DEPARTMENT OF MICROBIOLOGY, TUMOR AND CELL BIOLOGY
Karolinska Institutet, Stockholm, Sweden

# NK CELLS AND MISSING SELF RECOGNITION: GENETIC CONTROL, MHC CLASS I DEPENDENT EDUCATION AND POTENTIAL USE IN CANCER THERAPY

Stina L Wickström


Karolinska Institutet

Stockholm 2015

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB

ISBN 978-91-7676-046-8

The cover picture is drawn by Ronja Wickström 6 years old.

# NK CELLS AND MISSING SELF RECOGNITION: GENETIC CONTROL, MHC CLASS I DEPENDENT EDUCATION AND POTENTIAL USE IN CANCER THERAPY

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

By


**Stina L Wickström**

*Principal Supervisor:*
Maria H Johansson
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

*Co-supervisor(s):*
Klas Kärre
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Hanna Sjölin
Lund University
Department of Experimental Medical Science
Division of Immunology

*Opponent:*
Werner Held
University of Lausanne
Department of Ludwig Center for Cancer Research

*Examination Board:*
Yenan Bryceson
Karolinska Institutet
Department of Medicine
Division of Center for Infectious Medicine

Lisa Westerberg
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Kristoffer Hellstrand
University of Gothenburg
Sahlgrenska Cancer Center at Institute of Biomedicine

*Till mina underbara barn Ronja och Emil* ❤

# ABSTRACT

NK cells belong to the innate immune system and are important in the defense against virus infections and malignant cells. They mediate their effector functions via release of cytotoxic granules and by cytokine production which can influence the status of other (immune) cells. NK cells are regulated by germline encoded receptors, both activating and inhibitory, recognizing molecules that are induced upon infection or cellular stress and self ligands respectively. Ly49 receptors (Ly49r) make up the largest NK cell receptor family in mice. It contains both activating and inhibitory receptors most of which bind to major histocompatibility complex class I (MHC I) molecules. NK cells patrol tissues and inspect surrounding cells for alterations in activating ligands and MHC I expression, balancing the input for decision of response. If the activation exceeds the inhibition, the target cell is eliminated. This ability to sense loss of self MHC I is referred to as missing self recognition. It can be directed against virus infected cells and tumor cells which often downmodulate MHC I, while they upregulate activating ligands.

NK cells are educated via Ly49r-MHC I interactions to ensure self-tolerance and reactivity against aberrant cells. MHC I dependent education influences the NK cell population in at least two ways; modulation of responsiveness of each cell and skewing of the inhibitory receptor repertoire, i e the frequencies of NK cells expressing different combinations of Ly49r. The main aim of this thesis has been to study missing self recognition and MHC I dependent NK cell education and how these phenomena are influenced by different factors.

In paper I, we characterized a genetic defect leading to Impaired Missing Self Recognition, in a mouse strain that we have termed IMSR mice. These mice had originally been developed by targeting a non-classical MHC gene, but the defect and the IMSR defect segregated independently. The IMSR mice were found to have a normal number of NK cells, which retained some functions, while missing self rejection and some activation pathways were partly or completely impaired. This defect was found to be NK cell intrinsic; it was not due to total lack of inhibitory receptors function, nor lack of MHC dependent education.

In paper II and III we investigated how NK cells respond to altered inhibitory input from the environment in the host. Antibody mediated inhibitory receptor blockade was used as a tool to reduce the inhibitory input, which led to two different effects on the targeted NK cell populations 1) increased in vivo elimination of MHC $I^+$ tumor cells without breaking tolerance towards normal healthy cells (paper II) and 2) induction of hyporesponsiveness i e reduced in vitro responsiveness or reduced capacity to eliminate MHC $I^-$ spleen cells. Importantly, elimination of MHC $I^-$ tumor cells was maintained. This was also investigated in an adoptive transfer model where the NK cell responsiveness could be either increased or reduced, depending on the MHC I expression in the recipient host (paper III). In conclusion, we found that NK cells can retune their responsiveness upon altered inhibitory input, but that responsiveness levels are adapted to healthy cells, still allowing efficient killing of tumor cells of the same missing self phenotype.

In paper IV, we investigated whether skewing of the inhibitory receptor repertoire occurs already during NK cell development, before they reach the blood and the spleen. We found that the process leading to overrepresentation of NK cells expressing only one self MHC receptor is initiated during in the bone marrow already at the first NK cell developmental stage where inhibitory Ly49 receptors are expressed. This is most probably influenced both by selective proliferation and apoptosis.

# LIST OF SCIENTIFIC PAPERS

I. **Stina L Wickström**, Linda Öberg, Klas Kärre and Maria H Johansson.
A genetic defect in mice that impairs missing self recognition despite evidence for normal maturation and MHC class I-dependent NK cell education. *Journal of Immunology* 192:1577-1586, 2014.

