněk, což z nich činí slibnou buněčnou imunoterapii pro pacienty trpící AML. Imunoterapie založené na NK buňkách však čelí nepředvídaným problémům s účinností. Ukázalo se, že intracelulární Ca^{2+} signalizace hraje klíčovou roli pro cytotoxicitu NK buněk. Udržování složité rovnováhy intracelulární Ca^{2+} signalizace je životně důležité pro NK buňkami zprostředkované zabíjení cílových buněk. V komplexním mikroprostředí těla pacienta se NK buňky setkávají s různými podněty, které mohou potenciálně narušit rovnováhu intracelulární Ca^{2+} signalizace. Ukázalo se, že stimulace PRR ovlivňuje intracelulární Ca^{2+} a tak i celkovou cytotoxicitu NK buněk. Tato studie zkoumala dopad stimulace TLR na signalizaci Ca^{2+} a funkce NK buněk. Účinek stimulace TLR byl hodnocen měřením influxu intracelulárního Ca^{2+}, funkčního cytotoxicitckého a degranulačního testu, stejně jako analýzy genové exprese. Expozice TLR ligandům měla za následek zvýšení intracelulárních hladin Ca^{2+}, doprovázené snížením cytotoxické aktivity v nízkých poměrech efektorů k cílovým buňkám. Byl pozorován rostoucí trend v degranulaci. Kromě toho analýza genové exprese odhalila zvýšení exprese NFAT a Orai1 u NK buněk stimulovaných TLR ligandy. Tato zjištění naznačují, že nadměrná exprese DAMP molekul vyvolaná chemoterapií může narušit homeostázu Ca^{2+} adoptivně transferovaných NK buněk, snížit jejich cytotoxickou aktivitu a přispět tak k suboptimálním výsledkům NK buněčné imunoterapie u pacientů trpící AML.

Klíčová slova: AML, NK buňka, Ca^{2+}, TLR, cytotoxicita, chemoterapie, imunoterapie

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Abbreviations

aAPC	*artificial antigen presenting cell*
ADCC	*Antibody-dependent cellular cytotoxicity*
AIM2	*Absent in melanoma 2*
AML	*Acute myeloid leukemia*
AP	*Adaptor protein*
Bid	*BH3 interacting-domain death antagonist*
BM	*Bone marrow*
CAR	*Chimeric antigen receptor*
CRS	*Cytokine release syndrome*
CTL	*Cytotoxic T lymphocyte*
DAP10	*Hematopoietic cell signal transducer*
DAP12	*Transmembrane immune signalling adaptor*
DAMP	*Damage (Danger)-associated molecular patterns*
DAG	*Diacylglycerol*
DISC	*Death-inducing signalling complex*
DOCK	*Dedicator of cytokinesis*
EFG	*Epidermal growth factor*
ER	*Endoplasmic reticulum*
ERK	*Extracellular signal-regulated kinases*
Fas	*FS-7 associated surface antigen*
FDA	*U.S. Food and Drug Administration*
GM-CSF	*Granulocyte-macrophage colony-stimulating factor*
Grb2	*Growth factor receptor bound protein 2*
GvHD	*Graft versus host disease*
GvL	*Graft versus leukemia*
HLA	*Humal leukocyte antigen*
HPC	*Hematopoietic progenitor cell*
HSC	*Hematopoietic stem cell*
ICAM	*Intercellular adhesion molecule*
IFN	*Interferon*
IL	*Interleukin*
ILC	*Innate lymphoid cells*
IRF	*Interferon regulatory factor*
IS	*Immune synapse*
ITAM	*Immunoreceptor tyrosine-based activation motif*
ITIM	*Immunoreceptor tyrosine-based inhibitory motif*
KIR	*Killer-cell immunoglobulin-like receptor*
LAMP	*Lysosomal-associated membrane protein*
LFA	*Lymphocyte function-associated antigen*
LG	*Lytic granule*
LPS	*Lipopolysaccharide*
LSC	*Leukemic stem cell*
LYST	*Lysosomal trafficking regulator*
MAPK	*Mitogen-activated protein kinase*
MACPF	*Membrane attack complex/perforin*
MEK	*Mitogen-activated protein kinase kinase*
MHC	*Major histocompatibility complex*
MTOC	*Microtubule organizing centre*
MyD88	*Myeloid differentiation primary response 88*
NCR	*Natural cytotoxicity receptor*
NEMO	*Nuclear factor-κB essential modulator*
NFAT	*Nuclear factor of activated T cells*
NF-kB	*Nuclear factor kappa B*
NK	*Natural killer cell*
Nod-like receptor	*Nucleotide-binding oligomerization domain-like receptor*
Orai1	*Calcium release-activated calcium channel protein 1*
PAMP	*Pathogen-associated molecular pattern*
PBMC	*Peripheral blood mononuclear cell*
PD-1	*Programmed cell death 1*

PIP2	*Phosphatidylinositol 4,5-bisphosphate*
PLCγ	*Phospholipase C gamma*
PRR	*Pattern recognition receptor*
ROS	*Reactive oxygen species*
SET	*SET nuclear proto-oncogene*
SOCE	*Store-operated calcium entry*
SPL-76	*Lymphocyte cytosolic protein 2*
STIM	*Stromal interaction molecule*
Syk	*Tyrosine-protein kinase SYK; Spleen tyrosine kinase*
TGFβ	*Transforming growth factor-β*
TIGIT	*T cell immunoreceptor with Ig and ITIM domains*
TIR	*Toll-interleukin-1 receptor homology domain*
TNF	*Tumour necrosis factor*
TRIF	*TIR domain containing adaptor molecule 1*
TLR	*Toll-like receptor*
TME	*Tumour microenvironment*
Tyr	*Tyrosine*
Vav-1	*Vav guanine nucleotide exchange factor 1*
WASP	*Wiskott-Aldrich syndrome protein*
WAVE	*Wiskott-Aldrich syndrome protein family member 2*
WIP	*WAS/WASL-interacting protein*
Zap70	*Zeta-chain-associated protein kinase 70*

1 Introduction

1.1 NK cells

Natural killer (NK) cells are a highly specialized subset of lymphocytes, belonging to the innate lymphoid cells 1 (ILC1) family and are generally regarded as part of the innate immune system. They play a crucial role in surveillance and suppression of tumour cells as they can directly kill transformed or viral-infected cells (Maldonado-Bernal & Sánchez-Herrera, 2020), which lack HLA-I molecules or express ligands for NK cells activating receptors. NK cells are also able to sense microbial products via the expression of pattern recognition receptors (PRRs) and even present regulatory functions (Sivori et al., 2014). NK cells are potent producers of cytokines and chemokines recruiting other cells of innate and adaptive immune system and maintaining proinflammatory environment. The activation of NK cells differs whether they are activated by direct recognition of the target cell or by cytokine signalling. What divides NK cells from T lymphocytes is that the NK cells do not need prior sensitization to kill their targets (Maldonado-Bernal & Sánchez-Herrera, 2020).

NK cells are derived from the $CD34^{+}$ and mature into fully functioning cells in bone marrow (BM). Before maturation, NK cells undergo a process known as "licensing", where self-specific KIRs interact with self-MHC class I to achieve full functionality. Licensed NK cells exhibit heightened recognition and responsiveness toward MHC-I-specific tumour cells. Although the precise mechanism of licensing remains unclear, it has been understood to be an ongoing process, which can be altered by changes in the microenvironment. Conversely, unlicensed NK cells demonstrate increased responsiveness to targets expressing high levels of MHC-I, employing this as a strategy for immune evasion (Tu et al., 2016). After maturation, NK cells migrate to various sites and tissues of the body, and their function varies according to the individual microenvironment. They are defined by the expression of surface markers as $CD56^{+}$ (NCAM) $CD16^{+/-}$ (Fcγ-RIIIA) $CD3^{-}$ and $CD19^{-}$. However, NK cells showcase receptor plasticity according to their microenvironment. According to the expression of CD56 we can divide the NK cells into two major subpopulations: NK $CD56^{Bright}$ and NK $CD56^{Dim}$ (**Figure 1**). NK $CD56^{Bright}$ subpopulations represent only 10% of total peripheral blood NK cells and are generally regarded as regulators. They are characterized by poor cytotoxic activity and mainly by the production of cytokines such as IFN-γ, TNF-α, IL-5, IL-10, IL-13, and chemokines such as MIP-1α/β and RANTES. They are not generally found in peripheral blood but are mainly distributed in secondary lymphoid nodules (Maldonado-Bernal & Sánchez-Herrera, 2020). The NK $CD56^{Dim}$ subpopulations present 90% of peripheral blood NK cells. They exhibit potent

cytotoxic activity and cytokine secretion upon activation. CD56^{Dim} NK cells are generally defined by a CD16^{+}, KIR^{+} and NKG2A^{+} phenotype, capable of performing antibody-dependent cellular cytotoxicity (ADCC). This subpopulation is mainly found in peripheral blood and in inflamed tissue and presents potent cytokine production upon activation (De Maria et al., 2011; Sivori et al., 2014). The innate functions of NK cells make them an attractive target for cell-based immunotherapies directed towards the treatment of haematological malignant disorders (Xu & Niu, 2020).

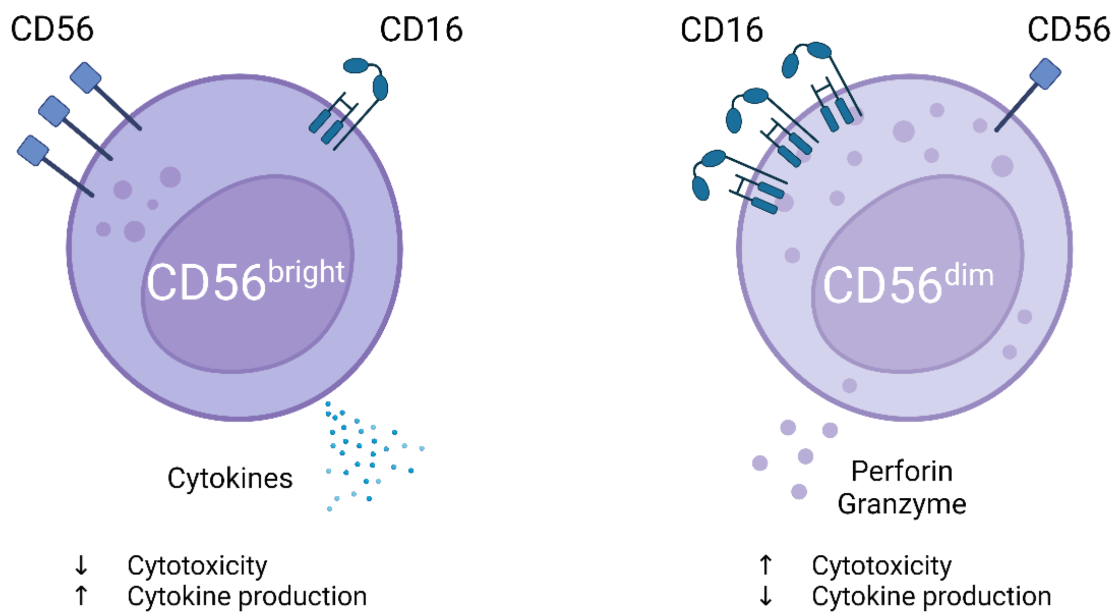

Figure 1 – Natural killer (NK) cell subpopulations based on CD56 and CD16 expression
Schematic summary highlighting key markers and effector functions defining NK CD56^{Bright} and NK CD56^{Dim} subpopulations (van Eeden et al., 2020).

1.2 Acute myeloid leukemia

Acute myeloid leukemia (AML) is a highly aggressive and heterogenous hematological cancer with an overall 5-year survival rate at only 28%. Furthermore, novel therapies are frequently lacking in effectiveness. AML is characterized by the accumulation of molecular and cytogenic mutations within hematopoietic stem (HSCs) and/or progenitor cells (HPCs), leading to the establishment of leukemic stem cells (LSCs). LSCs give rise to leukemic blasts that accumulate in the BM, displace normal HSCs, impair normal hematopoiesis, and spread to the peripheral blood and other organs such as spleen, liver, lymph nodes, testicles, and CNS. Individual patient survival depends on a number of factors ranging from age and gender to, chemotherapy or radiotherapy regimen to genetic factors. A hallmark of AML progression is significant shift of BM towards tumour-promoting microenvironment that supports and protects

LSCs and supresses invading immune cells (De Kouchkovsky & Abdul-Hay, 2016; N. A. Long et al., 2022; Prada-Arismendy et al., 2017). Several approaches have been designed, to harness the power of NK cells for the treatment of AML (Xu & Niu, 2020).

Even though NK cells seem to be an ideal effector cell for immunotherapy, the efficacy of treatment using NK cells is still rather low. Thus, novel approaches to current therapy or completely new protocols are desired to further improve patient care (Kaweme & Zhou, 2021). NK cell-based therapies are fully dependent on cytotoxicity and degranulation, processes that are tightly regulated via Ca^{2+} signalling. It has been shown that Ca^{2+} levels can be influenced by TLR signalling and since NK cells need to pass through proinflammatory environment, full of potential stimuli during adoptive transfer, this project chose to concentrate on the role of intracellular Ca^{2+} in connection with TLR signalling.

1.3 Cytotoxicity mechanisms

NK cells mediate target cell lysis via two distinct mechanisms. They induce death receptor-mediated apoptosis by expressing TRAIL and/or Fas ligand (FasL) or they release lytic granules, containing cytotoxic molecules such as perforin, granzyme and granulysin. The latter is a heavily regulated process initiated by the triggering of activating receptors (Prager & Watzl, 2019). Stimulation of these receptors results in an influx of intracellular Ca^{2+}, a crucial step subsequently leading to cytoskeletal rearrangements, exocytosis of lytic granules and target cell killing (Kaschek et al., 2021; Paul & Lal, 2017; Zhou et al., 2018).

1.3.1 Activating and inhibitory signals

The activation or inhibition of NK cells is regulated by an array of receptors that are germline-encoded and independent of RAG-mediated recombination (Lanier et al., 1986; Paul & Lal, 2017). Stimulation and subsequent activation or inhibition of NK cells depend on the balance of signals from two distinct types of receptors – activating and inhibitory receptors. Healthy cells express MHC class I molecules which are recognized by inhibitory receptors of NK cells, contributing to self-tolerance. On the contrary, viral infection or tumour development leads to cellular stress, DNA damage response, senescence program or tumour suppressor gene transcription. These processes lead to the upregulation of ligands for NK cell activating receptors and lower stimulation of inhibitory receptors. The signalling balance is shifted towards the activation of NK cells, resulting in cytokine production and NK cell-mediated lysis of the cell (Paul & Lal, 2017).

The most important regulation of NK cell activity is mediated by MHC-I specific inhibitory receptors, which triggers inhibition of cytotoxicity and activation (E. O. Long, 2008). Inhibitory

receptors signal through immunoreceptor tyrosine-based inhibitory motifs (ITIM) present in their cytoplasmic tails. Upon stimulation, the ITIM is phosphorylated and recruits phosphatases SHP-1, SHP-2 and SHIP. These phosphatases dephosphorylate ITAM bearing molecules and prevent downstream signalling of activating receptors (Paul & Lal, 2017). Upon binding to a cell, inhibitory receptors cluster at the immune synapse (IS). The level of inhibition corresponds with the amount of MHC molecules expressed on the bound cell. NK cell inhibitory receptors, in contrast to activating receptors, can signal independently. The signalling generally occurs through two main pathways. The first involves the engagement of SHP-1 and subsequent dephosphorylation of Vav-1 which is essential for Ca^{2+} mobilization, actin remodelling and synapse formation. The second pathway signals through the c-Abl mediated phosphorylation of the small adaptor protein Crk which dissociates from signalling complexes controlling cytoskeletal-remodelling. Crk is shown to be required for different steps of NK cell cytotoxicity (Kumar, 2018).

Killer cell immunoglobulin-like receptors (KIRs) represent one of the major families of human NK cell inhibitory receptors. These receptors recognise MHC class I molecules, precisely HLA-A, HLA-B and HLA-C, and bind to their peptide-binding region. KIRs are directly involved in the self-tolerance to healthy self-tissue. The second type of NK cell inhibitory receptors are CD94 (NKG2A) receptors binding to the non-classical HLA-E molecules (Paul & Lal, 2017). Signalling by inhibitory receptors also provides NK cells with proper responsiveness via a process termed licensing. NK cells lacking inhibitory receptors are hyporesponsive. Both types of receptors signals through the immunoreceptor Tyr-based inhibitory motif (ITIM) which recruits the Tyr phosphatases SHP-1 or SHP-2 (Kumar, 2018).

The lack of MHC class I molecules on the target cell surface is not sufficient for the activation of NK cell. Recognition of stress-induced molecules by NK cell activating receptors is required for full NK cell activation. As mentioned above, activating receptors can activate NK cell only in combination with other activating stimuli. The only difference is CD16, which alone can trigger NK cell degranulation (Bryceson et al., 2006, 2009). Majority of activating receptors signal through ITAMs. Receptor-ligand interaction leads to the phosphorylation of ITAM motifs on associated adaptor proteins (DAP10 or DAP12) by the Src family of kinases. This leads to the recruitment of tyrosine kinase Syk/Zap70, PI3K and the Grb2/Vav1/SPL-76 complex, resulting in the downstream activation of the MEK/ERK pathway. The recruited Syk kinase phosphorylates PLCγ which in response hydrolyses PIP_2 (phosphatidylinositol-4,5-bisphospate) and activates DAG pathway, leading to the activation of the NF-kB transcription factor. IP3 (inositol triphosphate) is also formed during PIP_2 hydrolysis, inducing the influx of intracellular

Ca^{2+} from the ER (endoplasmic reticulum) and subsequently activating the NFAT transcriptional factor. The result is the release of cytokines and chemokines as well as formation and release of cytotoxic granules by NK cells, leading to the lysis of the target cell (**Figure 2**) (Paul & Lal, 2017).

