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REGULATION OF CALCIUM FLUX BY ACTIVATING AND INHIBITORY RECEPTOR CROSSTALK IN NK CELLS

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Regulation of calcium flux by activating and inhibitory receptor crosstalk in NK cells

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Dedicated to Tumor Immunology

NK cell Rap song/Poem

''Let the real NK cell; please stand up
Let the real NK cell; please stand up
Mouse 3%, Humans 10%
Primates, Rats,
Never need antigen, never need stimulus
See the tumor, kill the virus
Let the real NK; please stand up
Let the real NK; please stand up
Balance and no imbalance
Not empty without me?
IL-X,Y,Z, IL15, mTOR, IFNy
IL-X, Y, Z, CD16, FcyR, ADCC
Let the real NK; please stand up
Let the real NK; please stand up
With the IR, they get educated
With the AR, they get activated
HLA, calcium, sex please
Quants, FBS, DC's
Let the real NK cell; please stand up
Let the real NK cell; please stand up''

ABSTRACT

The birth of a new born child kicks off the immune system to be on call and to respond promptly to threats from the environment. Natural Killer cells, classified as a member of the innate lymphoid cell family, express various activating, inhibitory, adhesion and cytokine receptors as well as MHC class I molecules on their surfaces, by which they interact with surrounding cells and perform various immunological function. The balance between the stimulation of these receptors determines the functional output of NK cells. NK cells are critically important for tumor control and viral clearance, but several basic cellular mechanisms and pathways regulating their function, both in mice and humans, remain largely unknown. This PhD thesis tries to explore a few aspects of these by studying NK cell proximal signaling downstream of activating and inhibitory receptors, and also explores the phenomenon of MHC class I signaling in NK cells. The work focuses mainly on mouse NK cells, but with a few outlooks into experiments with human NK cells as well.

The c-Abl non-receptor tyrosine kinase, a known pro-inflammatory protein in B and T cells, was proposed to function in inhibitory signaling in human NK cells. With the given parameters measured, we could find no role for c-Abl in murine NK cell education, using mice lacking c-Abl specifically in NK cells. The questions asked allowed us to design a novel real-time measurement of proximal inhibitory signaling using calcium flourometry as a read-out, which revealed no role of c-Abl in inhibitory signaling in murine NK cells. However, a possible role of c-Abl as a negative regulator of IFN-γ production was suggested. We further used this assay to show that upon *in vivo* dendritic cell depletion, NK cell calcium flux after triggering of the ITAM-dependent receptor NK1.1 was drastically reduced, suggesting that the DC crosstalk might be needed to maintain proximal signaling capacity in NK cells.

Further exploration of this assay led us to generate data to determine that inhibitory signaling operates in a quantitative and additive way in murine and human NK cells. Furthermore, $H2D^d$ ligand interaction in *cis*, in the NK cell membrane itself, compromised the ability of the Ly49A inhibitory receptor to transmit inhibitory signals inside the cell. We also provided evidence that IL-15, a vital cytokine for NK cell survival, ‘primes’ activating receptor signaling but does not directly affect the impact of inhibitory receptor signaling on these pathways. Finally, the MHC class I allele $H2K^b$ was shown to function as a signalling mediator that both synergized with activating receptor signaling and triggered calcium release by itself. In this case, a potential *cis* interaction with Ly49C did not seem to affect $H2K^b$ signal transmission.

I believe that the studies included in my thesis help us to understand the regulation of NK cell activity better. They also emphasise the usefulness of modifying and developing in-house methods to explore NK cell signaling properties. In the future, as the mechanisms that fine-tune NK cell activation and inhibition continue to be identified, NK cells can hopefully be used better as a prominent therapeutic tool in the clinic.

POPULAR SCIENTIFIC SUMMARY

Life on planet earth arose, probably from a collision of an asteroid/meteor on our planet 3-3.5 billion years ago. Simply due to the passage of time, life forms – microscopic and macroscopic – different kinds of organisms have come to life and disappeared, like the dinosaurs. But some life beings have survived and stayed, contributing to the amazing, ever-rejuvenating novelty to contribute to the variation of 'Life'. If we give it a thought about this process of life in general, it is quite simply fantastic and awe-inspiring. The YouTube videos on 'Symphony of Science' are quite sensational and my master thesis was a big motivational factor (http://www.ibg.uu.se/digitalAssets/167/c_167884-l_3-k_ganesan-sridharan-report.pdf).

The amazing human body constantly fights with the outside environment to keep the body at a check against pathogens, which constantly challenge the body. It is a constant war that goes on without our notice. We at a human being level do not feel the never-ending fight against factors that cause problems to our homeostasis and try to cause disease. Self-defending immunological mechanisms not only prevent dysbiosis, they also contribute to normal functioning of the human body. The different kinds of cells that sense 'danger' contribute to the healthiness of ourselves. Lymphocytes that reside in lymphoid tissues move and scan the body for signals of abnormality. This Doctor of Philosophy (PhD) thesis tries to understand basic cell biological mechanisms in one such cell-type, called Natural Killer Cells, in mice and in humans.

With emerging threats, such as Zika viral mother-to-child transmission, sporadic measles outbreaks, multi-drug resistant *M. tuberculosis*, malaria infections, resistance to HIV treatment, cancer-resistance to conventional therapies and metabolic diseases pointing towards immunological involvement, it is becoming increasingly important to study immunological mechanisms to counter attack signs of peril. Thus, scientifically for the benefit of humanity, governmental and non-governmental institutions could collaborate to support the burgeoning of immunological research through young and developing students who would like to choose career paths as scientists world-wide. With a never-ending enthusiasm, research in Immunology can be quite interesting. As my summer, high school students once said: 'To look at cells from our living bodies feels wow!'

LIST OF SCIENTIFIC PAPERS (INCLUDED IN THE THESIS)

I. Luu TT, **Ganesan S**, Wagner AK, Sarhan D, Meinke S, Garbi N, Hämmerling G, Alici E, Kärre K, Chambers BJ, Höglund P, Kadri N.
Independent control of natural killer cell responsiveness and homeostasis at steady-state by CD11c+ dendritic cells. *Scientific Reports*, 2016, 6:37996.

II. **Ganesan S**, Luu TT, Chambers BJ, Meinke S, Brodin P, Vivier E, Wetzel DM, Koleske AJ, Kadri N, Höglund P.
The Abl-1 kinase is dispensable for NK cell inhibitory signalling and is not involved in murine NK cell education. *Scandinavian Journal of Immunology*, 2017, 86:135.

III. **Ganesan S**, Höglund P.
Inhibitory receptor crosslinking quantitatively dampens calcium flux induced by activating receptor triggering in NK cells. *Frontiers in Immunology*, 2019, 9:3173.

IV. **Ganesan S**, Höglund P.
A novel costimulatory role for MHC class I molecules on NK1.1 signaling revealed by studies on Ca^{2+} flux in NK cells. *Manuscript*.

LIST OF SCIENTIFIC PAPERS (NOT INCLUDED IN THESIS)

I. Kadri N, Wagner AK, **Ganesan S**, Kärre K, Wickström S, Johansson MH, Höglund P.
Dynamic regulation of NK Cell responsiveness. *Current Topics in Microbiology and Immunology*. 2016, 395:95. Review.

LIST OF ABBREVIATIONS

NK	Natural killer
AR	Activating receptor
IR	Inhibitory receptor
HBSS	Hank's buffered salt solution
PBS	Phosphate buffered saline
SHP-1	Src homology region 2 domain containing phosphatase-1
SHIP	SH2-containing inositol phosphatase
c-Abl	Abelson murine leukemia viral oncogene homolog 1
IFN-γ	Interferon gamma
KIR	Killer cell immunoglobulin-like receptor
Ca^{2+}	Calcium
MHC	Major histocompatibility complex
DC	Dendritic cell
IL-15	Interleukin-15
ILC	Innate lymphoid cells
PCR	Polymerase chain reaction
CMV	Cytomegalovirus
Ly49	Ly49-proteins belonging to C-type lectin receptor family
TCR	T Cell Receptor
NKG	Natural Killer Gene Complex
HLA	Human Leukocyte Antigen

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1 INTRODUCTION

1.1 GENERAL

There have been many hollywood english movies made on viruses, infections, especially Ebola. Apparently, there have not been many movies made on cancer or immune cells, which this thesis is about. Thanks to a 3 to 3.5 billion-year life history in the face of planet earth, evolution has had time to shape the existing life-forms that we see today. The immune system is a hallmark of life, which, in the human body, actively and passively controls the response against various stressors, such as viral, bacterial, fungal and parasitic infections. However, immunity and inflammation has its drawbacks, being also involved in autoimmune diseases, obesity, diabetes, neurodegenerative diseases and cancer (*1*). In recent years, however, the immune system has been explored also to fight cancer, expanding the evolutionary horizons.