II. Gustaf Vahlne, Katja Lindholm, Anders Meier, **Stina Wickström**, Tadepally Lakshmikanth, Frank Brennan, Michael Wilken, Rikke Nielsen, Francois Romagne, Nicolai R. Wagtmann, Klas Kärre and Maria H. Johansson.
In vivo tumor cell rejection induced by NK cell inhibitory receptor blockade: maintained tolerance to normal cells even in the presence of IL-2.
*Eur. J. Immunology* 40: 813–823, 2010.

III. Arnika K Wagner*, **Stina L Wickström***, Rossana Tallerico, Sadia Salam, Tadepally Lakshmikanth, Hanna Brauner, Petter Höglund, Ennio Carbone, Maria H Johansson and Klas Kärre.
Retuning of mouse Natural Killer cells by interference with MHC class I sensing adjusts self tolerance but preserves anti-cancer response. Submitted 2014.

IV. Hanna Brauner, **Stina L Wickström**, Arnika K Wagner, Marjet Elemans, Ramit Mehr, Maria H Johansson, Klas Kärre
MHC class I dependent shaping of the NK cell receptor repertoire takes place already early during maturation in the bone marrow. Manuscript.

* Arnika K Wagner and Stina L Wickström contributed equally to the work

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADCC | Antibody dependent cellular cytotoxicity |
| $\beta_2$m | $\beta_2$-microglobulin |
| CLP | Common lymphoid progenitor |
| DAP-10,-12 | DNAX activating protein of 10 or 12KD |
| ELP | Early lymphoid progenitor |
| HSCT | Hematopoietic stem cell transplantation |
| HLA | Human leukocyte antigen |
| IFN | Interferon |
| IL ILC | Interleukin |
| IMSR | Innate Lymphoid cells |
| ITAM | Impaired missing self recognition |
| ITIM | Immunoreceptor tyrosine-based activation motif |
| KIR | Immunoreceptor tyrosine-based inhibitory motif |
| | Killer cell immunoglobulin-like receptor |
| KLRG1 | Killer cell lectin-like receptor G1 |
| KO | Knockout mouse |
| Ly49r | Ly49 receptor |
| NK cell | Natural killer cell |
| NKP | NK cell progenitor |
| MCMV | Murine cytomegalovirus |
| MHC | Major histocompatibility complex |
| PI3K | Phosphinositide 3-kinase |
| PLC-γ2 | Phospholipase C-γ2 |
| SHIP | SH2-containing inositol polyphosphate 5-phosphatase |
| SHP | SH-containing protein tyrosine phosphatase |
| TAP | Transporter associated with antigen processing |
| TF | Transcription factor |
| Tg | Transgenic |
| TRAIL | Tumor necrosis factor (TNF)-related apoptosis-inducing ligand |
| Wt | Wild type (mouse) |

# 1 INTRODUCTION

## 1.1 THE DISCOVERY OF NATURAL KILLER CELLS AND THEIR REGULATION BY MHC GENES

Natural Killer cells (NK cells) were discovered and described in 1974 by Rolf Kiessling, Hans Wigzell and Eva Klein after first having been being considered as "background noise" in assays searching for cytotoxic T cells in tumor bearing and normal individuals. They represented a new cell type detectable in non-immunized mice, with a rapidly occurring, natural in vitro cytotoxicity against YAC-1, a lymphoma induced by Moloney leukemia virus. (1). The follow up paper from the same group showed that these new cytotoxic cells could be found in mice from 3-10 weeks of age and were most abundant in the spleen (1-5% of spleen cells), although they could also be found in lymph nodes (LN) and bone marrow (BM) (2). They described their finding as follows: "*We have found a spontaneously occurring cell with cytolytic activity against in vitro grown Moloney Leukemia cells. It is present predominantly in the spleen of normal, young mice with limited activity to be found in other lymphoid organs….. At present we can only conclude that the present killer cells have a morphology of small lymphocytes*" cited from (1). Competition studies suggested that these new killer cells were mainly reactive against Moloney leukemia virus antigens (Ag). However, this was already proven to be incorrect by Herberman et al. the same year who in an independent but similar study in the mouse showed that natural cytotoxicity occurred against various tumor targets, not only virus induced (3). Both groups came to the conclusion that the cytotoxicity observed was not mediated by T cells, B cells or macrophages, nor was it likely to reflect so called antibody dependent cell mediated cytotoxicity (ADCC) (2, 4). These newly found cytotoxic cells were termed "natural" Killer cells (NK cells) in the paper of Kiessling et al., a name originally suggested by Eva Klein.