1.3.2 Calcium dependence of NK cell cytotoxicity

Stimulation of NK cell activating receptors induces the formation of an immune synapse, cytoskeletal rearrangements, and Ca^{2+} influx, all leading to the polarization of lytic granules towards the immune synapse and subsequent exocytosis (Galandrini et al., 2013; Martinet & Smyth, 2015). Ca^{2+} signals can be derived either by release from intracellular organelles or by influx of extracellular Ca^{2+} through Ca^{2+} permeable channels on the plasma membrane. It has been confirmed that Ca^{2+} signalling plays crucial role in NK cell cytotoxic activity. Strict Ca^{2+} dependence is a hallmark of NK cell-mediated cytotoxicity and granule secretion (Kaschek et al., 2021; Y. Li et al., 2022; Maul-Pavicic et al., 2011; Zhou et al., 2018).

As already stated, NK cells present an array of activating receptors carrying ITAM motifs capable of activating the NK cells upon stimulation. These receptors include the natural cytotoxic receptors and low affinity receptor FcyRIIA/CD16, another potent activating receptor, capable of interaction with the Fc part of antibodies. CD16 mediates recognition of opsonized target cells and induces antibody-dependent cellular cytotoxicity (ADCC), subsequently leading to the killing of opsonized cell either through the release of lytic granules or via death-receptor mediated killing (Ochoa et al., 2017). Triggering of these activating receptors leads to the activation of Vav1 and PLCγ. The PLCγ is critically involved in NK cell degranulation, cytotoxicity and cytokine signalling. PLCγ induces the cleavage of $PI(4,5)P_2$ located in the inner leaflet of the plasma membrane to create the membrane bound molecule DAG and soluble IP3. DAG induces the activation of NF-kB and Ras-MAPK pathway, while IP3 binds to the ER-localized IP3 receptor, inducing the release of the luminal store of Ca^{2+}. This release of Ca^{2+} ions is sensed by ER-localized proteins called STIM. These proteins, under normal circumstances, bind Ca^{2+}, but when most of the ions are released from the ER, STIM proteins dimerize and release bound Ca^{2+}, which activates them. They then interact with Orai1, a protein that forms a main part of plasma membrane-bound calcium release channel (CRACK), thus inducing the so-called store-operated Ca^{2+} entry, which is generally related to be the main entry route for Ca^{2+} ions (**Figure 2**) (Kaschek et al., 2021; E. O. Long et al., 2013). Orai1 forms a dominant Ca^{2+} channel, allowing the influx of extracellular Ca^{2+} into the cytosol of the NK cell (Maul-Pavicic et al., 2011). This Orai1-STIM1 system was found to be the main source of Ca^{2+} entry in CTL and

NK cells (Hogan et al., 2010). Mutations in both ORAI1 and STIM1 are connected with severe immunodeficiency, exhibited by defects in degranulation (Feske, 2009; Feske et al., 2006; Picard et al., 2009). Maul-Pavicic et al. (2011) demonstrated that STIM1-activated Orai1-mediated Ca^{2+} influx was required for NK cell cytotoxicity. Mice double knock-down of STIM1 and STIM2 completely abolished Ca^{2+} entry and revealed the importance of extracellular Ca^{2+} concentration for anti-tumour cytotoxicity. However, NK cell degranulation is still present even in the absence of extracellular Ca^{2+}, which highlights the importance of intracellular Ca^{2+} signalling for NK cell cytotoxicity (Freund-Brown et al., 2017). The rise in intracellular Ca^{2+} concentration affects various cellular processes, including the activation of different enzymes and proteins involved in F-actin cytoskeletal dynamics and the activation of the transcription factor NFAT which leads to the expression of IFN-γ and other pro-inflammatory molecules (Kaschek et al., 2021).

Furthermore, it has been demonstrated that maintaining an optimal level of Ca^{2+} is crucial for NK cell cytotoxicity. Zhou et al. (2018) observed a bell-shaped curve in the relationship between Ca^{2+} concentration and NK cell cytotoxic activity. This indicates that NK cells exhibit optimal effector function within a narrow range of Ca^{2+} concentrations. Deviations from this optimal range can impede NK cell-mediated killing of target cells. Notably, this phenomenon holds true for both extracellular and intracellular Ca^{2+} levels and Ca^{2+} influx, emphasizing the strict dependence of NK cell function on Ca^{2+} regulation (Zhou et al., 2018).

Keeping that in mind it was shown that enhanced Ca^{2+} influx led to unbalanced vesicle release, which further led to impairment of NK cell cytotoxicity. This means that enhanced Ca^{2+} signalling skew the regulation of lytic granule release, leading to the expulsion of all granules towards the first encountered target cell. Consequently, NK cells release a large number of granules towards a small number of target cells ultimately leading to a decrease in killing ability. This pinpoints the fact that optimized Ca^{2+} entry is extremely important for balanced vesicle release and NK cell-mediated cytotoxicity as disruption of Ca^{2+} dependent cytotoxicity can be crucial in the context of cell-based immunotherapy (Y. Li et al., 2022).

Despite the plethora of studies conducted to show the severe effects that impaired Ca^{2+} signalling has on NK cell cytotoxicity and degranulation, there has been little discussion towards the effect of enhanced Ca^{2+} signalling on NK cell effector functions.

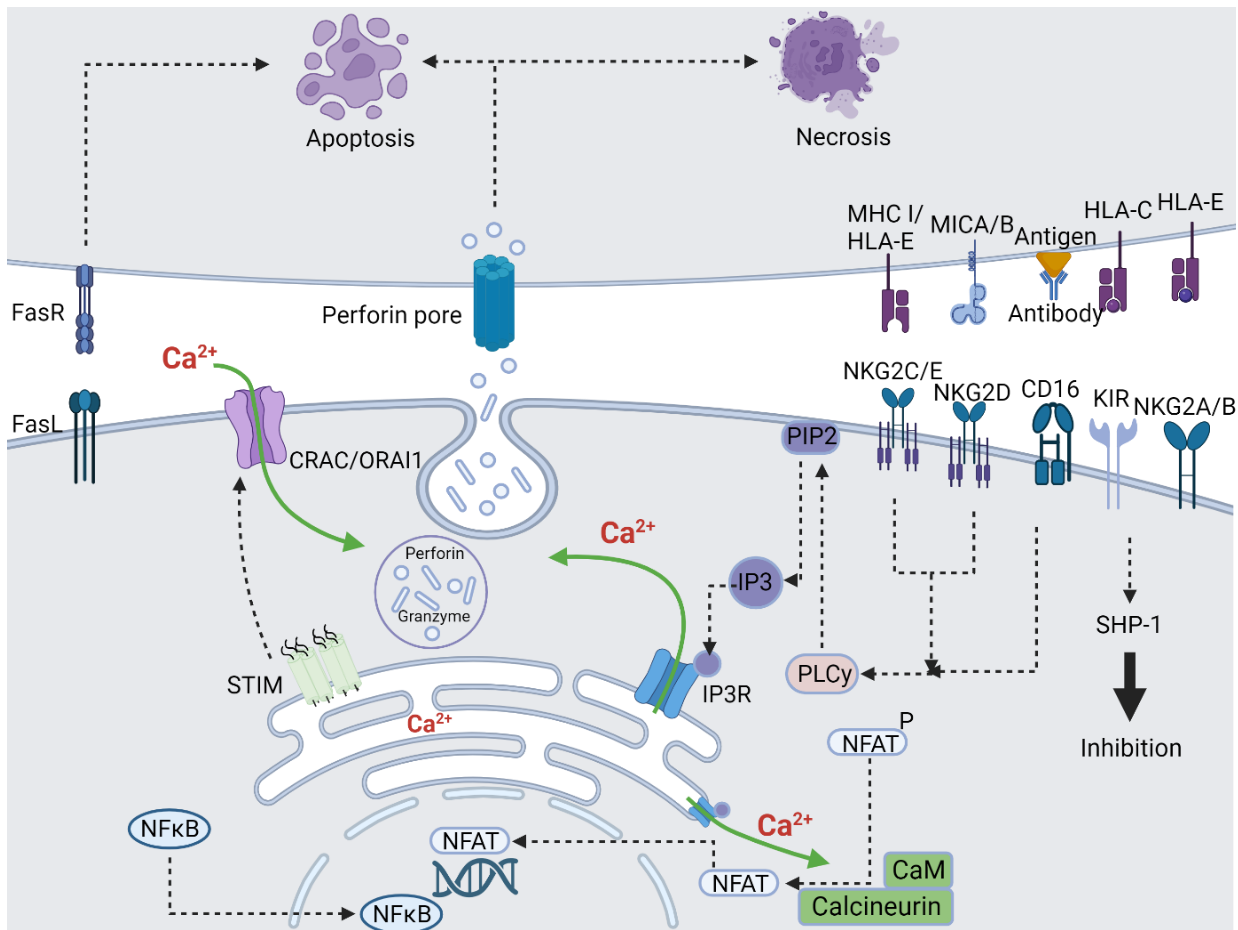

Figure 2 – Ca^{2+} as crucial mediator of NK cell degranulation
Activation receptor signalling initiates the activation of PLCγ, resulting in the hydrolyzation of PIP2 and formation of DAG and IP3. IP3 binds to its respective receptor on the endoplasmic reticulum, inducing release Ca^{2+} from the luminal stores. Decreases in Ca^{2+} levels are detected by STIM, prompting the interaction of STIM and Orai1, which initiates store-operated Ca^{2+} entry. This process leads to an increase in intracellular Ca^{2+} levels, subsequently triggering degranulation, and activation of the NFAT transcriptional factor (Kaschek et al., 2021).

1.3.3 Death receptors mediated cytotoxicity

As mentioned above NK cells can kill their target cells not only by lytic granule exocytosis but also by engaging the so-called death receptors. Death receptor-induced apoptosis of target cell is mediated by the induction of specialized surface molecules. We can distinguish three different receptor/ligand systems that can mediate cell apoptosis: TNF binding to TNF receptor -1 or -2, Fas ligand binding to CD95 (APO-1/Fas) receptor and TRAL interacting with different TRAIL receptors. NK cells can engage all three of these apoptosis inducing receptors which upon binding to their corresponding receptor on the target cell induce conformational changes and recruitment of adaptor proteins leading to the induction of apoptosis in the target cell (Paul & Lal, 2017; Prager & Watzl, 2019).

Fas ligand is a member of the TNF superfamily (Bodmer et al., 2002). It is expressed on the surface of NK cells and activated T cells. After the protein synthesis in the ER, it is stored in

secretory granules. These granules are distinct from the cytotoxic granules released during NK cell-mediated lysis of target cells, which means that the signal necessary for FasL externalization and surface expression differs from the signal inducing NK cell cytotoxic degranulation. FasL expression on the cell surface is short-lived as it quickly diffuses in the plasma membrane. The quick diffusion of FasL is constricted by LFA-1-mediated adhesion, increasing FasL's „life-span" on the surface of NK cell. After its externalization, FasL engages in multimerization of CD95 (APO-1/Fas) receptor on attached target cell. Interaction and multimerization of CD95 leads to activation of apoptotic signalling cascade, formation of death-inducing signalling complex and subsequent activation of caspases 8 and 10 (Prager & Watzl, 2019). Cleavage of FasL inhibits its cytotoxic activity and not only that, soluble FasL can in some cases inhibit apoptosis induced by membrane-bound FasL (Tanaka et al., 1998).

TRAIL exerts homology to FasL and to TNF (Bodmer et al., 2002). The expression of TRAIL on the surface of NK cells and T cells is induced by the presence and stimulation with IL-2, IL-15 or IL-12. TRAIL-mediated apoptosis was shown to be an important mechanism for NK cell-mediated lysis of viral infected cells. Unlike FasL, the process of TRAIL trafficking to the secretory lysosomes is unknown. It is also unclear, how binding of the target cell induces TRAIL expression inside the cytotoxic synapse. In the same manner as FasL, TRAIL can be cleaved from the cell membrane but unlike FasL, TRAIL retains its cytotoxic ability even in soluble form (Prager & Watzl, 2019).

The kinetics differ significantly when NK cells kill using lytic granules or when engaging the death-receptor mediated killing. Lytic granules are released within minutes of contact with the target cell, while death-receptor mediated killing can take up to 1-2 hours (J. Li et al., 2014). There are several possible explanations for what causes the different kinetics. As FasL and TRAIL are stored in different secretory molecules the timing scale of degranulation may differ. The externalization of CD107a, generally used as a marker of NK cell degranulation (Alter et al., 2004), can be seen within minutes of NK cell activation while accumulation of FasL on the cell surface was shown to start within 15-30 minutes after formation of the immune synapse (Prager & Watzl, 2019). It was also shown that the release of just 2-4 lytic granules is sufficient to induce lysis of target cell (Gwalani & Orange, 2018), but it may take longer to accumulate enough FasL molecules to fully stimulate CD95 and induce activation of apoptotic signalling. Here lies another reason. CD95 or TRAIL receptor stimulation induces multimerization of receptors, formation of DISC complex and subsequent assembly of larger signalling clusters ultimately leading to activation of caspase 8. Caspase 8 then activates caspase 3, inducing activation of the apoptotic program. Death-receptor signalling involves many regulated steps to sufficiently

induce cell death. On the other hand, granzyme B can directly target caspase 3, cleave it and induce very fast apoptosis of the target cell (Prager & Watzl, 2019).

1.4 Degranulation mechanism

The main mechanism of NK cell cytotoxicity is the direct secretion of lytic granules (LG) containing pore-forming and apoptosis inducing molecules. In this chapter we are going to cover the contents and molecular mechanisms regulating the development and secretion of lytic granules (Ham et al., 2022). Degranulation of lytic granules is a multistep process involving integrin-mediated adhesion to the target cell, formation of immunological synapse, granule convergence and polarization, and subsequent degranulation. Every step of this process is heavily regulated and controlled (Prager & Watzl, 2019).

Upon activation the NK cell induces the formation of so-called immune synapse (IS) with the target cell. This step is mediated by increased affinity interactions between integrins and their ligands on the surface of the target cell. Ultimately, NK cells secrete lytic granules directly towards the target cell in a process called cell-mediated cytotoxicity. Lytic granules are a specialized type of lysosomes containing apoptosis-inducing secretory proteins as well as lysosomal proteins. Therefore, LGs are also referred to as secretory lysosomes. In contrast to T-lymphocytes, NK cells constitutively express LGs, thus allowing NK cells to be primed for killing without any prior sensitization. These LGs, in the same manner as „normal" lysosomes, present an acidic environment with a pH between 5.1-5.4 and contain proteins with hydrolytic and degradative functions and common lysosomal soluble and transmembrane proteins like cathepsins and LAMP. Yet there are major distinctions between classical lysosomes and secretory LGs. The secretion process itself seems to be regulated by molecular machineries common to most cell types (Ham et al., 2022).

1.4.1 Cytotoxic molecules

NK cell LGs contain mainly apoptosis-inducing and pore-forming molecules such as perforin, granzymes and granulysin.

1.4.1.1 Perforin

A major component of NK cell lytic granules is a pore-forming protein called perforin. Perforin is synthesized as an inactive pro-enzyme in the ER and consists of 3 distinct domains: The N-terminal membrane attack complex perforin like (MACPF)-dependent cytolysin pore-forming domain, central epidermal growth factor (EFG) domain and C-terminal membrane and Ca^{2+} binding domain (Prager & Watzl, 2019). After synthesis perforin is transferred through the

Golgi to the LGs. The precise molecular mechanism by which perforin reaches the LG is not yet known but the LAMP1 (CD107a) and AP1 sorting complexes seem to play a crucial role in the transfer of perforin from trans-Golgi to the LG. Perforin undergoes proteolysis and glycosylation during its trafficking pathway. Perforin pore-forming activity is pH- and Ca^{2+} dependent since the binding is mediated by the calcium-binding C2 domain. The glycosylation is thought to inhibit perforin from oligomerization in the ER and Golgi where Ca^{2+} is viable and pH is neutral. Once it reaches LG, glycosylated C-terminal end is cleaved by cathepsin L and other proteases, forming an active form of perforin. After degranulation the perforin is no longer inhibited by lack of Ca^{2+} ions and acidic environment of LG and can oligomerize on the surface of the target cell, inducing the formation of perforin-mediated pores. These pores allow apoptosis-inducing proteins to enter the target cell and induce apoptosis as well as impose osmotic stress on the cell (Ham et al., 2022; Voskoboinik et al., 2015).

1.4.1.2 Granzymes

Granzymes are a family of apoptosis-inducing proteins belonging to the serine protease family commonly expressed in cytotoxic lymphocytes. We can distinguish 5 granzyme proteins, each exhibiting unique protease properties and substrate specificity: granzyme A, -B, -H -K and -M. Engagement of different granzyme proteins leads to the activation of a wide range of caspase-dependent and -independent apoptosis inducing pathways. However, most of our knowledge on the functionality of granzyme proteins comes from studying the mechanisms of granzyme A and B. NK cells release several different granzymes from their lytic granules and their individual functions will be briefly discussed (Prager & Watzl, 2019; Voskoboinik et al., 2015).