1.2 NK CELLS

The immune system consists of a complex mixture of components with complementary missions. Evolutionary older members, such as complement, acute phase proteins and phagocytes coexist with younger systems, primarily lymphocytes, in an intricate network of soluble factors and cellular interactions. This thesis is about Natural Killer cells (NK), one of several members of the lymphocyte group.

NK cells are the founding members of a family of innate group of lymphocytes (ILCs) (*2*). They are also referred to as large granular lymphocytes (LGL). NK cells differ from other lymphocytes, such as B and T lymphocytes, in a way that they do not depend on recombinase (RAG)-dependent clonal expression of receptors but rather on the expression of germline-encoded activating and inhibitory receptors for various ligands (*3*). They do, however, express RAG1/2 at some point in their natural lives (*4*), but they do not use these genes to recombine and re-arrange their surface receptors.

They express various kinds of activating receptors, inhibitory receptors, cytokine receptors, adhesion molecules and MHC class I molecules on their surface, which all interact with target ligands on surrounding cells (*5-9*). Not only do they express them, but also interact through them with other cells that leads them to perform immune regulatory function by means of cytotoxicity, cytokine secretion and granule secretion (*10*).

1.2.1 Biology and function of NK cells

NK cells are thought to move among the tissues of humans and mice, in which they have been shown to be involved in situations such as tumor detection and eradication and elimination of virus-infected host cells. Moreover, they have been shown to be involved as part of the immune orchestra, meaning that they affect the function also of other immune cells, in situations such as Herpesvirus infections, i.e. CMV (*11*), Pregnancy (*12*), HIV infection (*13*), Malaria (*14*), Rheumatoid arthritis (*15*), Obesity (*16*) and CNS injury (*17*). They are also helpful immune cells during bone marrow transplantations for the treatment of cancers, such as acute myeloid leukemia (*18, 19*). Actually, NK cells have been implicated in almost any disease (Pubmed 56851 articles as on 19th May 2019).

Mechanistically, NK cells kill other cells directly but also regulate the magnitude of the immune response by interacting with B cells (*20*), T cells (*21*), NKT cells (*22*) dendritic cells (DC) (*23*), T regulatory cells (*24*), red blood cells (*25*) and platelets (*26*). They comprise about 10-20% of peripheral lymphocytes in human blood (*27*) and 2-5% of spleen lymphocytes in mice (*28*). As members of the immune orchestra, they act, via numerous cytokines/granules/secretory mechanisms, as fine-tuners in the fulfilment of immune response during homeostasis or pathological states.

Previously, NK cells were classified as naïve lymphocytes involved only in spontaneous target recognition and they were not expected to be involved in a memory recall experiment. This view has been modified in the recent past. Many published reports have described a phenomenon called 'NK cell memory', during which NK cells perform better when re-exposed to certain antigens (*5, 29*). Thus, the borders between what was previously considered innate, or pre-existing static immunity and adaptive immunity are being challenged. A recent identification of so called "adaptive" NK cells, generated in a complex, hitherto not identified, interplay between NK cell education and CMV infections in humans (*30*), emphasizes this view.

1.2.2 NK cell activating receptors

As already alluded to, NK cells perform various cellular functions such as cytotoxicity, and cytokine secretion, by which they regulate innate and adaptive immune response against a stress challenge. It is a difficult task to summarize the receptors that they use, because there is a blurred definition of what is an activating receptor and what might be an adhesion receptor only. It is even more difficult to list all known ligands for these receptors, but I made a try and have included some of the known ligands (Table 1).

NK cells in the C57Bl/6 strain of *Mus musculus* express activating receptors such as NK1.1, NKp46, Ly49D, NKG2D, CD16, DNAM-1 and 2B4. In *Homo sapiens*, the receptors are somewhat different but to some extent overlapping. In human NK cells, NKp46, NKp30, NKp44, CD16, NKp80, NKG2D, DNAM-1 activating receptors are expressed. The well-known function of these receptors is to recognise ligands on surrounding cells that leads to NK cell activation and participate in a predominantly Th1 immune response with other immune cells (Table1).

Table 1. Activating receptors on NK cells

Receptors	**Species**	**Ligands**
NK1.1	Mouse	Murine cytomegalovirus (MCMV)-encoded protein, m12 (*31*)
NKp46	Human and mouse	Epa1, Epa6, and Epa7 (*32*)(*33, 34*)
Ly49D	Mouse	H-2D^d (*35*)
NKG2D	Human and mouse	H60, Rae-1 and Mult1 (Mice) and MICA, MICB and ULBP-1, 2, 3 (Humans) (*36-41*) (*42, 43*)
CD16	Human and mouse	IgG (Fc) (*44*)
DNAM-1	Human and mouse	The poliovirus receptor (PVR; CD155) and its family member nectin 2 (CD112) (*45*) (*46*)
2B4	Human and mouse	CD48 (*47*) (*48, 49*)

KLRG1	Human and mouse	E-Cadherin (*50*) (*51*)
Ly49H	Mouse	m157 protein mCMV (*52*)

1.2.3 NK cell inhibitory receptors

A key aspect of NK cells is their expression of a variety of inhibitory receptors that interact with ligands on surrounding/target cells. The function of inhibitory receptors is to act as 'check-points', or 'brakes', which impedes NK cell activation and thus reduce the overall NK cell immune response. The net balance between the stimulation of various receptors expressed on their surface ultimately determines whether NK cells are activated/inhibited and by this determines the kind of function they will perform. These dynamic interactions play a key role in the life-cycle of an NK cell. Mouse NK cells express variety of inhibitory receptors of the Ly49 family (*53-55*). The Ly49 receptors of most importance for this thesis are Ly49G2, Ly49A and Ly49C. NKG2A is another lectin-like receptor in mice, which is also a human receptor. Human NK cells express inhibitory receptors of the Killer cell Immunoglubulin-like (KIR) receptor family, in addition to NKG2A. The expression of inhibitory receptors is a central feature of NK cells, which is manifested by their expression in a variety of species, such as rat, mouse, horse, humans and non-human primates including chimpanzees, gorillas and oraguntans (*7*). A selection of inhibitory receptors in mice and humans are listed in Table 2

Table 2. Inhibitory NK cell receptors

Receptors	**Species**	**Main ligands**
Ly49C	Mouse	Mainly $H2K^b$ (*56-58*)
Ly49I	Mouse	Mainly $H2K^b$, $H2K^d$ (*56-58*)
Ly49A	Mouse	Mainly $H2D^d$ and $H2D^k$ (*56-58*)
Ly49G2	Mouse	Mainly $H2D^d$ and $H2L^d$ (*56-58*)
NKG2A	Human and mouse	MHC class I molecule $Qa\text{-}1^b$ (mouse) and HLA-E (human) (*57, 59-61*)
KIR2DL1	Human	HLA-C group 2 (*57, 62, 63*)
KIR2DL2/3	Human	HLA-C group 1 (*57, 62, 63*)
KIR3DL1	Human	HLA-Bw4 (*57, 64*)
KIR3DL2	Human	HLA-A3, HLA-A11, HLA-B27 (*57, 65*)

1.2.4 Missing self-hypothesis

It was an early notion that NK cells did not kill all cells but displayed some form of target cell selectivity. Klas Kärre noted that one common denominator for NK cell susceptibility was low expression of MHC class I on target cells. Whereas target cells with reduced levels of MHC class I expression were killed, those with high expression were protected (*66*). Kärre formulated the "missing self" hypothesis to explain both this relationship and the notion of "hybrid resistance" to parental grafts, in which NK cells had been implicated (*67*). According to this hypothesis, NK cells activate their cellular program for lytic and non-lytic functions only if MHC class I molecules were NOT present on targets (*68*). Thus, when NK cells sense

reduced or absolutely no levels of MHC class I on the surface of target cells, they see a 'rejection' phenotype and kill the cells (*68*).

The missing self-hypothesis placed MHC class I recognition as a major property of NK cells, stimulating further work on MHC class I as an "off signal". Missing self also placed MHC class I interaction as a central regulatory aspect of tumor/target cell recognition by NK cells. Missing self-rejection has been established and many early model systems showed that MHC class I molecules themselves were involved (*69*) and that missing self could be prevented by the restoration of self-MHC class I on the surface of their 'target' cells (*70*). The missing self-hypothesis has now embraced the clinical setting and has been shown to be important in solving problems that arise during allogeneic stem cell transplantation (*71-73*).

1.2.5 NK cell education

NK cells, through their inhibitory receptors, recognise and interact with versions of MHC class I on surrounding cells. As described in the previous section, this is the basic tenet of the missing self-model. However, the MHC class I interaction with NK cells is more complex than this. It was shown in the beginning of the 1990:ies that MHC class I molecules are critical for NK cells to gain their functional ability to act as potent cytotoxic machines (*74, 75*). The molecular basis for this "education" process, which is sort of similar, but only in a general sense, to positive selection of T cells in the thymus, is not clarified but has been shown to depend on MHC class I expression (*74-78*), Ly49 receptor expression in the mouse (*54*) and SHP-1 expression (*77, 79*).

Before, NK cell hyporesponsiveness in the absence of these components were thought to be aberrant to genetically modified model, but this view was challenged when it was shown that NK cells with no inhibitory receptors on the surface were found in both mice and humans. These inhibitory receptor-deficient NK cells have been found to be hyporesponsive, as expected from the genetic models (*76-78, 80, 81*). The entire dynamic process of NK cell tolerance referred to here has been called NK cell 'education', NK cell 'licensing' or NK cell 'disarming'. Although many NK cell researchers have their own versions of this process, in my humble opinion, NK cell education or licensing essentially mean the same phenomenon. Licensing is a bit fancier, which may be one reason this term has stuck with immunologists in general.

1.3 SIGNALLING IN THE IMMUNE SYSTEM

In order for a complex biological entity such as the amazing human body to function as one single unit, signalling in the form of neurotransmitters, hormones and cytokines, which transmit life-sustaining cues among cells, is essential (*82*). Cellular communication, or cross-talk, in this way occurs in all life forms to enable efficient function of tissue composed of individual cells. The DC-NK cross talk in mice and humans is one example (*23, 83, 84*).

1.3.1 NK cell activating receptor signaling

There have been many reports trying to understand the sophisticated machinery involving activating receptor signaling in NK cells in mice and humans. Activating receptors such as

NK1.1, NKp46, Ly49D and CD16 possess so called immunoreceptor tyrosine-based activation motifs (ITAM) in their cytoplasmic domains (*85*). The ITAM motifs, which is often supplied to the activating receptor by means of specific adaptor proteins such as DAP12 and FcεR1γ, is made up by a short peptide sequence (YxxL/Ix $_{(6\text{-}8)}$ YxxL/I) which, when activated, becomes phosphorylated by kinases that in turn leads to the recruitment of the Src-family kinases (SFK's) Lck, Fyn, Syk or ZAP-70 (*86, 87*). Furthermore, in the process there is agglomeration of signaling complexes consisting of SAP-family of adaptors that together result in the phosphorylation of PLC-γ1 and PLC-γ2. PLC-γ1 breaks down the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into Diacyl Glycerol (DAG) and Inositol 1,4,5-trisphosphate (IP_3) to release calcium ions from internal stores in the endoplasmic reticulum, mitochondria and calcium-containing granules in the cytoplasm (*88*).

This calcium release, which will be described in detail later, is a pivotal moment that takes place very early in the time interval of activating receptor proximal signaling and which further activates calcium-calmodulin-dependent activation of NFAT-1 and NFAT-2 (*89*). Subsequently, the signal translocates to the nucleus and results in gene transcription of IFN-γ and other important genes involved in NK effector responses to complete the cascade of signaling events over a specified time-course (*3, 90-95*). CD16 (FcγRIIIa) is an activating receptor expressed on NK cells binds to the Fc portion of antibodies bound on surface of other cells by which they get activated and perform 'Antibody dependent cellular cytotoxicity' (ADCC) (*95, 96*). NKG2D, an ubiquitously expressed receptor in NK cells from both mice and humans, surveys tissues for any kind of manifestation of stress. NKG2D signals differently than ITAM-associated receptors and uses an adaptor called DAP-10, which activates the cytotoxic program by means of an YXNM motif (X is any amino acid) in the transmembrane region of the adaptor (*41, 97-99*).

1.3.2 NK cell inhibitory receptor signaling

Inhibitory receptor (IR) expression on NK cells is an integral part of their character, development, function and homeostasis. IR's, when stimulated at the cell surface by natural means (a ligand *in-vivo*) or artificial means (antibody *invitro*) induce intracellular signaling that shut off activating signaling. The function of inhibitory signaling is thus to dampen activating receptor activity and thus reduce the overall NK cell response (*100*). There has been lot of work in the past years describing the components and mechanisms of IR signaling. SHP-1 and SHIP-1 have been the main protein molecules that are well characterised (*101, 102*). These molecules are phosphatases which dephosphorylate substrates such as Vav-1 that ultimately dampens AR signal transmission (*101, 103-113*). Though Vav-1 has been the well-described target of SHP-1 during inhibitory signaling, recent reports emerge that discover new targets of SHP-1 such as LAT, PLC-γ1 and PLC-γ2 (*109*). In addition, an intriguing inhibitory effect on proteosomal degradation has been proposed, expanding NK cell inhibiton to additional cellular processes (*109*).

1.3.3 Regulation of NK cell function by a signaling balance from cell surface receptors

From the previous overviews, it is obvious that NK cells, by virtue of their expression of activating and inhibitory receptors, is sensitive to a balanced input from various ligands on

surrounding cells which in turn then determines their final act in an immune response against any distress. This balance between positive and negative inputs is not unique to NK cells, even though it was first described in these cells, but is now known to occur in most cells and involving many receptor families. The Nobel Prize-winning research on immune checkpoint inhibitors is a good example, by which influencing the negative signals can potentiate the activating ones (*114, 115*).

Many reports in the past few years demonstrate that the intracellular signaling status of NK cells, in other words the net balance between activating and inhibitory receptor signalling, determines whether NK cells perform an activated response or do not respond at all (*94, 116-120*). The big picture of a response or no response is thus guided by fine-tuned, delicate and sophisticated perturbations in signaling networks at the molecular level. The understanding of how this balance is achieved is not known in any detail, and this is in fact been one driving theme of this thesis.

1.3.4 Calcium signaling in NK cells

The ion calcium is a versatile second messenger that forms an essential physiological component during proliferation, development, maturation, cytokine production, cytotoxicity and gene expression in biological organisms, especially in disease fighting machines (*121-124*). Calcium in cells, including lymphocytes, is stored in calcium-containing sources which include the endoplasmic reticulum, golgi apparatus, nucleus, mitochondria and lysosome-related vesicles. The net calcium content in a cell is regulated by the balance between sources providing calcium and the sinks quenching it away out of the cytosol from taking part in signaling (*125-127*). There are several proteins that regulate the transport of calcium in lymphocytes, for example SOCE (store-operated calcium entry), TRP, P2X and Cav channels. They become operational by voltage dependent mechanisms and enable the transfer of Ca^{2+} ions through differences in membrane potential across biological barriers (*128-130*).

Actin reorganisation and cytoskeletal rearrangements form an integral part of the life process in lymphocytes. TCR Microclusters (TCR MC) that form during activation consist of various scaffold and adaptor proteins forming a macromolecular complex to aid in signaling, without which calcium signaling does not occur. Thus, actin remodelling, MTOC (microtubule-organizing center) reorientation, TCR MC and Ca^{2+} signaling are linked to one another in a way that none of them function properly without the presence of other (*124*). Because calcium signaling is an integral part of the thesis, I will describe the fluxes of calcium in a bit more detail for background purposes.

a) Store-operated Ca^{2+} channels in the plasma membrane and ER: The CRAC (Ca^{2+} release-activated Ca^{2+}) channels in the plasma membrane appear to be the highly selective, major calcium entry and a prominent channel involved during antigen induced activation in T cells. Actually, not much is known about these in NK cells (*131*). Mutations in CRAC channel components results in SCID syndrome and elevates the risk of susceptibility to infections due to altered functions in T reg cell compartment (*132, 133*). CRAC channels are composed of two major proteins ORAI, also referred to as CRAC-membrane (CRACM) and STIM (stromal interaction molecule) (*134, 135*). ORAI has 3 isoforms OARI 1,2,3 and STIM has 2 isoforms 1 and 2 that have been described in mammalian cells (*136, 137*). CRAC channels are primarily required for the activation of nuclear factor of activated T cells

(NFAT) which in turn controls cytokine production, proliferation and immune fitness (*138, 139*).

b) InsP$_3$Rs and Ryanodine receptors in the ER: InsP$_3$Rs bind Ca^{2+} that is released during an activating receptor stimulated at the cell surface. There have been 3 isoforms described (*140*). The early Ca^{2+} signal (within 20 milliseconds after T cell activation) activated InsP$_3$Rs whereas NAADP and cADPR produced (within minutes of T cell activation) bind Ryanodine receptors help sustain Ca^{2+} signaling (*141*). Biochemical characterisation of these channels in NK cells warrants attention.

c) **TRP channels:** Transient receptor potential cation channel subfamily C, member 1 (TRPC 1) and TRPC 3, TRPM 2, 4, 7, proteins are expressed in T cells and affect T cell function, but their precise involvement in T cell competence remains partially unclear. To my knowledge, no papers describe these in NK cells but in T cells, they control Na^+ entry into the cell, thereby indirectly controlling Ca^{2+}-containing micro domains that participate in signaling (*142*).

d) **Purinergic ionotropic receptors and Ca$_V$ channels:** To add an additional layer of control over calcium signaling to modulate the function of lymphocytes, other channels have been described in T cells (*143-145*).