Granzyme proteins are synthetized as pro-enzyme molecules (zymogens). The zymogen contains signal peptide, which includes ER-specific signalling sequence guiding the pro-enzyme into the ER, and inhibitory dipeptide preventing the granzyme from reaching an active state (Voskoboinik et al., 2015). The pro-enzyme is translated into the lumen of the ER from where it is transferred to the cis-Golgi. Once there, it is further modified by adding mannose-6-phosphate (M6P) element. Through the induction of M6P receptor, the pro-enzyme is transferred to the endosome and finally to the LG (Griffiths & Isaaz, 1993). Granzymes are then modified to their active form by splicing off the inhibitory dipeptide via cathepsin C or H (D'Angelo et al., 2010; Meade et al., 2006). However, their activity is inhibited due to the low pH inside the granules and association with chondroitin sulphate proteoglycan serglycin (Raja et al., 2002).

Granzyme B is a serine protease with wide substrate specificity. The prominent targets for granzyme B are caspase-3 and caspase-7. Their cleavage results in activation and through series

of steps in caspase-dependent cell death (Adrain et al., 2005). In addition, granzyme B can also induce apoptosis by cleaving the BH3-only protein Bid, forming the so called tBid, which translocates into mitochondria and interacts with proteins Bax and/or Bak, causing disruption of mitochondrial membrane integrity, release of apoptotic factors and subsequent apoptosis of the cell (Heibein et al., 2000; Sutton et al., 2000).

Granzyme A presents a trypsin-like activity, inducing a fast form of cell death independent of caspase activity and resulting in the formation of large DNA fragments. Its activity induces the production of reactive oxygen species (ROS) from mitochondria, disrupting the mitochondrial electron transport and mitochondrial membrane potential. Human granzyme A was shown to express poor cytotoxic activity and may be more likely involved in promoting proinflammatory immune responses (Lieberman, 2010; Martinvalet et al., 2005).

Like granzyme A, granzyme K displays trypsin-like activity, inducing rapid caspase-independent cell death. Granzyme K activity induces ROS production, chromatin condensation and cleavage of the nucleosome assembly protein SET causing single-stranded DNA nicks. Granzyme K does not play an essential role in cell cytotoxicity, and it seems to be a stand-in option for granzyme A (Bouwman et al., 2021).

Granzyme M displays unusual enzyme specificity, inducing caspase-independent cell death by cleaving nucleophosmin, which is essential for cell viability. It was shown to also cleave and inactivate Serpin B9 (SB9), an inhibitor of granzyme B activity, showcasing that granzyme M promotes granzyme B-induced apoptosis. Granzyme M is predominantly expressed in NK cells, CD56+ T cells and γδ T cells (de Poot & Bovenschen, 2014; Mahrus et al., 2004).

Granzyme H is the last member of granzyme family, predominantly expressed in NK cells, and it’s shown to be homologous to granzyme B. Despite the homology it displays chymotrypsin-like activity. It was shown to interfere with viral replication in infected cells and inactivate viral 100K assembly protein, which inhibits the activity of granzyme B, thus promoting granzyme B-induced cell death. Apart from its anti-viral activities, granzyme H can induce cell death via depolarization of mitochondrial membrane, ROS induction, DNA degradation and chromatin condensation, which are independent of caspase Bid cleavage and do not induce cytochrome c release from mitochondria (Andrade et al., 2007; Fellows et al., 2007; Hou et al., 2008).

1.4.1.3 Granulysin

Granulysin is a part of the saposin-like protein family expressed in NK cells and pre-activated CTLs. It is synthetized as a precursor protein which is later cleaved into its 9-kDa

active form. The active form of granulysin exhibits pore-forming activity and is capable of inducing pores in the membranes of tumour cells, bacteria, fungi and parasites (Al-Wasaby et al., 2021; Krensky & Clayberger, 2009).

1.4.2 Lytic granule biogenesis and activation signals

The biogenesis of LGs is regulated by two main complexes: Adaptor protein 3 (AP3) complex and the CHS1/LYST protein. The involvement of AP3 complex seems to be crucial for the sorting of secretory lysosome-specific proteins such as LAMP (CD107a) and their trafficking from the endosome or trans-Golgi network. However, it is still unclear which exact proteins are dependent on AP3 regulatory functions (Peden et al., 2004; Robinson & Bonifacino, 2001). The CHS1/LYST protein is important for proper LG homeostasis and seems to regulate the fusion of lysosomes. Research on patients suffering from the mutations in CHS1/LYST protein showed that LGs gradually fuse together forming enlarged lysosomes impairing NK cell cytotoxicity (Chiang et al., 2017). Recent studies emphasize the importance of the optimal size and composition of LGs for efficient NK cell cytotoxicity (Gwalani & Orange, 2018), providing further evidence of the pivotal role of CHS1/LYST protein for LG homeostasis.

As already mentioned in a previous chapter activating receptor signalling triggers downstream signalling cascade, activating molecules Vav1 and PLCγ, leading to the release of Ca^{2+} from the ER and subsequent influx from the extracellular environment, which in turn influences F-actin cytoskeletal dynamics and activation of the transcriptional factor NFAT. The reorganization of the F-actin cytoskeleton is a crucial step for LG mediated cellular cytotoxicity. Activation of Vav-1 and DOCK2 or DOCK8 factors, induced by the stimulation of activation receptors, leads to the activation of Rho family of small GTP-binding proteins (Cdc42, Rac1 and RhoA), which regulates the activity of WASP and WAVE2 complexes, thus influencing the F-actin dynamics. Regulation of F-actin activity is critical for many steps in the development of NK cell-mediated cellular cytotoxicity including effects on the organization of the immune synapse (IS), clustering of activation receptors in the centre of the immune synapse, integrin-mediated adhesion, lytic granule convergence and the transit of lytic granules towards the IS. These signals ultimately lead to the formation of IS, LG convergence and microtubule organizing centre (MTOC) polarization towards the IS (Ham et al., 2022).

1.4.3 Immune synapse formation

Upon recognition of the target cell, NK cells induce the formation of cell-cell junction via the engagement of many transmembrane receptors with their respected ligands, thus forming a highly organised structure/connection called the immune synapse. NK cells require signalling

through the leukocyte function associated antigen-1 (LFA-1) to induce adhesion to the target cell. LFA-1 recognizes ICAM-1 molecules on the surface of the target cells. Stimulation of LFA-1 induces synapse formation, actin reorganization and granule polarization. Nevertheless, LFA-1 signalling alone, despite its ability to phosphorylate PLCγ and Syk kinase, fails to stimulate Ca^{2+} mobilization thereby is unable to trigger degranulation. The collective engagement of LFA-1 and a synergistic receptor pair represents the minimal requirements leading to NK cell degranulation (Kumar, 2018).

Stimulation of LFA-1 and other adhesion molecules initiates the formation of immune synapse into gasket-like structure limiting the diffusion of granular content, diminishing off-target escape of molecules and promoting direct granule-mediated cytotoxicity. During the formation of the immune synapse, adhesion molecules, including LFA-1, quickly segregate to the periphery of the synapse, thus creating a confined central zone for degranulation. There they contribute to adhesion as well as actin accumulation and polymerization. The accumulation of actin at the centre of the immune synapse is necessary for perforin accumulation and NK cell cytotoxicity. LFA-1 induction also controls the distribution of activation and inhibitory receptors in the immune synapse. The location of receptors changes upon LFA-1 depletion, suggesting a crucial role of LFA-1 in the organization of the immune synapse and the formation of organized receptor-ligand pairs (Kumar, 2018; Prager & Watzl, 2019).

The negative pH and the presence of Ca^{2+} in the immune synapse enable the aggregation of perforin monomers to form pores in the membrane of the target cell. The pore lumen is 13-20 nm which can be further increased by recruiting other perforin oligomers making it an easy entry site for granzyme B molecules that mediate apoptosis of the target cell (Law et al., 2010; Lopez, Jenkins, et al., 2013; Lopez, Susanto, et al., 2013).

1.4.4 Lytic granule trafficking

The first step of degranulation, after the formation of IS begins with the convergence of pre-synthesized LGs towards the MTOC. This process is initiated through the engagement of adhesion receptors such as LFA-1 in combination with other NK cell activating receptors. Convergence towards the MTOC not only prepares LGs for the direct secretion but also concentrates LG, thus minimalizing the off-target effects and ensuring sufficient LG secretion. The accumulation of LGs is dependent on dynein/dynactin interactions and movement along the microtubule network towards the MTOC. This process occurs independently of PI3K, MEK, and PLCγ signalling, taking place during the formation of the immune synapse, highlighting the significance of LFA-1 signalling. This implies that the convergence of LG is an early process

mediated by adhesion preceding commitment to cytotoxicity (James et al., 2013; Kuhn & Poenie, 2002; Mentlik et al., 2010; Orange, 2008).

Following LG convergence, the next step in LG trafficking is the polarization of MTOC with converged granules towards the maturing IS through F-actin reorganization. Polarization is initiated through signalling of clustered activating receptors after the commitment to kill the target cell. There are two proposed mechanisms towards how polarization of LG is mediated: The first is dynein-dependent cortical sliding mechanism, where dynein pulls on microtubules to bring the MTOC towards the IS; The second is a capture-shrinkage mechanism. In this case anchored dynein pulls on the microtubules causing their depolymerization which effectively pulls MTOC towards the IS. It is not clear which of these proposed mechanisms is utilized in the cell. Presently, the prevailing model assumes that both aforementioned mechanisms work in synergy. The regulation of LG polarization is thought to be conducted by several cytoskeletal regulatory proteins including small GTPase CDC42 which colocalizes with WASP to the MTOC after LG convergence (de la Roche et al., 2016; Hornak & Rieger, 2020; Orange et al., 2003; Stinchcombe et al., 2006).

After MTOC and LG polarization, the secretory lysosomes need to navigate through the dense F-actin network to finally interact and fuse with the plasma membrane releasing its cytotoxic cargo into the IS and towards the target cell. The proximity of LG is thought to be enough to offload the LG onto the F-actin network, however regulation of this process remains unclear. Movement of LG towards the plasma membrane is dependent on the non-muscle actin motor myosin IIA which is constitutively associated with LG through direct interaction with Rab25a or through the WASP/WIP complex. The interaction of myosin IIa with LGs is also dependent on the chaperone protein UNC-45A, which colocalizes with LGs and together they undergo polarization upon recognition of the target cell. UNC-45A increases binding of myosin IIA to the F-actin network and is crucial member for the trafficking of LGs towards the plasma membrane (Iizuka et al., 2015; Mace et al., 2012; Sanborn et al., 2009).

After the myosin IIA-mediated transfer, LGs dock at a plasma membrane and are prepared for the release towards the target cell. This process is mediated through small GTPase Rab27a which was shown to play a critical role for the last stages of NK cell degranulation. The function of Rab27a is mediated through the interaction with effector proteins such as Munc13-4. The crucial role of Rab27a is not restricted only for the regulation of LG docking, but also for the next step, the process called tethering. LGs make initial interaction with plasma membrane and are prepared for the final fusion. Tethering is mediated through Rab27a and interacting effector proteins Slp1, Slp2 and most importantly Munc13-4, which seems to be the crucial molecule

regulating LG tethering. Munc13-4 contains calcium-dependent C2-domains which facilitates binding with SNARE proteins and is thought to be a calcium sensor mediating local Ca^{2+} release, crucial for granule fusion (Bin et al., 2018; Feldmann et al., 2003; Holt et al., 2008; Ménasché et al., 2000).

Final steps of degranulation are mediated by the recruitment of SNARE complex proteins and formation of trans-SNARE complex. Afterwards, a fusion pore between LG and plasma membrane is formed and the content of granules is released finalizing NK cell degranulation (Ham et al., 2022).

1.4.5 NK cell protection from own lytic enzymes

Lytic granules are delivered to the plasma membrane where they release their cytotoxic molecules inside the immune synapse. Since these molecules are activated in the extracellular environment, the NK cells must protect themselves in order not to be killed. Firstly, NK cells express potent granzyme B inhibitor SB9 in their cytosol which may protect them from unintentional granzyme B entry . Secondly, it was shown that CD107a/LAMP1 molecule is exposed on the surface of the NK cell during exocytosis of lytic granules. CD107a inhibits perforin binding to the cell membrane and thereby avoids the cell damage. Therefore, CD107a is often used as a marker of NK cell degranulation. Another proposed mechanism is that cathepsin B, which also reaches the plasma membrane together with cytotoxic molecules, can inactivate perforin by cleavage and thereby inhibit its pore-forming activity. This mechanism can be utilized by some cancer cells that can secrete cathepsin B in order to protect them from granule-mediated cytotoxicity (Balaji et al., 2002; Bird et al., 1998; Cohnen et al., 2013; Khazen et al., 2016).

1.5 Toll-like receptors

PRRs are germ-line encoded proteins that recognize conserved pathogen associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). They stand at the start of the immune reaction and represent the key element of innate immune system, orchestrating the first recognition of immune stimuli and subsequent start of the immune response. Among these receptors we can find RIG-I-like receptors, Nod-like receptors, AIM2-like receptors, C-type lectin receptors, intracellular DNA and RNA sensors and Toll-like receptors which will be the focus of this chapter (Duan et al., 2022; Fitzgerald & Kagan, 2020).

Toll-like receptors are type I transmembrane proteins consisting of N-terminal domain formed by leucin-rich repeats, single transmembrane domain and cytosolic TIR domain which is highly similar to that of IL-1 receptor family. All TLRs are synthesized in the ER and then

transported to their destinations, either plasma or endosomal membranes. TLRs play a crucial role in the initiation of innate immune response, linking the innate and adaptive immunity (Duan et al., 2022; Fitzgerald & Kagan, 2020).

Upon recognition of their respected ligand, pathogen- or damage-derived molecules, TLRs initiates downstream signalling cascades via recruitment of adaptor proteins myeloid differentiation primary protein 88 (MyD88) or TIR domain containing adaptor molecule 1 (TRIF). Thus, activating nuclear factor kappa B (NF-kB) or/and IFN-inducing transcription factor IFN regulatory factor (IRF), leading to induction of inflammasome signalling pathways, production of inflammatory and antiviral cytokines, chemokines, defensins, type I interferons, co-stimulation and MHC molecules, and subsequent activation of adaptive immune cells (Duan et al., 2022).

TLRs are expressed on all innate immune cells and a large majority of non-hematopoietic cells, including NK cells (Duan et al., 2022). Apart from TLR-10 NK cells constitutionally express all human TLRs in various levels independently of the state of NK cell activation (Sivori et al., 2014). Receptors expressed by the NK cells work synergistically in order to activate, inhibit or regulate the NK cell activity according to the microenvironment, thus highlighting a role of TLRs. Along with other PRRs such as natural cytotoxicity receptors (NCRs), they are involved in the early response to pathogens, viral-infected cells or tumour cells. Stimulation of TLR signalling can induce NK cell-mediated killing of tumour/target cell and promote the production of pro-inflammatory cytokines and chemokines amplifying the immune response (Maldonado-Bernal & Sánchez-Herrera, 2020). TLR ligands can directly induce NK cell activation, cytokine production and even NK cell cytotoxicity (Noh et al., 2020), however it was shown that the microenvironment in which TLR-mediated NK cell activation occurs plays an important role in the manner of NK cell mediated cytotoxicity and in the regulatory functions of NK cells (Sivori et al., 2014).

1.5.1 TLRs and Ca^{2+} signalling

As already mentioned, Ca^{2+} signalling is crucial for NK cell degranulation and cytotoxicity among other physiological function that Ca^{2+} exhibits. Upon NK cell activation, Orai1 induces the store-operated calcium entry which was shown to regulate NF-kB transcriptional factor, regulating its activation and nuclear localization, and nuclear factor of activated T-cell (NFAT) (Berry et al., 2018). Incoming Ca^{2+} ions can interact with the molecule calmodulin, promoting its binding to the serine/threonine protein phosphatase enzyme calcineurin. This activates calcineurin, which dephosphorylate transcription factor NFAT. NFAT translocates into the

nucleus, where it promotes the expression of the genes further promoting NK cell activation, production of cytokines such as IFN-γ, TNF-α and IL-2, and it induces the expression of perforin and granzyme, promoting NK cell cytotoxic functions. NFAT signalling thus exerts a critical influence on NK cell effector function and is directly linked with Ca^{2+} signalling (Bendickova et al., 2017; Y.-J. Park et al., 2020).

It was found out that the engagement of PRRs can promote calcineurin-NFAT signalling (Goodridge et al., 2007), in particular certain TLRs, mostly mentioned is the engagement of TLR4 and TLR2. Thus, activation of TLR signalling is involved in the Ca^{2+} signalling and is capable of triggering Ca^{2+} mobilization. It was shown that TLR signalling can lead to the recruitment of Syk kinase and other signalling molecules, which subsequently activates PLCy followed by the increase of intracellular Ca^{2+} and activation of NFAT (Zanoni et al., 2009). Birla et al. (2022) has shown that Orai1 deficiency drastically decreased LPS-induced cytokine production, while simultaneously LPS-mediated TLR4 stimulation resulted in increased ORAI1 expression and increase in the basal level of Ca^{2+} and SOC after 3-4 hours of exposure. As we already covered very tight Ca^{2+} optimum is necessary for the NK cells to fully exert their effector functions and since TLRs can affect Ca^{2+} signalling, the question rises, how TLR stimulation does affect NK cell Ca^{2+} optimum and with that their ability to kill their target cells .

Adoptive transfer of NK cells is the most common type of NK cell-based immunotherapy for the treatment of Acute myeloid leukemia patients. This process is preceded by rounds of chemotherapy or radiotherapy and is usually followed by the transplantation of hematopoietic stem cells. NK cell immunotherapy options and protocols will be covered in more detail in the next chapter. Thus, NK cells are transferred to the patient who has already undergone chemotherapy treatment (Sivori et al., 2019). It was found out that chemotherapy or radiotherapy treatment induces the release of DAMPs from destroyed cells. The levels of DAMP proteins significantly increased following chemotherapy or radiotherapy treatment in comparison with healthy control patients. DAMPs act as a danger signal released by dying or necrotic cells but can also be actively secreted from cells upon external stimulation. These proteins are recognized and stimulate TLRs inducing signalling through TLR mediated pathways. Particularly, TLR2 and TLR4 are mainly triggered by a variety of endogenous DAMPs (Jang et al., 2020). Keeping that in mind, TLR signalling could be a crucial part of the NK cell immunotherapy problems, as it might be able to disrupt Ca^{2+} optimum in NK cells and thus disrupt their ability to efficiently kill their targets.

1.6 Clinical use of NK cells

Haematological malignancies represent heterogenous group of blood neoplasias, commonly characterized by abnormal production of blood cells. LSCs share many similarities with haematopoietic stem cells (HSCs) including their dependence on BM niches for their survival and resistance to chemotherapy, which can cause relapse and showcase limited treatment options (Méndez-Ferrer et al., 2020). Cancer immunotherapy works at a principle of activating body´s own immune system and targeting the reaction against cancer cells. In recent years the engagement of antibody- and cell-based protocols have become a hallmark of cancer immunotherapy (Hu et al., 2019). As already mentioned, NK cells are a crucial part of immunosurveillance in particular in the control of metastatic cells or hematological cancers. NK cells can detect and kill transformed cells and do not depend on specific neo-antigen recognition, making them an interesting target for cell-base cancer immunotherapy (Souza-Fonseca-Guimaraes et al., 2019). Thus, several protocols have been developed to harness innate power of NK cells for the treatment of patients suffering from leukemia or solid tumours (Sivori et al., 2021). NK cells are an attractive alternative to T cell immunotherapies since they preferentially target transformed cells and are able to kill without the need of prior sensitization (Souza-Fonseca-Guimaraes et al., 2019).

There are currently many different strategies designed to enhance cytotoxicity of NK cells against tumour cells (Ham et al., 2022). However, NK cell-based therapies face limitations. Cancer cells employ variety of escape strategies directed to delay, alter or even stop anti-tumour immunity (Hu et al., 2019). Cancer cells can evade NK cell detection by dysregulating the intricate balance between activating and inhibitory signalling, to allow the disease progression and metastasis to vital organs (Souza-Fonseca-Guimaraes et al., 2019). NK cells also present poor ability to reach tumour tissues, making them not viable for the treatment of solid tumours (Melero et al., 2014). Thus, most of NK cell-based protocols so far are directed against haematological malignancies. Further, the tumour microenvironment (TME) remains a major barrier for adoptive NK cells as it meddles with NK cell activation either through the expression of immunosuppressive cytokines such as TGFβ or interfering with receptor expression (Jedlička et al., 2022; Vitale et al., 2014).

To overcome these problems many strategies have been designed such as boosting effector function by immune stimulants, the adoptive transfer of *in vitro* expanded NK cells or transfer of genetically modified NK cells (Hu et al., 2019).

1.6.1 Adoptive transfer of NK cells

1.6.1.1 In vitro expanded NK cells

Adoptive therapies using CTL have been explored in the past decades and presently this approach has been used for the NK cells (Souza-Fonseca-Guimaraes et al., 2019). *In vitro* expanded NK cells, isolated from PBMCs, have been utilized in adoptive transfer as a form of cellular immunotherapy for AML patients undergoing hematopoietic stem cell transplantation. NK cells are commonly administered after the patient finished chemotherapy regimen (Hu et al., 2019). They offer several advantages in the adoptive cell therapy. Firstly, utilizing NK cells bypasses the necessity for antigen-specific T cells. Cytokine-release syndrome (CRS) and graft-versus-host disease (GvHD) represent major obstacles in adoptive T cell therapies. Clinical evidence suggests that allogenic NK cells are safe, with neither CRS nor GvHD being observed. Secondly, NK cells possess the ability to directly eliminate tumour cells and release proinflammatory cytokines, which can enhance the adaptive immune response. Lastly, NK cells are readily isolatable and modifiable, and they exhibit a comparatively short lifespan, reducing the likelihood of excessive expansion within the recipient's body (Paul & Lal, 2017; Souza-Fonseca-Guimaraes et al., 2019).

The source of NK cells for the expansion and subsequent transfer can be either from the patient itself (autologous) or isolated from healthy donor (allogenic). Transfer of allogenic NK cells is widely used as it mitigates the danger of NK cells being inhibited by the recognition of self-MHC molecules (Curti et al., 2011; Miller et al., 2005). Introduction of grafts with depleted αβ T-cells and B cells, but containing functioning NK cells can contribute to optimal graft-versus leukemia (GvL) effect mediated by donor NK cells, strongly reacting against HLA disparate leukemic cells, enhancing their clearance (Locatelli et al., 2018). Not surprisingly a plateau of studies using adoptive transfer of KIR-mismatched allogenic NK cells for the treatment of leukemia have been conducted (Mavers & Bertaina, 2018).

Culture of isolated NK cells together with cytokines such as IL-2 and IL-15 have been initially used in order to produce large numbers of alloreactive NK cells (H.-G. Klingemann & Martinson, 2004). However, this process fails to obtain desired number of functional cells. Subsequently, artificial antigen-presenting cells (aAPCs) were developed as feeder cells for the expansion of NK cells *in vitro*. This process in combination with cytokine infusion has been successfully utilized to manufacture large numbers of activated NK cells (Hu et al., 2019). However, it is important to consider that cytokine-prestimulation induces phenotypic changes that may influence trafficking and proliferation of NK cells (Terrén et al., 2018). Another option

is the use of clonal NK cell lines that have the ability to proliferate. The only FDA approved cell line so far, is the NK-92 cell line, derived from a patient suffering with a rare NK cell lymphoma. However, these cells need to be irradiated before adoptive transfer to mitigate their proliferation in the patient. NK-92 do not express CD16, thus is unable to be activated by the antibody-based therapy (H. Klingemann et al., 2016; Tonn et al., 2013). Third possible source are NK cells differentiated from human pluripotent stem cells (hPSCs), from umbilical cord blood (UCB), induced pluripotent stem cells (iPSCs) and human embryonic stem cells (hESCs). There is ongoing rapid research to fully harness these sources (Eguizabal et al., 2014).

1.6.1.2 CAR NK cells

Genetic modification of effector cells of the immune system is a promising approach to treat the advanced cancers that are refractory to conventional therapies. Thus, the introduction of chimeric antigen receptor (CAR) targeting cell surface antigen, can provide necessary targeting and specificity for otherwise „invisible" targets (Sivori et al., 2021). CAR is produced to transfer arbitrary antigen specificity to selected immune cell. They consist of an antigen-binding fragment fused to the T cell receptor and signalling components generating antigen-specific response (Souza-Fonseca-Guimaraes et al., 2019). CAR technology was originally applied on T cells and most of clinical data available today is from the use of CAR T cells which has become the predominant immunotherapy for number of malignant diseases (Maude et al., 2018; Neelapu et al., 2017; J. H. Park et al., 2018). Even though CAR T cells have made a revolution in the treatment of haematological disorders such as acute lymphoblastic leukemia and diffuse large B cell lymphoma, with FDA approved therapies (Pehlivan et al., 2018), there are still limitations. These are mostly connected to the toxicity of the CAR T cell therapy as hyperactive CAR T cells leads to side effects such as CRS and neurological toxicities (Brudno & Kochenderfer, 2016). Moreover, for the treatment of complex diseases such as acute myeloid leukemia the treatment has been less efficient as there is no specific antigen for the CAR T cells (Souza-Fonseca-Guimaraes et al., 2019).

CAR NK cells have become an interesting concept, linking NK cell innate ability to recognize malignant cells with specificity of CAR receptor. They are thought to possess several advantages in comparison with CTLs (Ham et al., 2022). Firstly, NK cells are easy to isolate and expand to large quantities and showcase relatively low life span, thus mitigating the danger of overexpansion of transferred cells. Secondly, NK cells produce mainly IFN-γ and GM-CSF, cytokines that are much less likely to induce cytokine release syndrome (CSR), a major complication connected with CAR-T cell therapy. Lastly, NK cells can recognize malignant cells

also with their innate receptors, thus NK cells are not obligated to rely solely on the targeting of CAR and can kill in CAR-dependent and -independent manner (Hu et al., 2019).

In recent years, studies has emerged, using iPSC as the source of NK cells for efficient off-the-shelf therapy, further improving CAR NK cell production and limiting donor variability (Y. Li et al., 2018). Introduction of these genetically enhanced lymphocytes is a great approach to further enhance potential of NK cell adoptive transfer, which may represent an efficient alternative to commonly used approaches while showcasing lower toxicity (Souza-Fonseca-Guimaraes et al., 2019). Yet, there are still substantial limitations with CAR-NK cells connected with the construction of functional CAR, the need for retroviral vectors and lack of targeting antigens in case of AML treatment (Hu et al., 2019).

1.6.2 Strategies amplifying NK cells effector functions

Mature NK cells isolated from peripheral blood of patients with AML display a low expression of major activating NK receptors, paralleled with an increased expression of CD94/NKG2A inhibitory receptors, low production of TNF-α and IFN-γ and an impaired cytolytic activity (Costello et al., 2002; Fauriat et al., 2005; Khaznadar et al., 2015; Stringaris et al., 2014). Thus, many strategies of immunotherapy either aim to boost immune activation by administration of cytokines and/or antibodies that help to improve anti-tumour immune response or trying to reverse immune suppression by targeting immune checkpoint molecules (Hu et al., 2019).

1.6.2.1 Cytokine-mediated activation of NK cells

Therapeutic approaches based on the use of cytokines able to directly stimulate and promote NK cell activation, persistence, and expansion have been tested in several preclinical and clinical studies (Sivori et al., 2021). Several cytokines such as IL-2, IL-15, IL-12, IL-18 and IL-21 have been used to enhance NK cell functions *in vivo*. Among these IL-2 and IL-15 stand out as the most used, showcasing the best clinical evidence (Paul & Lal, 2017).

IL-2 pioneered the use of cytokines in NK cell immunotherapy (Rosenberg et al., 1985); however, the use of IL-2 mediates unforeseen adverse reactions. The activation of vascular endothelium, leads to vascular leakage and organ injury and activation of Tregs, subsequently inducing immune suppression (Sivori et al., 2021).

Another cytokine that plays a crucial role in NK cell activation and is commonly used in therapeutical protocols is IL-15. IL-15 mediates NK cell activation without activation of Tregs and was shown to be potent mediator of NK cell antitumour responses (Sivori et al., 2021).

1.6.2.2 Antibody induced activation of NK cells

Apart from the use of cytokines to boost NK cell effector functions, several protocols harnessing the power of antibodies have been used, taking advantage of low affinity Fc receptor CD16, a major activating receptor on NK cells (Carotta, 2016). Tumour-specific monoclonal antibodies (mAbs) work by binding IgG Fc part with NK cell activating receptor CD16, inducing ADCC of opsonized cell. The effectiveness of mAbs to induce NK cell activation and antibody-dependent killing of the target cell have been proven. These molecules contain single-chain variable fragments (scFv) against both NK cell activating receptor and tumour associated antigen, drawing the target cell and NK cell in close proximity and inducing formation of immune synapse, resulting in efficient target cell killing (Hu et al., 2019). It has been demonstrated that CD16-directed bi-specific (CD16x19) and tri-specific (CD16x19x22) scFv agents directly stimulate NK cells and potentiate NK cell lytic activity and cytokine release (Bruenke et al., 2005; Gleason et al., 2012). Modified IL-15 crosslinker has been integrated into TriKE to enhance the survival and expansion of NK cells *in vivo* (Schmohl et al., 2016; Vallera et al., 2016). However, as the use of BiKEs and TriKEs have advanced the field of antibody-based therapies, there are still substantial limitations. For the treatment of AML, no clear marker has been found and thus the use of BiKEs and TriKEs face challenges in their ability to target NK cells towards myeloid blasts, enhancing NK cell capability to kill selected tumour cells (Hu et al., 2019).

Combination of antibody-based therapies together with TLR agonist in order to further enhance NK cell activation and effector function has recently been shown as efficient treatment option. Agonists against TLR2, TLR3, TLR7, TLR8 and TLR9 have been reported to have positive effect on *in vivo* NK cell activation and effector function against tumour cells or viral infection (Noh et al., 2020).

1.6.2.3 Immune checkpoint targeting

Different approach towards antibody-based NK cell immunotherapy is the targeting of immune checkpoints. Checkpoint-blockade therapies have revolutionized the field of immune therapies with first ones getting FDA approval in 2011. A number of studies is trying to harvest NK cell antibody-dependent cellular cytotoxicity together with checkpoint inhibitor therapies, which have been shown to be capable of enhancing tumour functions of tumour-infiltrating NK cells (Souza-Fonseca-Guimaraes et al., 2019). The purpose is to unblock NK cell inhibition induced by NK cell inhibitory receptors. Monoclonal antibodies blocking KIRs, major inhibitory receptors of NK cells, promoting lysis of MHC-I expressing tumour cells. Another target is the

inhibitory receptor NKG2A, which inhibition resulted in enhanced NK cell cytotoxicity against engrafted human primary leukemia *in vivo*. PD-1 and TIGIT are another inhibitory receptors that can be targeted by immune checkpoint mAb therapy to stop NK cell inhibition and promote killing of cancerous cells (Hu et al., 2019; Sivori et al., 2019, 2021).

2 Aims

Despite the potential of adoptive transfer of NK cells in treatment of AML, challenges in effectiveness persist. Intracellular Ca^{2+} signalling was shown to be a key player in regulation of NK cell cytotoxicity. Maintaining the delicate balance of intracellular Ca^{2+} signalling is vital for NK cell degranulation and effective cytotoxicity. NK cells express TLRs, which have been shown to induce downstream Ca^{2+} signalling pathways. Before adoptive NK cell transfer, the patients undergo chemotherapy regimen, inducing the release of DAMP proteins resulting in NK cells priming with a proinflammatory environment and an abundant PRRs stimuli. TLRs activation, inducing Ca^{2+} mobilization, disrupts the balance of Ca^{2+} signalling and subsequently leading to impairment of NK cell cytotoxicity. This project addresses the specific control of NK cells cytotoxic mechanism through following aims:

1) In the first part of the project, we aim to show that Ca^{2+} balance is extremely important to maintain effective NK cell cytotoxicity and that stimulation with certain TLR agonists can induce changes in intracellular Ca^{2+} levels of expanded NK cells.
2) Secondly, we hypothesise that the perturbation of Ca^{2+} levels is directly connected with disbalanced release of lytic granules leading to disruption of NK cell cytotoxicity.
3) Lastly, we aim to describe that exposure to TLR agonists can change NK cell metabolic activity and expression of genes connected to both effector functions and Ca^{2+} signalling.

Understanding the role of changes of intracellular levels of Ca^{2+} can ultimately lead to explanation to what is happening with the NK cells after the adoptive transfer and gives us insides into the mechanisms influencing the outcome of NK cell-based immunotherapy regiment.

NK cells are commonly transferred after the patient has finished his chemotherapy or radiotherapy regimen in order to boost efficacy of anticancer therapy. However, as we try to explain in this study, this can cause unforeseen disadvantages and affect efficacy of NK cell immunotherapy.

3 Materials and methods

3.1 Cell culture

3.1.1 Purification of NK cells from PBMCs

Human peripheral blood mononuclear cells were isolated from buffy coat obtained from healthy donors. The content of the buffy coat (30-60 ml) was transferred into 200 ml flask and diluted with phosphate-buffered saline with ethylenediaminetetraacetic acid (PBS-EDTA) to a final volume of 120 ml. Diluted blood is than carefully layered on top of 15 ml of Lymphoprep (Scintilla; 7861) in 50 ml falcon tube and centrifuged for 30 minutes (800 g, 20 °C, acceleration 5 and deceleration 0). The layer of PBMCs was then carefully transferred to new 50 ml falcon tube and PBS-EDTA was added to a final volume of 50 ml. The cells were centrifuged for 10 minutes (300 g, 20 °C, acceleration max, deceleration max). The pelleted cells were resuspended in 50 ml of PBS-EDTA and centrifuged for 20 minutes (200 g, 24°C, acceleration 9, deceleration 9). This step was repeated twice in total. Pelleted PBMCs were resuspended in PBS, counted using Automated Cell Counter TC20 (BioRad) using trypan blue dye (Trypan Blue Solution 20x, Merc Life Science; T8154-20ML) and prepared for immunomagnetic selection (Miltenyi Biotec).

The desired number of PBMCs (10^7 cells) was centrifuged (5 min., 300 g, 24 °C, acceleration 9, deceleration 9), resuspended in buffer (10^7 cells/40 µl), mixed with 10 µl of biotin-conjugated antibody cocktail and incubated at 2-8 °C for 5 minutes. After incubation 30 µl of buffer together with 20 µl of magnetic beads was added. The cells were incubated in 2-8 °C for another 10 minutes. NK cells were separated using AutoMACS (Milteneyi Biotec). After separation the negative population of NK cells was centrifuged (5 min., 300 g, 24 °C, acceleration 9, deceleration 9), resuspended in X-VIVO 20 media supplemented with gentamicin (Lonza; E04-448Q) and 5% of human serum (Capricorn, HUM-3B/20) and seeded.