1.4 DENDRITIC CELL AND NK CELL CROSSTALK

NK cells, like all other cells in the immune system, constantly interact with other immune cells, either for their self-survival or to fine-tune a general immune response. DC's are particularly implicated in cellular interactions by means of their immunostimulatory properties, in particular in the initiation of immune responses. For NK cells, DC interactions are widely studied. For example, Flt3-ligand expanded DC's mediate controlled expansion of NK cells which in turn regulate the regression of MHC class I negative non-immunogenic mesothelioma (AK7) tumors (*23*). To investigate the molecular mechanism of DC-mediated NK cell activation, a study used a genetic approach to deplete DC's by an inducible system based on diphtheria toxin-guided depletion of CD11c$^+$ high-expressing DC's and assessed NK function and phenotype (*83*). The study confirmed the non-essential role of macrophages and metallophilic macrophages and showed that NK cell-mediated responses to a bacterial pathogen, such as *Listeria monocytogenes*, required the persistence of CD11c$^+$ high DCs. The *in vivo* site of DC-NK interaction was found to be secondary lymphoid organs such as draining lymph nodes during infection. The study also confirmed that TLR, IFN-I or anti-CD40 stimulation of DC's led to the upregulation of IL-15Rα on DC's, for the efficient trans-presentation of IL-15 to NK leading to NK competency. Thus, the molecule that regulates NK cell survival, homeostasis, proliferation and function constantly provided by DC was proposed to be IL-15 (*83*).

The molecular nature of IL-15's influence on NK cells has been the topic of many investigations. One study showed that the activation state induced by IL-15 in mouse NK cells was characterized by the induction of several markers related to metabolism (*146*). It was also proven that mTOR activity is under the strict control of developmental and inflammatory cues. In addition, it was shown that IL-15 activates mTOR S6 phosphorylation

and activates pSTAT5. Also, mTOR-/- NK cells had reduced capacity to respond to IL-15 since they had reduced CD122 surface expression. Thus, IL-15 regulates metabolic program of NK cells, thereby providing a survival advantage during a cytokine challenge by reshaping mTOR activity (*146*).

IL-15 also has remarkable effect on human NK cells (*147*) and was proposed to activate stress-related pathways, thus leading them to show potent anti-tumor ability (*148*). This led to the understanding that IL-15 induced mTOR activation, acts as a rheostat in bolstering the NK cell fitness during metabolic changes, putting mTOR as a pivotal cytoplasmic protein in regulating NK cell survival, proliferation and function (*149*) downstream of IL-15 signaling provided by DC's. A study trying to place together the function of IL-15 in regulating metabolic function and mitochondrial mechanism, the authors first proved that Mcl-1 is directly under the influence of IL-15. They show that Mcl-1 is expressed all stages of NK development. They convincingly proved that Mcl-1 induced NK cells contribute to IFN-γ /IL-6-mediated inflammation (*150*). Thus, DC-NK cross-talk, in which IL-15 is a key mediator, has been a matter of extensive investigation due to its benefit in NK cell activation. DC's provide IL-15 by means of trans-presentation to NK cells which in turn activates metabolic machinery to regulate PI3K-AKT-mTOR-Mcl-1 axis and activity for the NK cell to survive which ultimately decides the NK cell effector response.

1.5 MHC CLASS I EXPRESSION ON NK CELLS THEMSELVES: CIS-INTERACTION PARTNERS OF LY49 RECEPTORS AND SIGNALING PROPERTIES

In the last paper of this thesis, we study a property of MHC class I molecules that has not been very well studied, their intrinsic capacity to activate NK cells. As alluded to, and very important for the missing self-principle, MHC class I molecules are expressed by almost all cells in mice and humans, except for RBC's, some anucleated cells. While there have been huge efforts to characterize how MHC class I on surrounding cells affect NK cell activation and inhibition to understand NK cell effector responses, not so much has been devoted to NK cell MHC class I. Apparently, NK cells, being nucleated cells, also express MHC class I themselves and it has become clear that this intrinsic MHC class I expression has unique effects on NK cell function, at least in mice.

In early work from my supervisor's laboratory, the Ly49A^{+} receptor on NK cells expressing the H2D^{d} ligand themselves behaved differently from the same NK cell lacking the ligand (*151*). Subsequent work, also from other laboratories, showed that H2D^{d} and Ly49A indeed interact in *cis* in the NK cell surface membrane, which has profound effects on many aspects of Ly49A biology, including expression level, display of H2D^{d}-binding motifs and capacity to be inhibited by H2D^{d} on other cells (*107, 152, 153*). Not only Ly49A but also some other Ly49 receptors, including H2K^{b}, display *cis*-binding to relevant H2 ligands (*154-156*). No major role for *cis* interactions between KIR receptors and HLA on human NK cells have been demonstrated so far.

While the above-mentioned *cis* interaction has been extensively studied, there has not been many papers describing other roles for MHC class I in NK cells such as signaling. One set of studies indicate that MHC class I molecules can indeed influence intracellular signaling. Upon cross-linking MHC class I on Jurkat T cells, induction of a delayed calcium release and tyrosine phosphorylation was seen (*157*). Two studies have reported on MHC

class I signaling on human NK cells and reported that it induced apoptosis (*158*) or inhibited NK cell function (*159*). The possibility that MHC class I expression on NK cells may have several functions is interesting and suggests a more complex regulation than previously thought.

2 KNOWLEDGE GAPS FORMING THE BASIS OF MY THESIS WORK

During the time that I started as a PhD student, which feels almost like a decade ago, there were many things known and many things unknown. I myself, based on the discussion with others and with some amount of critical thinking, knew that there was much left to be discovered in NK cells during the start of this thesis. What specifically was not known then, is now evident from several papers. A retrospective analysis of the contributions by this thesis and also by several other research groups provides to some extent the 'knowledge gap' that existed few years ago in NK cell field, which is now more complete than before. Below is a summary of a few knowledge gaps from 2012, which I tried to fill in during my PhD work:

- DC's constantly contact NK cells as an important interaction that determined the anti-pathogenic and anti-carcinogenic response of NK cells, as reviewed above. The regulation of this impact needed further study and the fine-tuned regulation of DC-NK cross-talk pertaining to homeostasis and NK cell function was not known. An attempt had been tried to study NK cells in DC-depleted mice, but the early model suffered from a short kinetics frame of DC depletion *in vivo* for technical reasons. Thus, the more long-term effects of DC depletion on NK cell properties were not completely understood, including the role of IL-15 in such an influence. Moreover, would such an impact affect early signaling in NK cells?

- Though it was known that Ly49 receptors sense MHC class I by receptor-ligand interaction and this was crucial for 'tolerance' induction (*68, 77, 160, 161*), the precise molecular contributions to inhibitory signaling downstream of Ly49 receptors was not known. SHP-1 and SHIP-1 had been identified to control inhibitory signaling, but if other layers of mediators were involved was not known. The c-Abl kinase was proposed to be involved in human inhibitory signaling (*162*), which was a surprise. No data was obtained from mice, but provided a suggestive involvement of this molecule in NK cell education and in inhibitory signaling.

- No assay had been developed to study inhibitory signaling in NK cells and, thus, the nature of the inhibitory input in terms of inhibitory strength and other dynamic aspects was not well understood. Calcium flux assays had been used to demonstrate the interplay between activating receptor signaling in human NK cells (*163*). Earlier, synergy between inhibitory receptors on NK cells had been identified at the level of cytotoxicity (*164*), but the interplay between activating and inibitory receptors warranted further exploration. We postulated that the use of the calcium flux assay would be useful in measurement of proximal activating and inhibitory signaling in mice and human NK cells. This might help to test if NK cell inhibition was quantiative or threshold-based.

- The role of MHC class I on NK cells had been studied in light of the *cis* interaction with Ly49 but there were also some notes in the literature that MHC class I molecules as such could be involved in regulating NK cell function. To be honest, this question was not really defined as a knowledge gap during our early discussions, but was formulated after a

serendipitous finding I made that crosslinking of $H2K^b$ on NK cells provide a costimulatory signal to NK1.1 stimulation. The questions then were formulated to what extent there is a direct function of MHC class I on NK cells? Can they signal internally into the NK cell and contribute to function? In which way could the *cis* interaction and the signaling properties overlap?

3 AIMS OF THE THESIS

1) To use a model for genetic depletion of DC to study how this would affect NK cell development, homeostasis and function. Specifically, was there en effect on early NK cell signaling?

2) To conditionally deplete the c-Abl kinase in NK cells using a Cre-LoxP system, to ask if the c-Abl gene was involved in NK cell education and inhibitory signaling.

3) To establish an assay, bassed on calcium flux, to study the dynamics of inhibitory signaling in NK cells, with a focus on the inhibitory capacity of different inhibitory recpetors and the way they would work additively.

4) To investigate the role of MHC class I crosslinking on NK cell signaling, with a focus on the possible costimulatory role on NK1.1-mediated Ca^{2+} flux.

4 RESULTS

4.1 LOSS OF CALCIUM SIGNALING UPON DC DEPLETION IN MOUSE NK CELLS

In the first aim, my specific contribution was rather limited, but, I believe, represented an important piece of the puzzle in the interpretations on the effects of DC depletion on NK cell function. The reason I was involved was that the main focus on my work then was to set up a calcium flux-based assay that could help us to interpret activating and inhibitory receptor signaling cross-talk. As the work in the DC-depleted mice proceeded, led by my co-supervisor Kadri and fellow PhD student Luu, a question came up at what levels the functional consequences of DC depletion acted? It became obvious then to use the Ca^{2+} flux assay to determine the ability of NK cells from DC-depleted mice and thus, extend the usefulness of this assay to various other projects as well.

With my co-supervisors Bryceson and Douagi, I got in touch with the calcium assay protocol and set it up as a regular working technique in our group. The main early focus of this strategy was to measure proximal signalling input from activating receptors and it was easy to think that this technique could be used to measure NK cell Ca^{2+} flux response, as a proxy of NK cell activation, upon DC depletion. We did this by crosslinking NK1.1 using antibodies. Even though NK1.1 expression was similar in NK cells from mice with and without DC's, cross-linking NK1.1 at the surface of NK cells from *in vivo* DC-depleted mice showed a drastic reduction of intracellular calcium release *ex-vivo* (**Paper 1**). DC withdrawal thus impacted the capacity of NK cells to flux calcium. These data were consistent with the other data from the paper, showing reduced cytokine release and degranulation and also increased apoptosis and effects on NK cell maturation (**Paper 1**). We concluded from the study that DC depletion has rapid and profound effects on both homeostasis and function of NK cells, which seemed to be caused by reduced IL-15 availability and, consequently, impaired intracellular signaling.

4.2 C-ABL IS A NEGATIVE IFN-GAMMA REGULATOR IN NK CELLS BUT IS NOT INVOLVED IN NK CELL EDUCATION

Based on the article from Eric Longs group (*162*) we set out to test the function of the c-Abl kinase, more specifically c-Abl-1, in mouse NK cells by a 'loss-of-function' approach: to generate c-Abl-deficient NK cells specifically by use of a Cre-Lox system **(Paper 2).** Upon characterization of the mouse, we found that there were equal numbers of the major lymphocyte subsets in the spleen and that the maturation status of NK cells was unaltered**.** When we measured activating receptors by a flow cytometry panel, no major differences were seen. YAC-1 killing was also normal by c-Abl knock-out NK cells. We concluded that deficiency of c-Abl did not seem to affect NK cell homeostasis.

When we measured the ability of c-Abl-deficient NK cells to produce IFN- γ, we found a slightly enhanced such capacity in knockout NK cells in comparison to controls, after activating receptor triggering. This was unexpected, as our hypothesis was reduced function. Finally, we tested specifically if inhibitory signaling was affected in the absence of c-Abl. To

test this, we used the calcium flux assay and added antibodies to inhibitory receptors into the assay. The results showed that c-Abl deletion had no direct impact on the capacity of Ly49A, Ly49G2 or NKG2A to dampen calcium release downstream of NK1.1-triggering **(Paper 2).**

4.3 ADDITIVE EFFECTS OF ACTIVATING AND INHIBITORY RECEPTOR CROSSLINKING IN NK CELLS

We used the assay based on real-time calcium fluorometry by combining activating and inhibitory receptors with fluorescent antibodies and used it to only assess AR signaling. Several conclusions were drawn in that study. First, we could show that NK cell activating receptors NK1.1 and NKp46 signal differently in-terms of kinetics and the amount of calcium release (**Paper 3**). Secondly, our experimental data suggested that NK1.1 and NKp46 signaling is quantitative, meaning that to certain extent, the intracellular signals were correlated to the amount of external input on the surface, using antibody titrations as a surrogate. Thirdly, NK1.1 and NKp46 synergised with each other exhibiting an elevated release of calcium ions from internal stores compared to when NK1.1 and NKp46 were activated separately. What remains to be explored is to which extent these two receptors exhibit diversity also in *in vivo* settings, what controls the differences we saw, whether other activating receptors possessing ITAM-domains show differences and which is the nature of signals generated by activating receptors without ITAM motifs?

There are articles studying the mechanism behind IR signaling but there has been no major effort towards putting together data on the nature of inhibitory receptor signaling in mice and humans in a comprehensive manner. With our calcium flux assay, a major goal was to study inhibitory signaling by coupling inhibitory and activating receptor stimulation simultaneously. We thus had to look for reducing effects of AR triggering and to measure and quantify the inhibitory impact on calcium flux.

We found in **Paper 3** that both Ly49A and Ly49G2 exhibited a quantitative nature of inhibitory signaling in mouse NK cells, meaning that to a certain extent the external input on the surface of NK cells determines the nature of inhibition of calcium release (**Paper 3**). We could also imply a synergy between Ly49A and Ly49G2 towards the inhibition of calcium release, in comparison to when only one inhibitory receptor was crosslinked. Then, we used this assay on human NK cells from 6 normal healthy donors. We found that both $CD57^{high}NKG2A^{+}$ and $CD57^{low}NKG2A^{+}$ NK cells exhibited a quantitative nature of inhibitory signaling. Anti-CD94/NKG2A stimulation, which was used in the human setting, could quantitatively inhibit NKp46 activation (**Paper 3**). We also extended the analysis to $CD57^{low}$ NK cells and found that these NK cells fluxed calcium significantly less than $CD57^{high}$ NK cells. The reason for this observation, in my mind, could be due to gain of maturation status with CD57 acquisition. Further questions that arise: what is the mechanism behind the quantitative nature of inhibition and can it be explored to boost NK cell therapy in cancer patients?

4.4 PROPERTIES OF NK CELLS THAT MIGHT AFFECT INHIBITORY CAPACITY OF LY49 RECEPTORS: CIS INTERACTION AND IL-15 PRIMING

About 10% of C57Bl/6 NK cells are $Ly49A^{+}$. It is also one of the receptors that has been quite well studied and we found in our model that it transmitted very strong inhibitory

signaling to NK cells from C57Bl/6 mice. Moreover, Ly49A is also known to interact with H2D^{d+} in *cis*, which has been suggested to affect its function. To test this in our model of calcium signaling, I coupled Ly49A and NK1.1 with co-crosslinking antibodies in our calcium flux assay and tested it on both mice expressing H2D^{d} and in mice that did not express this MHC class I molecule, such as C57Bl/6, and performed a parallel series of antibody titration experiments. I titrated anti-Ly49A antibody from 1:200 to 1:6400 in both strains and found that Ly49A^{+} NK cells from C57Bl/6 mice exhibited powerful inhibition at higher concentrations and the nature of inhibition was quantitative. In contrast, in H2D^{d+} mice, Ly49A^{+} NK cells did not exhibit any trace of inhibition, at any antibody concentration, despite similar staining intensityin key concentrations. As a control receptor, I titrated anti-Ly49G2 antibody and found that anti-Ly49G2^{+} NK cells in both C57Bl/6 and H2D^{d+} mice showed powerful quantitative inhibitory effects **(Paper 3).** Thus, presumably due to the *cis* interaction between the receptor Ly49A and its ligand H2D^{d}, at the surface of H2D^{d+} NK cells, the antibody used to crosslink Ly49A receptor cannot efficiently trigger an inhibitory signal. This was independent on the staining intensity. This observation is quite remarkable since this raises an interesting question as to how Ly49A^{+} NK cells are 'licensed' *in vivo*. In addition, one could test is whether also other *cis*-interacting activating and inhibitory receptors exhibit similar relationships?