3.1.2 NK cell expansion

Isolated NK cells were standardly seeded into 6-well plate (SARSTEDT; 83.3920.500) together with irradiated K562 (irK562) cells as feeders in ratio of 1:5. The used standard seeding concentration was $2x10^5$ NK cells to $1x10^6$ irK562 cells per 1 ml of complete X-VIVO 20 media in total volume of 2 ml. Complete X-VIVO 20 media was used for the whole cultivation. The standard length of NK cell expansion took 14 days. Cytokines were administered at the start of the cultivation and were added at each passage – 5 µl of IL-2 (200 IU/ µl. RnD; 202-IL-500) and

20 µl of IL-15 (1 ng/ µl, RnD; 247-ILB-025) per 1 ml of media. Cells were passaged every two days during the process of NK cell expansion as shown in **table 1.**

Table 1 – Passage schedule

No. of passage	Media taken [ml]	Media added [ml]	Cytokines added	Total volume
0	-	-	IL-2 + IL-15 per 2 ml	2 ml
1	-	1	IL-2 + IL-15 per 1 ml	3 ml
2	1	2	IL-2 + IL-15 per 2 ml	4 ml
3	2	2	IL-2 + IL-15 per 2 ml	4 ml
4	2	3	IL-2 + IL-15 per 3 ml	5 ml

3.1.3 K562 cell culture

Leukemic K562 cell line was purchased from American Type Culture Collection. IMDM media (Gibco; 12440061), supplemented with 10% Fetal Bovine Serum (Biosera; F9665-500ML) and 1% PS (Gibco; 15140122), was used for leukemic cell mono-cultures. The cells were passaged every two days to a maximum of 14 passages.

Irradiated K562 cells were used as feeder cells in a culture with NK cells. The K562 cells were irradiated with 100 Gy in a concentration of $3x10^5$ cells per ml. After irradiation the cells were cryopreserved in liquid nitrogen.

3.2 TLR ligands

TLR ligands were purchased from InvivoGen, reconstituted, and stored according to the manufacturer's instructions as shown in **table 2**.

NK cells were exposed to TLR ligands either directly during assay – Cytosolic Ca^{2+} influx measurement assay; or prior an individual assay for 18h, 3h and 1h respectively. TLR ligands were diluted to their working concentrations as shown in **table 2.**

Table 2 – Toll-like receptor ligands properties

Name	Specificity	CAS number (Cat. Code)	Reconstitution	Storage	Working concentration

Pam3CSK4	TLR2/1	tlrl-pms	1 mg/ml in endotoxin-free water	1 month in 4 °C; 6 months in -20 °C	10 ng/ml
PGN-SA	TLR2	tlrl-pgns2	200 µg/ml in endotoxin-free water	1 year in -20 °C	10 ng/ml
Pam2CSK4	TLR2/6	tlrl-pm2s-1	1 mg/ml in endotoxin-free water	1 month in 4 °C; 6 months in -20 °C	10 ng/ml
Poly A:U	TLR3	tlrl-pau	1 mg/ml in sterile physiological water	1 month in 2-8 °C; 1 year in -20 °C	100 µg/ml
CRX-527	TLR4	tlrl-crx527	1 mg/ml in DMSO	6 months in -20 °C	10 ng/ml
ODN 2395	TLR9	tlrl-2395	1 mg/285 µl in endotoxin-free water	6 months in -20 °C	35,24 µg/ml (5µM)

3.3 Calcium depletion treatment

BAPTA, AM, a cell-permeant Ca^{2+} chelator (ThermoFisher; B1205), was used to deplete intracellular Ca^{2+} levels. NK cells were treated with 50 µM BAPTA, AM for 20 minutes at 37 °C. After incubation the cells were centrifuged (5 min., 300 g, 20 °C, acceleration 9, deceleration9) and washed in PBS prior cytotoxic assay.

For the study of extracellular Ca^{2+} depletion, the NK cells were incubated in MEM suspension, no calcium, no glutamine (Gibco; 11380037) supplemented with 5% of human serum, 20 minutes prior cytotoxic assay. The cells were left in the media for the entirety of the assay; viz Calcein AM cytotoxic assay. The obtained data were analysed using excel and plotted in GraphPad prims 9.

3.4 Cytosolic calcium influx measurement assay

NK cells were loaded with 10mM FURA Red AM, a Ca^{2+} indicator dye (10mM; ThermoFisher; F3020, F3021), in X-VIVO 15 without Phenol red and Gentamicin (Lonza; BEVP02-061Q). FURA Red AM is excited at 406 nm and emits at 670 nm when bound to Ca^{2+} and excited at 532 nm and emits at 670 nm when unbound. The FURA Red ratio – fluorescence intensity of bound to Ca^{2+} /fluorescent intensity of unbound to Ca^{2+} - shows changes in intracellular Ca^{2+} levels. NK cells were stained with FURA Red AM at concentration of $4x10^6$ cells/ml and incubated for 30 minutes at 37 °C in the dark. After incubation cells were

centrifuged (5 min., 300 g, 20 °C, acceleration 9, deceleration9) and washed in PBS to remove the extracellular dye and afterwards resuspended in X-VIVO 20 (w/o phenol red) at concentration of $2x10^6$/ml and were kept at 37 °C in the dark. After staining cells were loaded (150 µl) into tempered 96-well black plate (Schoeller Pharma Praha s.r.o.; 237108) and fluorescence was measured at Spark reader (Tecan), also tempered to 37 °C. The reagents were added (50 µl) through the Spark reader (Tecan) injector system and tempered to ensure stable cell temperature. Addition of X-VIVO 15 media was used as a baseline negative control, and Ionomycin (1,5 µl/ml; Sigma-Aldrich; I9657-1MG), ionophore that raises cytoplasmic Ca^{2+} levels, was used as a positive control. Intracellular Ca^{2+} fluorescence intensities were recorded for 2 minutes and reagents were added after the initial 50 seconds. Each measurement was performed 3 times on 3 different donors. The time change of ration between bound (ex. 406 nm) and unbound (ex. 532 nm) FURA Red was normalized to 1 and plotted into a graph. The Ca^{2+} influx rate ($\Delta R/\Delta t$) was calculated 10 seconds (t_{10}) after addition of reagent (t_0) using the equation $(R[t_{10}] - R[t_0])/(t_{10} - t_0)$, with the R being the FURA Red ratio. The area under curve analysis was assessed using GraphPad prims 9 analytics function. All data was analysed using excel and graphs were plotted using GraphPad prims 9. All obtained data were in triplicates (n=3).

3.5 Calcein AM cytotoxic assay

The K562 target cells were stained with Calcein AM, which is a cell-permanent dye (Invitrogen; 1CH2-002113). The dye was diluted according to the manufacturer´s instructions. The cells were left to incubate with Calcein AM for 30 minutes in the dark in X-VIVO 20 media (without added serum). K562 cells were then washed twice in complete X-VIVO 20 media and left to rest another 30 minutes. Meanwhile effector NK cells were serially diluted into 96-well round-bottom plate (SARSTEDT; 83.3925.500) in triplicates for total amount of $45x10^3$, $15x10^3$, $5x10^3$ and $1,6x10^3$ cells per well. The target cells were then added to each well in total amount of $5x10^3$ cells per well. Cells were incubated for 4 hours in 37 °C. Complete X-VIVO 20 media was used as a blank. Target cell without the addition of effector cells was used as a control of spontaneous cell death. For maximal cell death control were used target cells that were heat-killed on a thermos block at 70 °C for 10 minutes. After the incubation 60 µl of supernatant was taken from each well and transferred into new 96-well flat-bottomed plate (SARSTEDT; 83.3924.500). The fluorescence was measured at Spark reader (Tecan; excitation 488 nm, emission 520 nm). Data were then processed by excel and GraphPad Prism9. All obtained data were in triplicates (n = 3).

3.6 Degranulation assay

The K562 target cells were stained with CellTrace™ Violet (Invitrogen; C34557), which is a cell labelling dye. The dye was diluted according to the manufacturer's instructions. The cells were left to incubate for 20 minutes in the dark. Whilst the target cells were being stained, the effector cells were loaded into 96-well round-bottom plate (SARSTEDT; 83.3925.500) in triplicates at total amount of $25x10^4$ cells per well. After incubation, targets cells were washed once with PBS and added to desired wells at total amount of $5x10^4$ cells per well creating a 5:1 effector: target cell ratio. After 1 hour of incubation, Ionomycin (1,5 µl/ml; Sigma-Aldrich; I9657-1MG) and anti-CD107a antibody (2,5 µl/ well) were added. Cells were left to incubate for another 2 hours for total of 3-hour incubation. Then were transferred to FACS tubes, stained with LIVE/DEAD dye, and analysed using flow cytometry at Cytek Aurora (Cytekbio). Final data were analysed with FlowJo (BD). All obtained data were in triplicates (n = 3).

3.7 SeaHorse metabolic assay

For the measurement of bioenergetics parameters, cells were pretreated with TLR ligands in the same manner as described above. 24 hours prior the assay the plates were loaded with poly-D-lysine (20 µl/well; Gibco; A3890401), rehydrated with Agilent Seahorse XF Calibrant (200 µl/well, 103059-000) and incubated in thermostat without CO_2. After 24-hour incubation, the Seahorse XF Cell Mito Stress Test (HPST; 103010-100) was performed using Seahorse XFp Analyser (Agilent Technologies, Santa Clara, CA, USA). Test was performed using pre-treated expanded NK cells using manufacturer protocol. Untreated expanded NK cells were used as a control. The data were analysed using Agilent Seahorse Analytics website and GraphPad Prism9.

3.8 RNA isolation and qPCR

3.8.1 RNA isolation

RNA was isolated from minimum of $3x10^6$ cells. Cells were centrifuged (5 min., 400 g, 25 °C, acceleration 9, deceleration 9) in 1,5 ml Eppendorf tubes. The pellets were resuspended in 350 ml of TRIzol reagent (Invitrogen; 15596018) and stored in -80 °C or processed immediately. For RNA isolation the Quick-RNA MagBead kit (Zymo Research; R2132) was used according to the manufacturer´s instructions. RNA was eluted using 50 µl of DNase/RNase free water and measured using NanoDrop One (ThermoFisher). The samples were stored in -80°C or processed immediately.

3.8.2 Reverse transcription

The reverse transcription was assessed using 150 ng per reaction of isolated RNA in 0,2 ml Eppendorf tubes. RNA samples were diluted in RNase-free water to a desired volume of 11 µl per reaction. In the first step, 1 µl of 50 µM Oligo(dT)20 primer (Invitrogen; 18418020) and 1 µl of 10 mM dNTP mix (Invitrogen; 10297018) were added to each sample to form a final volume of 13 µl. The RNA-primer mixtures were inserted into Thermal Cycler (Biometra) and heated to 65 °C for 5 minutes and chilled onto the frozen cooler rack for 1 minute to complete the denaturation step. In the second step, 4 µl of 5x SSIV Buffer, 1 µl of 100 mM DTT, 1 µl of RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen; 10777019) and 1 µl of SuperScript^{TM} IV Reverse Transcriptase (Invitrogen; 18090200) was added to each reaction to a final volume of 20 µl. Samples were incubated at 50 °C for 10 minutes and then heated to 85 °C for another 10 minutes in Thermal Cycler (Biometra) to complete the reaction. The acquired cDNA was stored at -20 °C or used immediately for qPCR reaction.

3.8.3 qPCR

The polymerase chain reaction was performed using FrameStar 384 Well Skirted PCR Plate (Azenta; 4ti-0381). For every gene an individual premix was made using 5 µl of TaqMan Gene Expression MasterMix (Applied Biosystems) and 0,5 µl of TaqMan probe as listed in **table 3**. Samples were diluted in RT-PCR grate water (Invitrogen; AM9935) to a concentration of 6 ng/µl. 1 µl of diluted cDNA and 3,5 µl of RT-PCR grate water (Invitrogen; AM9935) was added to each well to form a final volume of 10 µl. The plate was sealed using a LightCycler 480 Sealing Foil (ROCHE s.r.o.; 04729757001) to protect against evaporation.

The qPCR analysis was conducted using the LightCycler480 System from Roche. The procedure commenced with a 5-minute incubation at 95 °C, followed by 45 polymerization cycles occurring at 95 °C, 60 °C, and 72 °C, each lasting 10 seconds. Post-amplification, the plate underwent a cooling phase down to 40 °C. Data analysis employed the delta-deltaCT method, computing the fold change in expression through the $2^{-\Delta\Delta Ct}$ formula. This formula is based on the difference between the ΔCt of the sample and the ΔCt of the control, where ΔCt is calculated as the disparity between the Ct values of the gene of interest and the housekeeping gene.

Table 3 – List of TaqMan hybridization probes used for qPCR

Gene	Cat. number	Manufacturer
GAPDH	Hs02758991_g1	Applied Biosystems
GZMB	Hs00188051_m1	Applied Biosystems

IFNG	Hs00989291_m1	Applied Biosystems
TNF	Hs00174128_m1	Applied Biosystems
ORAI1	Hs03046013_m1	Applied Biosystems
STIM1	Hs00963373_m1	Applied Biosystems
PRF1	Hs00169473_m1	Applied Biosystems
NFATC1	Hs00542675_m1	Applied Biosystems
NFATC2	Hs00905451_m1	Applied Biosystems
TLR1	Hs00413978_m1	Applied Biosystems
TLR2	Hs00152932_m	Applied Biosystems
TLR3	Hs01551079_g1	Applied Biosystems
TLR4	Hs00152939_m	Applied Biosystems
TLR6	Hs01039989_s1	Applied Biosystems
TLR9	Hs00370913_s1	Applied Biosystems

3.9 Flow cytometry

To assess purity of freshly isolated and expanded NK cells, samples were processed on spectral flow cytometry system Aurora (Cytek) using the antibody panel described in **table 4**. The degranulation of NK cells was measured using the panel described in **table 5**.

Table 4 – Antibody flow cytometry panel for NK cell purity

Marker	Fluorochrome	Cat. number	Manufacturer
CD3	Spark blue 550	344852	Biolegend
CD14	Alexa fluor 594	325630	Biolegend
CD16	BV650	302042	Biolegend
CD19	BV570	302236	Biolegend
CD45	Alexa fluor 647	304018	Biolegend
CD56	APC fire 750	362554	Biolegend
LIVE/DEAD	Blue	L34962	Invitrogen

Table 5 – Antibody flow cytometry panel for NK cell degranulation

Marker	Fluorochrome	Cat. number	Manufacturer
CD56	APC fire 750	362554	Biolegend
CD16	BV650	325630	Biolegend
CD107a	PE	555801	BD
LIVE/DEAD	Blue	L34962	Invitrogen

To stain the cells for assays, the cells were transferred to FACS tubes and centrifuged (5 min, 600g, RT, acceleration 9, deceleration 9). The samples were washed twice in 1 ml of PBS to get rid of serum from media. Premix of antibodies was made in flow cytometry staining buffer (PBS, 10% FBS and 0,1% NaN_5 in distilled water). After PBS was drained, the premix was added to the samples, cells were briefly vortexed and left to stain for 20 minutes in the dark.

After 20 minutes the cells were washed and resuspended in Cell Wash (BD; 349524). When needed the cells were fixed with 100 µl/sample of IC Fixation buffer (BD; 00-8222-49). Prior measurement the cells were resuspended in Cell Wash. Data were processed by FlowJo.

All schemes were created in BioRender.

The data were statistically compared using a paired t-test, and the P-value limit for statistically significant differences between groups was set to 0.05 ($P < 0.01$ **). All statistical analyses were performed using Prism 9.5.1 (GraphPad Software, La Jolla, CA, USA) software.

4 Results

4.1 Assessment of purity and phenotype of expanded NK cells

The purity of natural killer (NK) cells was evaluated post-isolation and following a 14-day expansion using flow cytometry. Freshly isolated NK cells exhibited an average purity of 95%, while NK cells after the 14-day expansion displayed an average purity of 98%. **Figure 3A** illustrates the typical phenotype of freshly isolated NK cells, with the predominant population being the CD56dim CD16+ subset, known for its involvement in cytotoxic reactions against target cells. The CD56bright CD16- subset, constituting the remainder of the NK cells, lacks the CD16 receptor and thus the ability to induce antibody-dependent cellular cytotoxicity (ADCC). Nonetheless, these cells are potent cytokine producers and possess regulatory functions. During the 14-day expansion period, NK cells were cultured in the presence of cytokines (IL-2, IL-15) and co-cultivated with irradiated K562 cells (irK562) at a 1:5 ratio to enhance growth. This expansion induced a notable shift in the phenotype of NK cells, as depicted in **Figure 3B**, with the percentage of CD56bright NK cells increasing during expansion. Notably, the majority of CD56bright NK cells post-expansion expressed the CD16 receptor. These expanded NK cells were subsequently utilized in all subsequent experiments. (The complete gating strategy for assessing the purity of both freshly isolated and expanded NK cells is provided in the supplementary material – **Figure S1** and **Figure S2**, respectively).