Another important factor for NK cell function, as mentioned, is IL-15. We realised that we could ask, by using our inhibitory signaling assay, if NK cell priming by IL-15 would potentially work by affecting the inhibitory receptor function? Thus, after the addition of IL-15 to NK cell cultures, would the balance between activation and inhibition be affected? The series of experiments revealed that IL-15 did indeed prime NK cells upon NK1.1 triggering, leading to enhanced calcium release, but the relative inhibitory capacity of Ly49A and Ly49G2 on NK1.1 signaling seemed to remain unaltered. Thus, IL-15 seems to influence activating receptor signaling but not the impact of inhibitory receptor signaling **(Paper 2).**

4.5 CROSSLINKING MHC CLASS I ON THE NK CELL LEADS TO AUGMENTED CALCIUM FLUX

In our inhibitory receptor calcium assay, when needed a control of Ly49 receptor crosslinking and tested MHC class I, expecting this molecule not to signal. In contrast, when we co-crosslinked NK1.1 with the MHC class I molecule H2K^{b}, we observed an additive calcium release response, both with a higher peak and a sustained response. When H2K^{b} was triggered alone, we could observe a delayed slow and transient release of calcium in NK cells for 150-200 seconds **(Paper 4).** When we triggered another MHC class I molecule, H2D^{b}, we could observe a similar, delayed slow and transient release of calcium ions. However, H2D^{b} did not synergise with the NK1.1 receptor to the same extent as H2K^{b}. In H2D^{d+} mice, H2D^{d} crosslinking responded in a similar manner to H2K^{b} when triggered alone or coupled with NK1.1.

Thus, all MHC class I allelles we tested, when triggered alone, displayed a similar delayed late slow transient calcium release response. They also all provided a costimulatory effect on NK1.1 crosslinking, but with different effect, **(Paper 4).** Next, it is known in the NK cell field that H2K^{b} and Ly49C interact in *cis* on the surface of NK cells. Wanting to know whether this interaction interfered with H2K^{b} and NK1.1 signaling, we stained for Ly49C (using an antibody that did not crosslink the receptor) and studied the effects of

crosslinking H2K^b and NK1.1 at the same time. The conclusion from this experiment was that Ly49C$^+$ and Ly49C$^-$ NK cells showed a similar response, suggesting that the *cis*-interaction with Ly49C did not affect the costimulatory capacity of H2K^b.

We next investigated if extracellular calcium ions during signaling played a role in signaling. We thus performed the experiment in the presence of either HBSS or PBS, containing Ca^{2+} or not. The results showed that in HBSS, calcium flux was stronger compared to in the absence of Ca^{2+} (PBS) but the magnitude of the NK1.1+H2K^b co-crosslinking was still the same. Two more sets of data were generated: First, we confirmed, by including an antibody against Ly49G2 in the triggering, that the stimulatory effect of H2K^b was subject to inhibition. Next, the obvious question was whether IFN-γ and degranulation was augmented upon NK1.1+H2K^b activation in a time-dependent manner. Interestingly, H2K^b triggering did NOT lead to an augmented IFN-γ response. Similar results were obtained for CD107a.

5 DISCUSSION

5.1 LOSS OF NK CELL FUNCTION UPON DC DEPLETION

It is quite well known that immune cells regulate NK cell function, homeostasis and development. Our data suggested that DC's continuously interact with NK to provide them with vital life-sustaining support in the form of IL-15 **(Paper 1)**. There have been reports stating that this interaction happens in lymph nodes in mice during infections (*83*). Our study added a significant aspect since we show that this cellular interaction happens also during normal NK cell homeostasis, in a disease-free steady state. It is quite a difficult question to test whether this is also true in humans and other mammals, even if there are *in vitro* models to suggest that the DC-NK cell interplay is important also here. Moreover, if other internal organs than lymph nodes provide cellular niches for these interactions is not known, but it is a relevant question given the idea of NK cell migration and retuning to maintain homeostasis and a functional capacity to response properly to insults (*165*).

DC's provide IL-15 to NK by means of *trans*-presentation IL-15 and IL-15 receptor α chain to the β and γ chain of the IL-15 receptor expressed on NK cells. It is likely that a constant signal from this cross-presentation provides the effect that we identified. Because IL-15 is a growth and survival factor for NK cells, the effects on homeostasis was predictable. In contrast, the rapid loss of function, preceding the homeostatic effects, was less obvious. An interesting effect of IL-15 stimulation is IL-15 priming, which seems to be a more rapid early effect that precede proliferative effects (*149*). From our data, we thus predict that the loss of NK cell function after DC depletion is likely due to loss of NK cell priming, which is then suggested to take place continuously. In addition, our data suggest that it might operate by downtuning intracellular signaling, manifested by a reduced calcium flux. We do not know whether other NK cell receptors also exhibit a similar loss of ability in calcium signaling since we only tested NK1.1 triggering. However, it should be mentioned that we do not know whether the loss of calcium signaling is a direct effect of DC removal, and loss of IL-15, or if the DC removal has other effects that in turn effect calcium signaling.

Further experiments would have to address these issues, for example by testing these mice during viral or bacterial infections. Furthermore, it is of interest to ask if also other cells, when removed from the vicinity of NK cells, for example neutrophils, show similar effects on NK cells? A more philosophical question is why the calcium fluxing ability in NK cells would be regulated by DC's? Furthermore, to tie this question to the *in vivo* effects on NK cell education is of interest. We did observe, in **Paper 1**, that the Ly49 receptor repertoire was altered. To which extent this was an effect of NK cell apoptosis and accumulation of immature NK cells or influenced by NK cell education, remains to be investigated, including if there is a role of MHC class I on DC for the effects we observed.

5.2 C-ABL AS A NEGATIVE REGULATOR OF IFN-GAMMA PRODUCTION

The c-Abl non-receptor tyrosine kinase was experimentally shown to be involved in B and T lymphocyte development. Not only restricted for its role in differentiation, it was also shown to regulate antigen responsive functions and to humoral immunity. Not long ago, c-Abl knock

out chimaeras showed unaltered functions of NK cells (*166*). c-Abl was then shown to be involved in human NK cell function and in particular inhibitory signaling (*162*). Thus, we postulated that in mouse NK cells, c-Abl regulated the formation of a complex consisting of c-Abl, Crk and c-Cbl and C3G, which would in turn play a role in NK cell education (*8*). We had lots of early problems in generating mice with c-Abl knocked out in NK cells. We never achieved complete deletion in our first model, so we had to switch to an alternative NKp46-driven Cre model. Eventually, I persisted and in the end gained control of the model system to generate c-Abl-deficient NK cells.

The *modus operandi* showed that our approach generated mice with a deficient c-Abl gene. Thus, c-Abl mRNA was seen in $CD3^{+}$ cells but not in NK cells. Nevertheless, our mice did not show any homeostatic effects in NK cells and did not affect the acquisition of maturation markers CD27 and CD11b, which suggested either compensatory effects of the c-Abl-deficiency by some unknown mechanism that regulated CD27 and CD11b positivity, or that c-Abl deficiency is indeed not involved in NK cell maturation. When desperately looking for a phenotype in c-Abl knock out NK cells, I stumbled upon IFN-γ protein upregulation after NK1.1 and $NKp46^{+}$IL-15 triggering. Activating receptor expression was tested and found to be normal. We did not confirm that this was a transcriptional effect since we did not check for IFN-γ mRNA levels. Not only the frequency of IFN-γ-positive cells was enhanced, also MFI levels were slightly increased in c-Abl fl/fl NK cells.

The question we decided to test was whether we could directly show the role of c-Abl in inhibitory receptor signaling. This attempt represented, *de facto*, the first time I was able to directly visualize NK cell inhibition using flow cytometry, exploring the idea of gating on flourescently labeled antibodies again inhibitory receptors and studying calcium flux in IR-positive and IR-negative cells in the same sample. We thus concluded our study by showing that c-Abl was not critically involved in inhibitory signaling leading to calcium flux.

Having generated these data, we decided to publish the data. However, I believe that this study also raises interesting questions for the future. Perhaps the most important being by which mechanism deficiency in c-Abl leads to elevated IFN-γ responses in NK cells? We did not pursue this in the present study, because we did not consider the difference being large enough to merit several additional years of work. However, if time permits and additional readouts can be developed, this finding might nevertheless give interesting new leads into NK cell activation. Another question is if there is really a fundamental difference between mouse and human NK cells in the involvement of c-Abl, or if the fact that we study c-Abl-deficiency during NK cell developments lead to compensatory mechanisms? In order to study this, one would have to develop assays to test the role of c-Abl in mature normal NK cells, similarly to what was done in humans. Finally, there is an interesting recent study in which NK cells from Crk/CrkL double-deficient mice showed reduced functionality. It is unclear how this study might relate to ours, but if c-Abl and Crk somehow co-regulate IFN-γ secretion in mice, the two studies imply that the pathway that we were interested in might affect NK cell function. Finally, *in vivo* challenges of mice with an NK cell-specific c-Abl knockout could be attempted. Perhaps a more robust *in vivo* challenge, for example with CMV, might reveal a more pronounced phenotype.