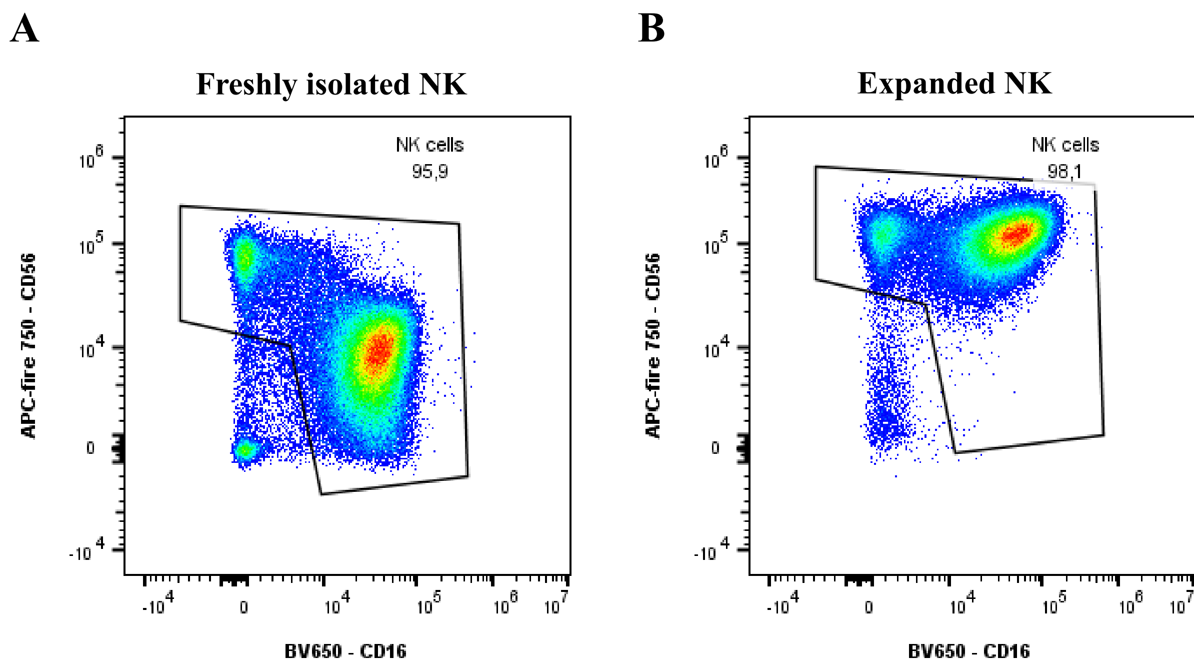

Figure 3 – Phenotype of freshly isolated NK cells and NK cells after 14-day expansion assessed by flow cytometry
(A) Typical phenotype of freshly isolated NK cells shown as cytometry scatter plot. (B) Typical phenotype of expanded NK cells shown as cytometry scatter plot.

4.2 Calcium depletion affects NK cell cytotoxicity

To showcase the role Ca^{2+} in effector functions of expanded NK cells, the cells were depleted of either intracellular or extracellular Ca2+ using BAPTA, AM and MEM, suspension, no calcium, no glutamine medium respectively, prior cytotoxic assay. The K562 cells were used as targets cells to analyse cytotoxic potential of NK cells. K562 cell line represent an ideal target for NK cells, due to the lack of MHC-I molecules on their surface. The lack of Fas on the K562 cells makes them an ideal target to study LG-mediated killing and thus any impairment of cytotoxicity caused by Ca^{2+} depletion would be observable.

As shown in **Figure 4**, significant impairment of NK cell cytotoxicity was observed upon Ca^{2+} depletion using BAPTA AM. As hypothesised, cells treated with BAPTA, AM, a cell-permeant Ca^{2+} chelator depleting intracellular Ca^{2+}, caused significant reduction of NK cell cytotoxicity in all ratios in comparison to untreated control (**Figure 4A**). NK cells co-cultivated in Ca^{2+} free medium for the duration of the cytotoxic assay, also displayed impairment of cytotoxicity (**Figure 4B**). However, the negative effect of the treatment can be observed in lower effector-to-target ratio. Thus, highlighting that initial triggering of intracellular Ca^{2+} influx is crucial for cytotoxicity of expanded NK cells, with extracellular Ca^{2+} having a minor role in comparison.

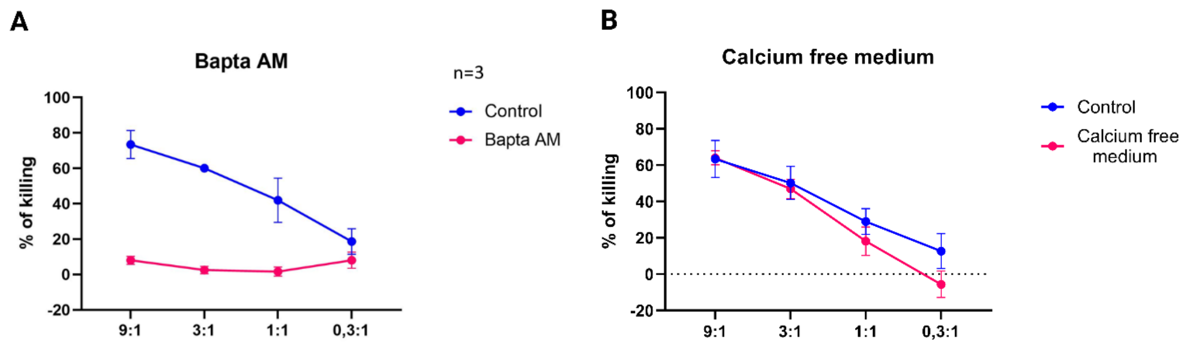

Figure 4 – Depletion of intracellular and extracellular Ca2+ in expanded NK cells affects NK cell cytotoxicity
Calcein AM cytotoxic assay was performed with NK cells as effector cells and K562 cells as targets in 9:1, 3:1, 1:1 and 0,3:1 ratio. (A) NK cells were treated with 50 µM BAPTA, AM 20 minutes prior cytotoxic assay. (B) NK cells were put in MEM, suspension, no calcium, no glutamine medium 20 minutes prior and for the entirety of the cytotoxic assay.

4.3 TLR ligands induces changes in intracellular calcium levels

After establishing the vital role of intracellular Ca^{2+} for NK cell cytotoxicity, the Ca^{2+} influx assay was performed in order to assess how stimulation of TLRs on expanded NK cells affects intracellular Ca^{2+} levels (**Figure 5**). NK cells were stained with Fura Red AM for 30 minutes and prepared for measurement. Reagents were added after initial 50s of the measurement, using build-in injector system. Ionomycin, ionophore that raises cytoplasmic Ca^{2+} levels, was used as a positive control to assess the reliability of the measurement. Addition of media was used as a negative control.

As expected, stimulation with TLR2 heterodimers Pam2CSK4 (TLR2/6) and Pam3CSK4 (TLR1/2) as well as TLR3 ligand Poly A:U prompted significant elevation in intracellular Ca^{2+} levels (**Figure 5A**). Interestingly, contrary to expectations, introduction of TLR4 ligand CRX-527 showed no effect on intracellular Ca^{2+} levels, in a same manner as addition of PGN-SA and ODN2395, ligands of TLR2 and TLR9, respectively (**Figure 5B**). Statistical analysis was assessed using calculation of the relative Ca^{2+} influx rate (**Figure 5C**) and area under curve (**Figure 5D**).

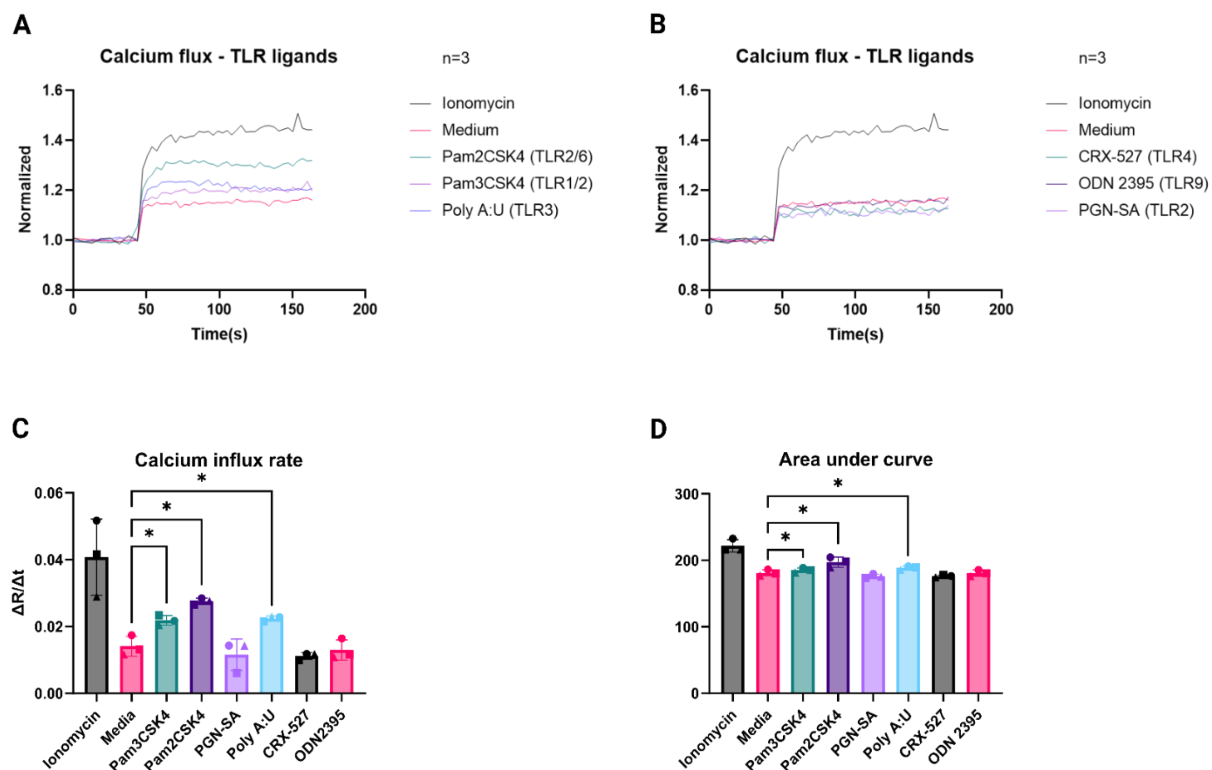

Figure 5 – Stimulation with Pam2CSK4, Pam3CSK4 and Poly A:U triggers increase in intracellular Ca^{2+} levels
*Expanded NK cells were stained with 10mM FURA Red, AM for 30 minutes and rested for 20 minutes. Fluorescence was measured at TECAN Spark. The time change of FURA Red ratio values were normalized to 1 and plotted. Ionomycin was used as a positive control and media was used as a negative control in all measurements. Reagents were added using built-in injector system. (A) Stimulation with Pam2CSK4 – 10 ng/ml, Pam3CSK4 – 10 ng/ml and Poly A:U – 100 ng/ml ligands. (B) Stimulation with PGN-SA – 10 ng/ml, CRX-527 – 10 ng/ml and ODN2395 – 37,25 µg/ml ligands. (C) Representative graphs of Ca^{2+} influx rate after addition of reagents. (D) Representative graphs of Area under curve calculation for every respected reagent. Data were tested for significance using paired t-test: *p < 0.05; **p < 0.01; ***p < 0.001; ns, no significant difference.*

4.4 Stimulation of TLRs influence NK cell cytotoxicity

Experiments described in **Figure 6** were performed in order to determine the effects of TLR stimulation, and subsequent rise in intracellular Ca^{2+}, on NK cell cytotoxicity. NK cells were pre-treated with TLR ligands for 18 hours, 3 hours and 1 hour prior cytotoxic assay, respectively. K562 cells were again used as a target cells. The assay was performed in four different ratios of effector to target cells; 9:1, 3:1, 1:1 and 0,3:1.

Pre-treatment with Pam3SCK4 (TLR1/2) ligand significantly increased NK cell cytotoxicity after 18-hour stimulation in 9:1 ratio and in 1:1 ratio regardless of the time of pre-stimulation (**Figure 6A**). Interestingly, 3-hour pre-treatment caused downregulation of NK cell cytotoxicity in 0.3:1 ratio. Comparatively, Pam2CSK4 (TLR2/6) enhanced NK cell cytotoxicity in 1:1 ratio upon 18-hour and 3-hour pre-treatment, but similarly to Pam3CSK4, the cytotoxicity in 0.3:1 ratio was once again lowered by 3-hour pre-stimulation (**Figure 6B**). Stimulation with PGN-SA (TLR2) showcase variable outcomes in the case of NK cell cytotoxicity (**Figure 6C**). Continuing in the observed trend, 3-hour pretreatment with Poly A:U (TLR3) ligand resulted in increased

NK cell cytotoxicity in higher ratios; however, unlike with Pam3CSK4 and Pam2CSK4, no observable reduction of NK cell cytotoxic activity has been observed in the lowest ratio (**Figure 6D**). Interestingly, pre-stimulation with TLR4 ligand CRX-527 induced a significant decrease in NK cell cytotoxicity in 3:1 and 0.3:1 ratio, with the trend noticed in all ratios, regardless of the time of pre-stimulation (**Figure 6E**). Lastly, stimulation with ODN2395, similarly to PGN-SA, yielded variable outcomes, most likely as a result of high donor variability (**Figure 6F**).

Pre-stimulation with ligands, shown to induce elevation in intracellular Ca^{2+} levels, increased NK cell cytotoxicity, regardless of the pre-stimulation time. However, in lowest ratio of effector-to-target cells, the effectivity of NK cells to kill their targets is significantly impaired (**Figure 6A**; **6B**; **6D**). On a contrary, stimulation with of TLR4 resulted in surprising decrease in NK cell cytotoxicity with trend being spotted across all ratios (**Figure 6E**). Donor variability is a crucial factor, which needs to be taken into account when interpreting these data.

Four TLR ligands have been selected for further analysis according to the results of Ca^{2+} influx and cytotoxicity assay; namely Pam3CSK4, Pam2CSK4, Poly A:U and CRX-527.

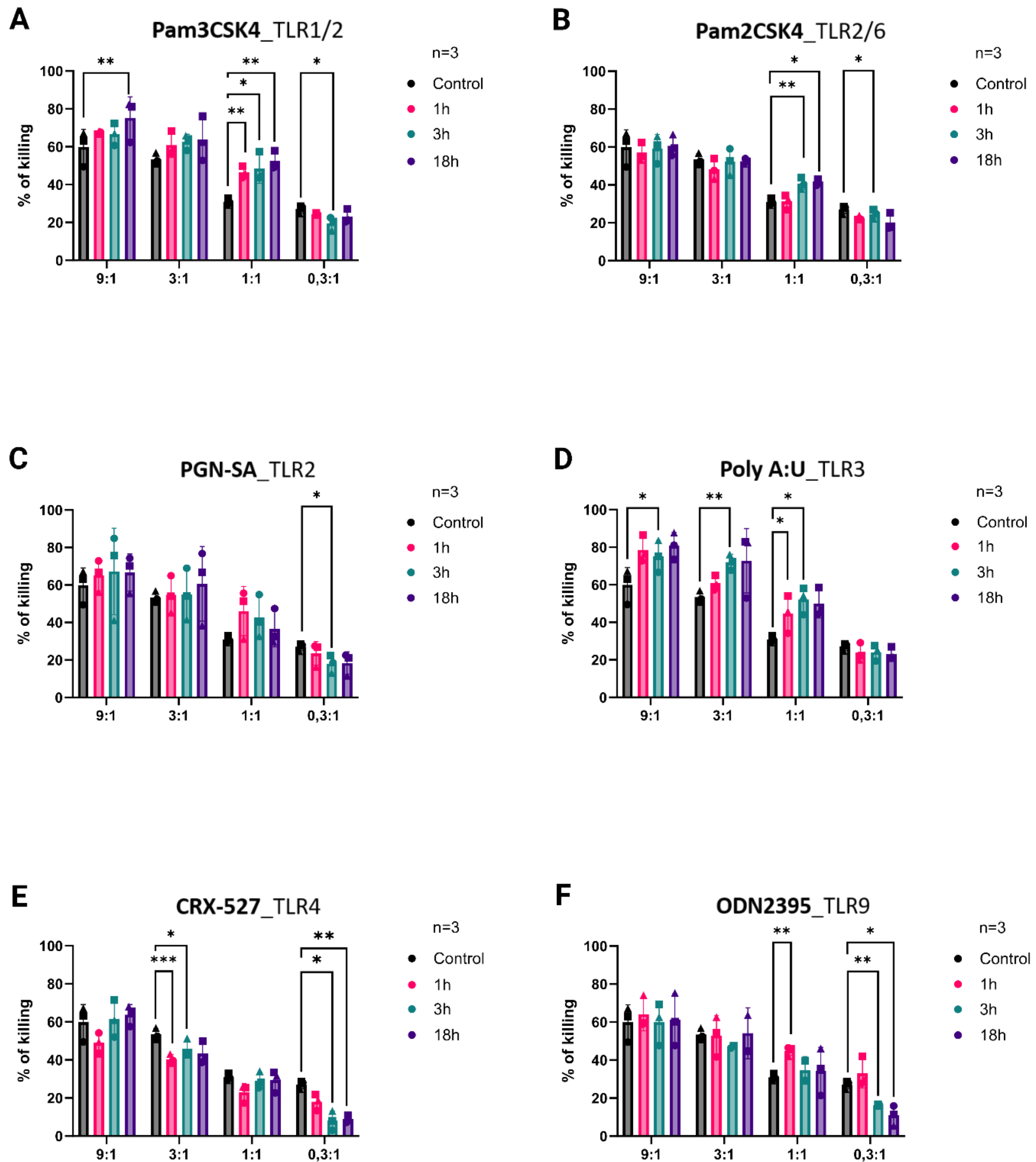

Figure 6 – Stimulation of expanded NK cell with TLR ligands significantly affects NK cell ability to kill target cells
*Above we show the results of Calcein AM cytotoxic assay. TLR ligands: (A) Pam3CSK4 – 10 ng/ml (B) Pam2CSK4 – 10 ng/ml (C) PGN-SA – 10 ng/ml (D) Poly A:U – 100 ng/ml (E) CRX-527 – 10 ng/ml (F) ODN2395 – 37,25 µg/ml were administered 18 hours, 3 hours and 1 hour respectively, prior to addition of K562 cells. Cytotoxic ability was tested in 9:1, 3:1, 1:1 and 0,3:1 of effectors to target cells ratios. TLR-treated cells were compared to untreated NK cells. Statistical significance was tested using paired t-test: *p < 0.05; **p < 0.01; ***p < 0.001; ns, no significant difference.*

4.5 TLR stimulation does not affect NK cell degranulation

NK cell killing ability and Ca^{2+} signalling is tightly linked with degranulation; **Figure 7** displays a set of experiments conducted in order to determine the effects of designated TLR

ligand pretreatment on degranulation of NK cells. Control samples included NK cells treated with ionomycin to induce maximal degranulation, unstimulated NK cells representing spontaneous degranulation, and NK cells co-cultivated with K562 cells. NK cells were exposed to TLR ligands for durations of 18 hours, 3 hours, and 1 hour, respectively. To evaluate the impact of treatment, cells were either co-cultivated with K562 cells or cultured without additional stimuli throughout the assay. Subsequently, cells were stained with monoclonal antibodies, analysed using flow cytometry and presented as fraction of CD107a+ cells.

Stimulation with Pam3CSK4 appeared to enhance NK cell degranulation in co-cultivation with K562 cells, with a consistent trend observed across all three pre-treatment time-points (18-hour, 3-hour, and 1-hour stimulation), although achieving statistical significance only after 1-hour stimulation (**Figure 7A**). Pre-treatment with Pam2CSK4 ligand led to an elevation in spontaneous degranulation compared to the control after 1-hour stimulation (**Figure 7B**). Notably, stimulation with the CRX-527 ligand elicited significant enhancement in degranulation when co-cultured with K562 cells, particularly pronounced after 3-hour pre-treatment (**Figure 7C**), despite its previously established role in impairing NK cell cytotoxicity. Conversely, a 1-hour pretreatment with Poly A:U resulted in a reduction in spontaneous granule release (**Figure 7D**). However, it cannot be conclusively stated that stimulation of NK cells with TLR ligands impacted spontaneous degranulation, as no other treatment option, apart from Pam2CSK4, exhibited any apparent interference.

Nevertheless, it is essential to acknowledge the influence of donor variability on all observed results. Contrary to initial expectations, stimulation of NK cells with TLR ligands did not induce significant alterations in NK cell degranulation.

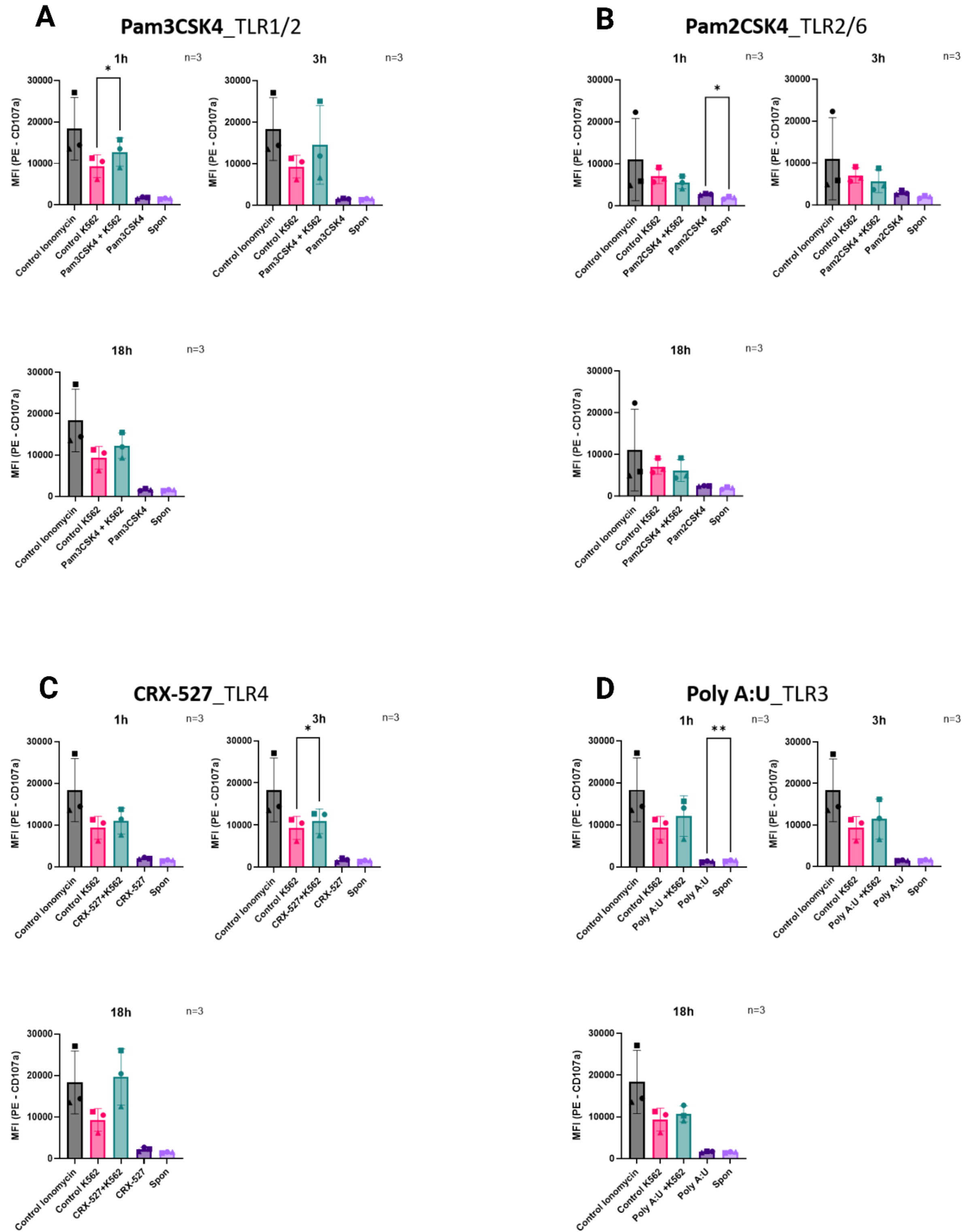

Figure 7 – Selected TLR ligand do not significantly alter NK cell degranulation
*Above we show results of degranulation assay assessed via flow cytometry. Degranulation was represented as median fluorescence intensity (MFI) of CD107a+ fraction – cells in CD56+ CD16 population. Treated NK cells were compared to untreated NK cells, which was either co-cultivated with K562 cells or without any stimuli, respectively. Ionomycin (1,5 µl/ml) stimulation was used as a control of maximal degranulation in all experiments. Statistical significance was tested using paired t-test: *p < 0.05; **p < 0.01; ***p < 0.001; ns, no significant difference.*

4.6 NK cell metabolism is affected by stimulation of TLR

Immunometabolism plays a crucial role in regulating the function of NK cells. The metabolic pathways are tightly linked to their effector functions, including cytotoxicity and cytokine production. The experiment presented in **Figure 8** aimed to elucidate the impact of TLR pre-treatment on NK cell metabolism. NK cells were subjected to treatment with TLR ligands, following the protocol outlined in previous experiments.

Stimulation with Pam3CSK4 for durations of 1 hour and 3 hours respectively resulted in a significant enhancement of basal respiration. However, prolonged stimulation for 18 hours led to a return of basal respiration to near-basal levels or even lower. Notably, due to donor variability, the observed downregulation was not statistically significant (**Figure 8A**). Exposure to Pam2CSK4 did not significantly impact basal respiration; however, a slight increase was observed upon 1-hour stimulation (**Figure 8B**). Additionally, CRX-527 treatment led to significantly reduced basal respiration upon 3-hour stimulation (**Figure 8C**). Similarly to Pam3CSK4, 18-hour stimulation with Poly A:U exhibited a significant downregulation of basal respiration. However, in contrast, short-term exposure did not yield a significant effect on NK cell basal respiration (**Figure 8D**). Thus, long-term exposure to two TLR ligands, elevating intracellular Ca^{2+} levels, Pam3CSK4 and Poly A:U, lead to downregulation of NK cell metabolism.

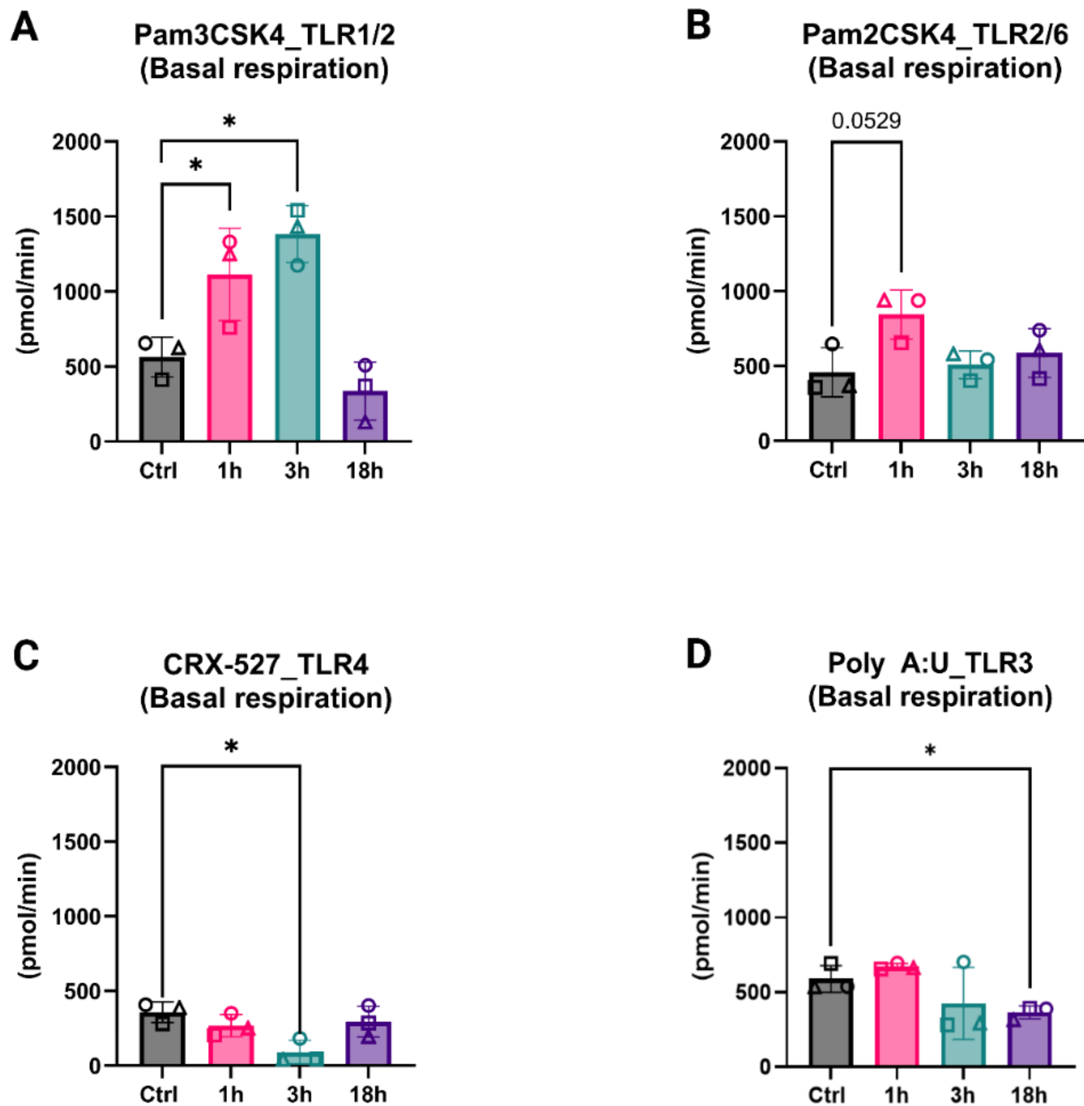

Figure 8 – Prolonged exposure to Pam3CSK4 and Poly A:U led to impaired basal respiration of expanded NK cells
*Above we show results of Seahorse Mito Stress Assay. The cells were treated with TLR ligands (A) Pam3CSK4 – 10 ng/ml (B) Pam2CSK4 – 10 ng/ml (C) CRX-527 – 10 ng/ml and (D) Poly A:U – 100 ng/ml. The figure shows the basal respiration of the treated cells compared to untreated NK cells. Statistical significance was tested using paired t-test: *p < 0.05; **p < 0.01; ***p < 0.001; ns, no significant difference.*

4.7 Gene expression changes upon stimulation with selected TLR ligands

In order to gain further inside into how stimulation of TLRs over time influence NK cell Ca^{2+} signalling and effector functions, the expression of several key genes was assessed via qPCR (**Figure 9**).

Stimulation with all TLR ligands resulted in elevated expression of effector genes (Perforin and Granzyme B), indicating their role in NK cell cytotoxicity. Notably, these genes exhibited a similar time-dependent pattern of upregulation, underscoring the joint contribution of respective

ligands to NK cell cytotoxicity. Interestingly, a distinct pattern emerged, with expression of effector genes peaking following short-term stimulation with Pam3CSK4 and Poly A:U, followed by a decline over time. Similarly, the expression of cytokines, IFN-γ and TNF-α, displayed a downward trend over time upon stimulation with Pam3CSK4 and Poly A:U. However, contrary to expectations the expression of cytokines did not seem to be substantially elevated in short-term stimulation.

TLR signalling upregulated the transcriptional factors NFATC1 and NFATC2, particularly evident following pre-treatment with Pam3CSK4 and Poly A:U. Notably, long-term stimulation with CRX-527 increased expression of both NFAT genes. Given NFAT's direct association with Ca^{2+} signalling, we evaluated the expression of Ca^{2+}-related genes Orai1 and STIM1. Consistently, Orai1 expression significantly increased following stimulation with all TLR ligands, regardless of pre-stimulation duration. Remarkably, a correlational pattern emerged between the expression of Orai1 and effector genes (Perforin and Granzyme B), as well as NFATC2, suggesting a link between Ca^{2+}-related genes and those involved in NK cell cytotoxicity. This highlights the multifaceted influence of TLR signalling on Ca^{2+} dynamics, not only through direct induction of Ca^{2+} influx but also by modulating Ca^{2+} pathways at the mRNA level, potentially impacting genes associated with NK cell cytotoxicity.

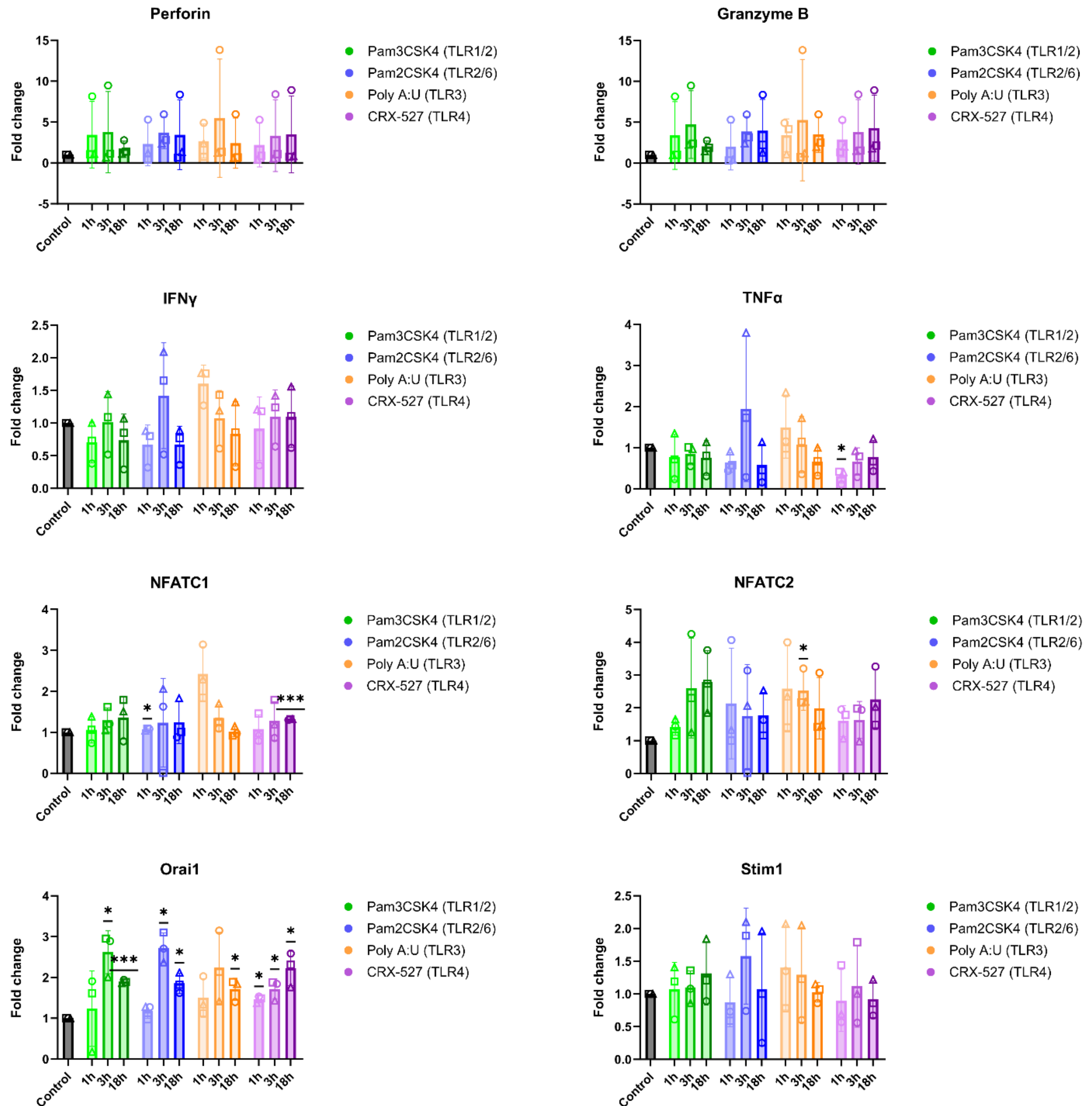

Figure 9 – TLR stimulation changed expression profiles of expanded NK cells
*Relative expression of mRNA of genes related to NK cell effector function and Ca^{2+} signalling was assessed via qPCR. The figure depicts expression of NK cells treated with TLR ligands (Pam3CSK4 – 10 ng/ml; Pam2CSK4 – 10 ng/ml; CRX-527 – 10 ng/ml; Poly A:U – 100 ng/ml) compared to untreated (Control) NK cells. TLR ligands were administered 18 hours, 3 hours and 1 hour prior to RNA isolation, respectively. Delta-deltaCT analysis was performed using GAPDH as a house-keeping gene. Statistical significance was tested using paired t-test: *p < 0.05; **p < 0.01; ***p < 0.001; ns, no significant difference.*