5.3 STUDIES OF THE INTERPLAY BETWEEN ACTIVATING AND INHIBITORY RECEPTORS ON CALCIUM SIGNALING

Activating receptors NK1.1 and NKp46 are supposed to be very similar ITAM-dependent receptors. However, upon testing of the receptors with the calcium flux assay, I could find that the two molecules actually signal differently, in terms of kinetics and the amount of calcium ions released. This is quite striking. The result brings out the wonderness of biological heterogeneity. Though they are supposed to signal similarly, they are still very different. What might determine this difference is not easy to answer. One possibility is that they exist in different types of multi-protein clusters in the cell membrane, and their close partners affect recruitment of signaling adaptors or inhibitory phosphatases. Nevertheless, even though not similar, they still synergise in their activation, which makes it even more interesting. It is known that receptors synergise for better NK function (*163*), but this was the first time shown in mouse NK cells. It remains to be tested for other receptors and ITAM versus non-ITAM receptors. In addition, receptor signaling has always been discussed as a threshold. My results show that signaling might not operate in terms of thresholds, at least not at the level of calcium release, but that it is quantitative in nature. By using a different technique, I could actually bring a different type of concept to the field, which is challenging to the knowledge that exists. What is really true, is based on the context as to what is the question and what is the method.

It was known that inhibitory receptors synergise for a killing phenotype (*164*), but using a calcium assay to prove that such a synergy exists in primary mouse NK cells is quite novel. What are the mechanisms behind such a delicate balance that makes tiny changes affecting it? Kinases and phosphatases operate in such signaling balances and it is likely that the same balance determines the quantitative effects that we have seen. To experimentally demonstrate the recruitment of kinases being involved in phosphorylation and phosphatases involved in dephosphorylation in real-time is difficult. Again, is inhibitory receptor synergy in general or seen only for a few receptors and if also other functions in NK cells are regulated in this way remains to be seen. Also, how is apparent quantitative and dynamic receptor signaling consistent with previous work suggesting signaling threshold involving inhibitory input by human HLA (*167*)? Perhaps signaling operate with multiple switches, the first being quantitative and more downstream, there might be those with more threshold-like operations. Finding experimental models to test this is a challenge but might be worthwhile.

5.4 LIGAND-RECEPTOR INTERACTION IN *CIS* AND IL-15 PRIMING: EFFECTS ON INHIBITORY SIGNALING

Ly49A^{+} NK cells from H2D^{d+} mice perform better cytotoxic function and respond better with activating receptor stimulus compared to Ly49A^{+} NK cells from mice lacking H2D^{d} (*168*). The plausible explanation for this phenomenon is that Ly49A^{+} NK cells are better "educated" in the presence of a strong MHC class I ligand (*8*). How MHC class I-guided NK cell education is regulated is not known. One suggestion is that education is mediated by MHC class I on other cells. On the other hand, it has also been suggested that, in the case of Ly49A at least, the interaction with H2D^{d} in *cis* is the primary mediator of their better functional phenotype (*153*). The explanation is elegant: The *cis* interaction with H2D^{d} prevents Ly49A from moving into the activating immune synapse, thus allowing an ITAM-triggered signal to

proceed into function. In $H2D^{d-}$ NK cells, on the other hand, the 'free' Ly49A receptors moves into the synapse upon NK cell activation, where it provides signaling and shuts off killing. Why such 'unengaged receptors' would signal spontaneously is not known. The model also cannot explain escape from nonspecific inhibition by receptors that does not bind MHC class I in *cis*.

It is a consensus in the field that inhibitory receptor signaling by Ly49 receptors is crucial for gain of functionality by NK cells during NK cell education (*54*). Not only this is important for education but also is important for self-tolerance which controls their non-responsiveness to self-tissues. In light of this, the results in my hands, that $Ly49A^{+}$ and $Ly49A^{-}$ NK cells from $H2D^{d+}$ mice responded similarly in a calcium flux experiment despite co-crosslinking of Ly49A was indeed surprising. This suggested that IR signaling is incapacitated in $Ly49A^{+}H2D^{d+}$ NK cells through Ly49A receptor. This was specific for Ly49A, since $Ly49G2^{+}$ NK cells elicited a perfect inhibitory signaling response. This observation raised several thoughts. How do then, $Ly49A^{+}$ NK cells become educated or licensed or maintain self-tolerance? *A priori*, this experimental results poses a serious challenge to the NK cell licensing theory. Or maybe it provides support for the model proposed by Chalifour et al, that the only thing that matters is that Ly49A interacts with $H2D^{d}$ on the NK cell itself? On the other hand, perhaps the data is even more difficult to understand from the point of view of missing self. How could $Ly49A^{+}$ NK cells from $H2D^{d+}$ mice, which represents the most educated and most functional subset we know of, be useful in missing self-recognition if it cannot sense $H2D^{d}$ on other cells? However, my result is purely based on antibody crosslinking and as such being an *in vitro* observation. Maybe *in vivo*, or in a cell-cell interaction, the entire event takes place in a very different manner, which is to be explored in the future. Therefore, I cannot rule out the possibility, that there are multiple interactions controlling the signaling of the Ly49A receptor which enables them to be better educated and gain better function *in vivo*.

IL-15 has become a famous molecule in the recent past due to its potent action on several aspects of NK cell function including development and priming. (*83, 149, 169*). It has not been properly investigated if IL-15 somehow intersects with NK cell inhibition. My result with IL-15 showed that it primes NK cells to an activated NK cell calcium signaling but that it does not intersect with NK cell inhibition.

5.5 MHC CLASS I SIGNALS FEED INTO PROXIMAL ACTIVATING RECEPTOR SIGNALING IN NK CELLS

Through a process of natural selection, MHC class I molecules not only enable host cells to sense dysbiosis, they also crucially function as a tolerance inducing molecule by defining 'self' *versus* 'non-self'. As they are ubiquitously expressed in almost every tissue, they control inflammatory insult on a global scale. Though signaling by MHC class I molecules has been shown to be present in Jurkat T cells and human NK cells, our data is the first to study this in real-time by a calcium flux assay. $H2K^{b}$ cross-talk with NK1.1 is new to mouse NK cell biology and raises several new thoughts. Some of them are related to the *in vivo* relevance. Does $H2K^{b}$ interact with some ligand *in vivo* that might, in collaboration with NK1.1, provide enhanced NK cell activation in spleen, bone marrow or lymph nodes? Or is there in fact no external ligand to $H2K^{b}$ on surrounding cells, but it operates simply by some form of *cis*-binding (but apparently not to Ly49C)? Irrespective, MHC class I influence on

proximal activating receptor signaling is not only new but also fascinating. However, if the laws that govern NK cell education, or missing self, should be rewritten or not must be put on hold. It is important to bear in mind that ours is an *in vitro* result. Nevertheless, even if it cannot be excluded that we have been studying an artefact (for example due to antibody binding that causes a cell to leak calcium internally), the inhibitory influence on MHC class I signaling effects were inhibitable by Ly49G2 crosslinking, suggesting that the signal is real.

One mechanistic alternative we have discussed is that the costimulatory effect is not a direct effect of $H2K^b$ crosslinking, but an indirect effect caused by binding of the Fc part of the anti-$H2K^b$ antibody to the FcγRIIb (CD16) on the NK cell itself. Thus, our results would then explain a synergistic signaling response between two ITAM-encoded receptors: NK1.1 and CD16. We tried to block CD16 using either clone 2.4G2 or clone 93 to study this. While NK1.1+$H2K^b$ co-crosslinking was still additive in the presence of blocking antibodies, the results were inconclusive. For 2.4G2, we were not able to stain for CD16, making it uncertain if this particular antibody actually worked. Clone 93 did stain the NK cells, but we have not yet performed a sufficient number of experiments to say for sure that the $H2K^b$ co-stimulating effect is unaffected. Perhaps arguing against a pure CD16 effect would be the unusual kinetics of the Ca^{2+} flux, which is not really characteristic of an ITAM response (rapid onset and then decline) similar to what we have seen for NK1.1 and NKp46 triggering (slow onset and sustained slope). Further work is required to settle this important point.

A final few points should be made. 1) Various calcium channels in the cells could differentially initiate, stabilize and terminate calcium signaling upon $H2K^b$ and NK1.1+$H2K^b$ signaling. So far, we have only tried Ca^{2+}-free buffers and found that the additive effects was still seen despite lack of extracellular calcium, but inhibitors or other types of targeting of various protein channels involved in calcium signaling could be used to further investigate the source of calcium for the $H2K^b$ effect. 2) There are some discrepancies in the literature regarding the outcome of MHC class I crosslinking. In NK cells, it has been shown that MHC class I cross-linking leads to cell death and to NK cell inhibition. The methods used were different, however, and it would be good to test MHC class I cross-linking in human NK cells by calcium assay to know whether this scenario exists in humans or is it specific to mice. Finally, I would like to comment on the fact that there was no elevation of IFN-γ or CD107a expression when NK1.1 stimulation was compared to NK1.1+$H2K^b$ stimulation. This could be due to differences in how the crosslinker was added during the two setups; soluble *versus* plate-bound. Additional work is required and novel technical platforms should be set up to test this.

6 MATERIALS AND METHODS

Each paper in this thesis contain detailed materials and methods where key information can be found regarding how the experiments were performed. Here, I make a brief recollection of the methods and discuss advantages and drawbacks with the methods used.

6.1 MICE

All mouse strains were bred and maintained at the animal facility in Karolinska Institutet, PKL, Karolinska University Hospital Huddinge, according to international and institutional guidelines. The following mouse strains represent the major focus:

iNcrCre+/-: These mice were obtained in collaboration with Dr. Eric Vivier, Centre d'Immunologie de Marseille-Luminy, Université de la Méditerranée UM 631, Marseille, France (*79*). They were bred in heterozygous state.

c-Abl floxed/floxed: These mice were obtained in collaboration with Dr. Dawn Wetzel, Children's Hospital of Dallas, Dallas, Texas, USA and Dr. Anthony Koleske, Yale University, New Haven, Connecticut, USA (*170*). Similarly, to iNcrCre mice, c-Abl mice were bred in a heterozygous state.

C57Bl/6: This is a standard inbred mouse strain, which was purchased either from Jackson Laboratories or Charles River laboratories. They were maintained by standard inbreeding.

Single $H2D^d$ mice: These mice were generated by breeding MHC class I-deficient mice with C57Bl/6 mice expressing an $H2D^d$ transgene (*69*) and selecting for lack of $H2K^b$ and $H2D^b$ but expression of $H2D^d$. They have been described in detail before (*76*).

CD11c.DOG mice: Mice with the diphtheria toxin gene driven by the CD11c promoter and with a special property of mediating deletion for longer times (*171*). The mice were obtained in a collaboration with Natalio Garbi, Heidelberg, Germany.

Advantages:
The mouse is a mammal that has its genome mapped, so gene-protein function can be correctly associated. Germ-line knock outs can also be synthetically designed and tested in-vivo. The mouse is easy to manipulate. Several observations in physiology are similar to humans. For NK cells, functionality is similar in principal, though they may have some dissimilar molecules. Many NK cell observations have been replicated in humans

Disadvantages:
Observation found in mice have to be re-tested in humans to translate knowledge to human diseases. The mouse is still not a human. It has a shorter lifespan compared to humans and they are inbred, which makes the genetics completely different. They are also kept in clean conditions, which is very different from human. Mice have to be genotyped by conventional

methods, which is time consuming. There are also ethical issues where or not it is correct, or defendable, to use mice in biomedical research.

6.2 CYTOKINE STIMULATION ASSAY

24-well plates were incubated at 4°C in PBS with 20 ug/ml of purified anti-NK1.1 and anti-NKp46 antibodies. Fresh splenocyte solution was prepared and NK cells were isolated using the MACS Miltenyi Biotec negative isolation kit. Approximately 1-1,5x10^5 NK cells were stained with antibodies at 4°C in PBS for 30 mins. Both the plate and the cells were then washed with PBS to remove excess antibodies. NK cells were placed in the wells with antibody and stimulated for 2, 4 and 6 hrs at 37°C in 10% FCS+RPMI. To stain for CD107a, anti-CD107aPE antibodies (1:200 or 1:300) were added during the start of stimulation. Golgi Plug (1:1000) was added to prevent cytokine leakage during stimulation. PMA (phorbol-12-myristate-13-acetate) was used as positive control for stimulation and PBS as negative control. BD Cytofix/Cytoperm kit was used to perform intracellular detection of IFN-γ using anti-IFN-γ PE /anti-IFN-γ PE-Texas Red.

Advantages:
An antibody targeted against a specific receptor NK1.1 can be used, studying a particular pathway. The effector functions, IFN-γ release and CD107a (a surrogate marker of cytotoxicity) can be studied in individual NK cells, providing a measure of heterogeneity. Can be controlled for a specific time point. Cells can also be fixed and analyzed later.

Disadvantages:
Stimulation is an artificial *in vitro* response that does not mimic the complexity of a cell-cell interaction. It also does not mimic the *in vivo* environment when many interactions determine NK activation and inhibition.

6.3 CALCIUM FLUX ANALYSIS ON NK CELLS

Isolated mouse NK cell suspensions were prepared using a negative isolation kit from MACS Miltenyi. Purity check and cell counting was performed using Neubauer chamber Bürker, 0.100 mm depth, 0.0025 mm^2. NK cells were subsequently incubated with aqua live/dead antibodies and antibodies against cell surface receptors at 4°C for 30 mins in HBSS+ 2% FCS. After staining, NK cells were washed and then stained for 30 mins at 4°C in HBSS+2%FCS and probenecid was added during incubation with Fluo-4 and Fura-Red. Cells were rested on ice until further analysis. About 20000 to 25000 NK cells were analysed by the LSR Fortessa. During acquisition, fluorescence was acquired for 30 seconds, then the crosslinker was added and data was further acquired for 300 secs (mice) and 240 secs (humans) in total.

Advantages:
Responses from triggering any specific receptor using primary antibodies and secondary antibodies. It can be used to measure both activation and inhibitory signaling. The response can be controlled for a specific time point and real-time responses can be quantified.

Disadvantages:
Intracellular analysis of signaling molecules by flow cytometry cannot be performed since live cells are needed. The method is a bit cumbersome and subject to rather large variability. Requires high purity of NK cells which can be expensive to achieve.

6.4 REAL-TIME PCR

RNA extraction by Qiagen RNA extraction kit from isolated NK cells by MACS Miltenyi kit. RNA – cDNA conversion was performed by Promega kit. Converted DNA was amplified by Real-Time Polymerase Chain Reaction by an in-house protocol with reagents from common suppliers. Amplified cDNA was check for c-Abl mRNA in comparison to actin controls. Values obtained upon amplification were exported to MS-excel and data was plotted on Graph Pad prism.

Advantages:
Quantifies precisely the mRNA content of any gene of interest. It is highly sensitive and provide quantitative measures.

Disadvantages:
Might be prone to artifacts and contaminations. Cumbersome with many careful steps which makes it time-consuming. Depends on a house-keeping gene as control, which might be subject to variations. May not directly reflect protein expression.

6.5 ^{51}CHROMIUM RELEASE ASSAY

Target cells, in this case YAC-1, were incubated with ^{51}Cr for 1 hr and then washed in PBS. Negatively isolated pure NK cells were mixed with YAC-1 cells at various E:T ratios and incubated for 4 hrs at 37°C. After incubation, cell culture supernatants were subject to analysis by a γ-counter and percentage of killing was calculated by a mathematical formula.

Advantages:
Gives an estimate of NK cell killing capacity against real target cells compared to antibody-stimulation assays. Standard method to to measure NK spontaneous killing.

Disadvantages:
Only measures killing capacity as an average in a population of NK cell and the function of individual NK cells cannot be determined. Requires radioactive isotopes and is not quantitative.

7 FUTURE PROSPECTS

The work from this thesis, which of course is based on the huge body of knowledge that already existed, explored many aspects of proximal activating and inhibitory receptor signaling in NK cells, both mice and humans, which was not available before. c-Abl as a signaling protein was only known to be pro-inflammatory. As a twister, the role of c-Abl as an inhibitor of IFN-γ in mice NK cells is quite new. Is it true also in humans? Which is the mechanism underlying this effect? Can it be used to treat people with cancer? Moreover, a forward genetic screen to identify negative regulators of effector functions in NK cells could be of relevance to NK cell biology and to NK cell therapy-related measures.

Proximal signaling, being quantitative and synergistic in both mice and humans, gives researchers a new understanding and a possibility to develop novel methods to explore dynamics of signaling in real-time. To-date, estimation of phosphoproteins in a calcium fluorometric experiment is not possible. Experts in cytometry might be able to design brand new machines that can investigate this problem. Reaching an increased depth into the complicated nature of signaling in cells will be useful to people working in many areas of biological research.

The role of MHC class I as a signaling mediator in activating receptor signaling is a teaser. The use of antibodies against MHC class I was intended as a negative control and I did not expect this result. Can this finding be used to explore the role of MHC class I and its interactions with Ly49 and KIR receptors in NK cells a bit more *in-vivo*? I think that the role of MHC class I in NK cell biology is still very much under-estimated. There is so much to be done yet in the lab. So: what's the next experiment to test?

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