5 Discussion

In the context of tumour immunosurveillance, NK cells play a critical role due to their innate ability to recognize and eliminate transformed cells, without reliance on specific markers. This characteristic makes them an intriguing option for immunotherapy of AML (Xu & Niu, 2020), where the absence of leukemia-specific markers has since hindered the use of CAR T cells (Haubner et al., 2019). (Rubnitz et al., 2010b) demonstrated successful engraftment in pediatric AML patients through adoptive transfer of haploidentical NK cells combined with low-dose immunosuppression. Similarly, Bednarski et al. (2022) reported that *ex vivo* cultured donor-derived NK cells maintain enhanced anti-leukemic responsiveness, expand substantially post-infusion, persist in patients, and sustain anti-leukemic activity without exogenous cytokines, with observed remissions in a subset of patients.

Despite promising initial results, the efficacy of adoptive NK cell transfer appears to be modest, with limited successful clinical studies (Bachanova et al., 2014; Björklund et al., 2018; Curti et al., 2011; Fehniger et al., 2018; Iliopoulou et al., 2010; Lee et al., 2016; Miller et al., 2005; Nguyen et al., 2019; Parkhurst et al., 2011; Rubnitz et al., 2010; Sakamoto et al., 2015; Stern et al., 2013). A major challenge lies in the leukemic microenvironment, which exhibits protective properties, supports immune cell evasion, and contributes to relapse (Jedlička et al., 2022). However, we propose that another limiting factor influencing adoptively transferred NK cells is the disruption of intracellular Ca^{2+} balance, which may contribute to their impaired killing ability.

The crucial role of Ca^{2+} in NK cell cytotoxic activity was initially demonstrated by Leibson et al. in 1990, and later confirmed by Maul-Pavicic et al. (2011), who highlighted the necessity of Ca^{2+} for NK cell function. Maul-Pavicic's work elucidated that Orai1-mediated store-operated calcium entry (SOCE) is essential for NK cell cytotoxicity, as well as for chemokine and cytokine production. Notably, Orai1-deficient NK cells failed to effectively eliminate their targets. However, contrary findings were reported by Freund-Brown et al. in 2017, who demonstrated that murine NK cells can operate without SOCE, underscoring the critical role of intracellular Ca^{2+} for NK cell degranulation. These finding were based on STIM1/2 double knock-out NK cells for *in vivo* studies and NK cells presented with Ca^{2+}-free media to use *in vitro*. In contrast, Zhou et al. in 2018 suggested that NK cell cytotoxicity may indeed rely on SOCE, albeit the Ca^{2+} concentration needed for sufficient NK cell degranulation seems to be at very low levels, nonetheless necessary. These findings challenge the conclusions drawn by Freund-Brown et al. (2017). Consistent with these observations, our results indicate that the

absence of extracellular Ca^{2+} impairs NK cell cytotoxicity, although it does not completely abolish it (**Figure 3B**). Furthermore, our data confirm the importance of intracellular Ca^{2+} signalling for NK cell degranulation, as depicted in **Figure 3A**. As noted by Zhou et al. (2018), NK cells exhibit a "bell-shaped optimum" of intracellular Ca^{2+} concentration, where deviations from this optimal range result in impaired killing ability, indicating that tight Ca^{2+} balance is crucial for NK cells to exhibit their effector functions.

Adoptive transfer of NK cells is utilized as a supportive therapy for patients after completion of chemotherapy or radiotherapy regimens (Xu & Niu, 2020). These treatments elicit rapid production of immunostimulatory molecules, including damage-associated molecular pattern (DAMP) molecules, as documented by Galassi et al. (2024). These DAMP molecules serve as potent stimuli for TLRs. Studies have demonstrated the involvement of TLR signalling in Ca^{2+} pathways. Jin et al. (2016) reported that TLR2 induction by respected ligand activates NFAT, a transcription factor that is directly modulated by Ca^{2+} binding to calcineurin and subsequent dephosphorylation of NFAT, thereby establishing a direct link between TLR-induced Ca^{2+} signalling and NFAT activation. Furthermore, TLR4 signalling promotes calcineurin-NFAT activation via the recruitment of Syk kinase as reviewed in (Bendickova et al., 2017). Adding to this, Birla et al. (2022) noted that acute TLR4 stimulation in astrocytes did not induce Ca^{2+} influx; however, prolonged exposure to lipopolysaccharide (LPS), a TLR4 ligand, resulted in increased expression of Orai1 and STIM1 and elevated basal Ca^{2+} levels, directly linking TLR signalling with Ca^{2+} signalling pathways. Consistent with these findings, our results (**Figure 5A**) demonstrate a significant increase in intracellular Ca^{2+} levels upon exposure to TLR2 and TLR3 ligands. In line with the observations by Birla et al. (2022), acute stimulation with a TLR4 ligand did not induce Ca^{2+} influx in expanded NK cells (**Figure 5B**). These results validate the direct association between TLRs and Ca^{2+} signalling in NK cells, underscoring their potential to influence cytosolic Ca^{2+} levels. Consequently, we propose that DAMP molecules induced by chemotherapy may be recognized by TLRs on adoptively transferred NK cells, leading to an increase in cytosolic Ca^{2+} levels and subsequently influencing the Ca^{2+} balance of NK cells.

To further study the effects of TLR mediated-Ca^{2+} influx on NK cell effector functions we performed cytotoxicity assay. The NK cells were exposed to TLR ligands for 18 hours, 3 hours and 1 hour respectively. TLR2 and TLR3 ligands were shown to enhance NK cell cytotoxicity in all ratios except 0,3:1, where the cytotoxic activity was actually impaired in case of TLR2 and TLR4 ligands (**Figure 6A**; **6B**; **6C**) or not effected in case of TLR3 ligand (**Figure 6D**). This seemingly random pattern however could be explained. NK cells are known to perform serial killing of their targets (Bhat & Watzl, 2007). Schwarz et al. (2013) suggested that increased Ca^{2+}

influx can impair NK cell ability to kill multiple targets. They hypothesized that enhanced Ca^{2+} influx can lead to unbalanced release of lytic granules (LGs), leading to NK cells expelling all pre-made granules towards the first target and failing to kill the next. This was later confirmed by the study of Y. Li et al. (2022). They showed that treatment with small molecule inhibitor UNC1999, caused increased Ca^{2+} influx, which was connected with significantly increased degranulation but impaired cytotoxic activity. We hypothesize that impaired killing ability of NK cells in 0,3:1 ratio is a result of this mechanism as the cells are forced to kill multiple targets. However, this hypothesis would need to be confirmed in future experiments, such as serial killing assay, which would give us precise knowledge about NK cell killing ability, when forced to kill multiple targets upon TLR stimulation. Interestingly prolonged exposure to TLR4 ligand significantly impaired NK cell killing ability in almost all ratios (**Figure 6E**), suggesting that TLR4 affects NK cell cytotoxic activity in different mechanism compared to TLR2 and TLR3, where we can observe comparable trends.

In order to delve deeper into the impact of TLR-mediated Ca^{2+} influx on NK cell effector functions, we performed cytotoxicity assays. NK cells were exposed to TLR ligands for varying durations: 18 hours, 3 hours, and 1 hour, respectively. Our findings revealed that TLR2 and TLR3 ligands enhanced NK cell cytotoxicity across most ratios, with the exception of the 0.3:1 ratio, where TLR2 ligands actually impaired cytotoxic activity (**Figure 6A**; **6B**), or had no effect in the case of TLR3 ligands (**Figure 6D**). Although this pattern may initially seem arbitrary, an explanation can be proposed. NK cells known to engage in serial killing of their targets. Schwarz et al. (2013) proposed that increased Ca^{2+} influx could disrupt NK cell capacity to effectively kill multiple targets. They hypothesized that increased Ca^{2+} influx might lead to an unbalanced release of lytic granules (LGs), causing NK cells to expel all pre-formed granules toward the initial target and consequently fail to engage subsequent targets. This hypothesis was subsequently supported by Y. Li et al. (2022), who demonstrated that treatment with the small molecule inhibitor UNC1999 resulted in increased Ca^{2+} influx, accompanied by significantly enhanced degranulation but impaired cytotoxic activity. We speculate that the impaired killing ability of NK cells in the 0.3:1 ratio may be attributable to this mechanism, as the cells are compelled to engage with multiple targets. However, this hypothesis warrants validation in future experiments. Interestingly, prolonged exposure to a TLR4 ligand markedly impaired NK cell killing ability across nearly all ratios (**Figure 6E**), suggesting that TLR4 exerts distinct effects on NK cell cytotoxic activity compared to TLR2 and TLR3, where comparable trends are observed.

Upon evaluating the results of Ca^{2+} flux measurements and cytotoxic assays, we selected four TLR ligands (Pam3CSK4 – TLR1/2, Pam2CSK4 – TLR2/6, Poly A:U – TLR3, CRX-527 – TLR4) for further investigation. Our next objective was to assess the degranulation of NK cells treated with these specific TLR ligands. While previous studies have indicated that degranulation and cytotoxicity may not always exhibit a direct positive correlation (Y. Li et al., 2022), our analysis did not reveal significant interference of NK cell degranulation following stimulation with TLRs. Nonetheless, a noticeable trend suggesting an increase in NK cell degranulation was observed upon stimulation with TLR1/2, TLR3, and TLR4 ligands, regardless of the duration of pre-stimulation (**Figure 7A**; **7C**; **7D**). It is noteworthy that our results were influenced by donor variability, posing challenges for further interpretation. Nonetheless, these findings appear to be consistent with our initial hypothesis.

The role of immunometabolism in regulating the function of NK cells has gained increasing attention within the scientific community. Metabolic pathways within NK cells are linked to their effector functions, including cytotoxicity and cytokine production (Wang et al., 2020). NK cells undergo metabolic reprogramming in response to environmental stimuli, such as cytokines or cellular stress, to meet the energetic and biosynthetic demands associated with their activation and proliferation (O'Brien & Finlay, 2019). To investigate the impact of TLR pre-stimulation on NK cell metabolism, we conducted Seahorse Cell Mito Stress Tests. Our findings reveal that short-term exposure to TLR ligands augments NK cell basal respiration, whereas prolonged exposure results in downregulation (**Figure 8A**; **8D**). This reduction in basal respiration signifies a metabolic state characterized by reduced energy production and altered cellular functionality. Consequently, prolonged exposure of transferred NK cells to TLR ligands may lead to metabolic downregulation, potentially impairing their effector functions. Although our data does not directly demonstrate functional impairment dependent on the duration of ligand stimulation, we propose that repeated stimuli could substantially modulate NK cell metabolic activity, potentially resulting in functional deficits. However, further studies are warranted to validate this hypothesis, as no data are currently available to confirm this claim.

Lastly, we sought to examine the impact of TLR pretreatment on the expression levels of key molecules critical for NK cell effector functions and Ca^{2+} signalling pathways. We specifically focused on a select group of molecules to elucidate the effects of TLR treatment, including granzyme B, perforin, IFN-γ, TNF-α, NFATC1, NFATC2, Orai1, and STIM1. Granzyme B and perforin are pivotal effector molecules central to NK cell cytotoxic activity, while IFN-γ and TNF-α serve as major effector cytokines secreted by NK cells. Moreover, the Orai1-STIM1 pathway represents a crucial calcium-inducing pathway in NK cells, as

demonstrated in previous studies (Maul-Pavicic et al., 2011). Notably, NFAT is a transcription factor directly activated by Ca^{2+} signalling, and its connection to TLR signalling has been established in prior research (Bendickova et al., 2017; Birla et al., 2022; Zanoni et al., 2009).

Our analysis of gene expression by qPCR, as depicted in **Figure 9**, revealed a notable increase in the expression levels of perforin and granzyme B following TLR pretreatment. This observed trend was consistent for both genes, suggesting a joint role in NK cell effector functions. These findings align with our initial expectations. However, contrary to our anticipations, TLR stimulation did not result in a significant upregulation of effector cytokine expression. A similar trend was evident across various treatments in the expression levels of IFN-γ and TNF-α. Interestingly, these results diverge from those reported in other studies, which have demonstrated an increase in both IFN-γ and TNF-α expression following TLR stimulation (Lauzon et al., 2006; Pisegna et al., 2004; Sawaki et al., 2007).

We further observed (**Figure 9**) a notable increase in the expression of NFATC1 and NFATC2 genes following TLR pretreatment, particularly evident upon stimulation with TLR1/2 and TLR3 ligands. Additionally, prolonged exposure to TLR4 ligand significantly enhanced the expression of NFATC1. These findings are consistent with prior research by Birla et a., (2022); Jin et al. (2016) and Zanoni et al. (2009), which demonstrated that TLR stimulation influences the expression of Ca^{2+}-related genes in expanded NK cells. Our data also revealed a significant upregulation in the expression of Orai1, aligning with previous findings reported by Birla et al. (2022). Orai1 serves as a predominant Ca^{2+} channel directly involved in NK cell cytotoxic activity and NFAT activation, as documented by Maul-Pavicic et al. (2011). However, the expression level of STIM1 remained unaffected by TLR stimulation, although it is important to note the variability observed in STIM1 data. As showed by Z. Li et al. (2011), shifting the expression of Orai1 or STIM1 decreases Ca^{2+} influx, further resulting in decreased cytotoxicity. The availability of STIM1 decreases upon elevation of Orai1 levels, leading to disruption of store-operated calcium entry (SOCE) and impairment of NK cell cytotoxic activity, suggesting that TLR stimulation leads to decrease of Ca^{2+} influx. However, these findings appear to contradict both our own data and existing literature. Our study reveals a consistent phenotype across our data, indicating impaired NK cell effector functions due to elevated intracellular Ca^{2+} levels aligned with previous research by Y. Li et al. (2022) and Schwarz et al. (2013). As previously stated by Zhou et al., NK cells demonstrate a characteristic "bell-shaped" response to Ca^{2+} levels which can be disrupted by either decrease or increase of intracellular Ca^{2+}. Whereas decrease leads to reduced LG exocytosis, elevated Ca^{2+} levels induce unbalanced release of LGs,

resulting with only first few target cell lysis. Ultimately, both disruptions result in a decline in NK cell killing ability, as elucidated by Schwarz.

Investigating the serial killing capability of expanded NK cells following exposure to TLR ligands would offer valuable insights and assessing the cytosolic Ca^{2+} levels before and after prolonged TLR ligand exposure would reinforce our hypothesis. Additionally, expanding the donor pool for our measurements would enhance statistical robustness and mitigate the impact of donor variability on our findings.

6 Conclusion

Our study underscores the critical role of intracellular Ca^{2+} in regulation of NK cell cytotoxicity. Furthermore, our findings reveal that TLR stimulation leads to an elevation of intracellular Ca^{2+} levels, implicating TLRs as inducers of this crucial signalling pathway. Interestingly, while TLR stimulation increased cytotoxicity in higher effector-to-target ratios, a decrease was observed in lower ratios, suggesting impaired effector functions during multiple target killing. Moreover, although TLR stimulation did not significantly increase NK cell degranulation, a trend towards elevation was observed, indicating potential modulation of effector functions. Notably, prolonged exposure to TLR2 and TLR3 ligands resulted in the downregulation of NK cell metabolism, revealing an interplay between TLR signalling and NK cell immunometabolism. Additionally, TLR stimulation induced the upregulation of genes directly associated with Ca^{2+} signalling, including the transcriptional factor NFAT and the major Ca^{2+} channel Orai1, which orchestrates store-operated Ca^{2+} entry.

Collectively, our findings elucidate propose that prolonged exposure to TLR ligands in proinflammatory environment of the patient results in the enhancement of intracellular Ca^{2+}, ultimately leading to functional impairment and unsatisfying results of clinical trials.

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