One of the first published studies on human NK cells by Pross et al. showed that natural cytotoxicity varied between different donors but was stable over time in the same individual. This study also suggested that the cytotoxicity of human NK cells can at least in part be mediated through ADCC, since removal of Fc receptor expressing cells and lack of antibody (ab) coating of target cells in some settings could abolish the natural cytotoxicity (4a). A third paper from Kiessling et al. in 1975 investigated the genetic control behind the natural cytotoxicity and its correlation with tumor resistance in vivo. By injection of YAC-1 cells subcutaneously into several $F_1$ crosses between A/Sn (from which YAC-1 cells had originated) and other mouse strains with different MHC I ($H\text{-}2^a$ or $H\text{-}2^b$) they found that rejection efficiency against tumors as well as levels of NK cytotoxicity were both controlled by genes linked to the H-2 locus. In 1977 the first connection between NK cells and radioresistant rejection of bone marrow (BM) grafts was published (5). It was known from before mainly through studies of Cudkowicz et al that $F_1$ hybrid mice rejected not only completely allogeneic but also parental hematopoietic grafts via a poorly characterized mechanism that was radioresistant. The 1977 study

showed that mouse NK cells and BM graft resistance shared many common features; both phenomena emerged at the age of three weeks, both were quite resistant to total body irradiation, both were sensitive to depletion of cells in the bone marrow and both could be suppressed by repeated injection of parental spleen cells in to $F_1$ hybrids.

Already at this stage within five years of the discovery, speculations on the importance of NK cells in regulation of hematopoiesis and tumor surveillance were put forward. After that, the NK cell field became influenced by the mainstream interest in immunology at that time, and many studies addressed the capability of NK cells to produce and respond to different cytokines as well as their involvement and role in infections. However, the key principles behind their specificity remained elusive.

In the 80´s Klas Kärre et al. postulated the "missing self" hypothesis to explain how MHC genes could control hybrid resistance mediated by NK cells. It was based on the assumption that NK cells expressed inhibitory receptors that recognized self MHC I molecules; when an NK cell failed to recognize some self MHC I molecules e g on a grafted parental bone marrow cell, this would lead to NK cell activation. NK cells would thus recognize "missing self", rather than the presence of a foreign antigen. To further investigate and test the missing self hypothesis they showed that in vivo rejection of lymphoma cells was strongly dependent on the expression of syngeneic H-2 molecules: $H\text{-}2^-$ tumor cell variants failed to grow in syngeneic mice while $H\text{-}2^+$ tumor cells seemed resistant to rejection (6). This phenomenon was T cell independent but NK cell dependent; an even stronger reduction of tumor burden was observed when $H\text{-}2^-$ cells were inoculated into athymic nude mice, while they grew out when mice were NK cell depleted (7). The missing self hypothesis was later further strengthened by studies of MHC I transfected tumor cells and MHC I transgenic mice, and most importantly, by identification of inhibitory MHC I specific receptors, both in mice (8, 9)and humans (10, 11).

## 1.2 NK CELLS TODAY

Today, we understand that NK cells belong to a special branch of the innate immune system containing different types of innate lymphoid cells (further discussed below) which can initiate a rapid immune response to viral infections and intracellular pathogens ((12-17)(18)). NK cells are also important in tumor surveillance, in elimination of virus infected cells and reactions associated with hematopoietic grafts, such as host-versus-graft rejection and graft vs leukemia reactivity (19-22). In addition, they are of importance during reproduction: they accumulate in the maternal-fetal interface in the placenta and influence the development of the fetus, most probably by taking part in the regulation of angiogenesis and blood flow to the placenta (23).

NK cells are characterized as bone marrow derived large granular lymphocytes that constitute about 3-5% of spleen cells in the mouse and 5-15% of human peripheral blood (24, 25). Cell surface markers are used to identify NK cells: they are characterized by absence of CD3, TCR and Ig in combination with expression of NK1.1 or Dx5 in mice,

and of CD56 in humans (26-28). In both species, NK cells can be identified by expression of NKp46 (however, NKp46 can also be expressed by other ILC populations, see below (29, 30).

Natural Killer cells are found also throughout the body in different tissues, for example bone marrow, spleen, lymph nodes, liver, lung, pancreas, joints and placenta. They can display organ specific functions at different sites ((26, 27) and reviewed in (31, 32)).

It is usually considered that two main criteria need to be fulfilled in order to classify a cell as part of the adaptive immune system 1) the receptor used to recognize the antigen should be generated by gene rearrangement and 2) the repertoire should be further selected by clonal deletion, survival and expansion and ultimately generate immunological memory. Each NK cell expresses a variety of receptors, both activating and inhibitory, and is regulated by a balance between these two different types of receptors (reviewed in (33)). NK cell receptor genes are germ line encoded, they do not go through rearrangement to achieve diversity and specificity, as required for assembling B and T cell receptors (reviewed in (34)). Regarding the second criterion there is a debate in the NK cell field. The original view was that the whole (or most of the) NK cell population can be rapidly activated upon stimulation to mediate effector functions and proliferate, but without differences between subsets or clones, and without subsequent generation of immunological memory. They would thus give the same response in the second encounter with the same pathogen while B and T cells exhibit a much faster, more potent response during a secondary infection. But more recent studies, mainly in relation to virus infections, indicate that certain NK cell subsets can expand, resulting in long-lived effector cells that act more efficiently in a second encounter of the same pathogen (see section 1.10 below). However, NK cells are still usually referred to as being part of the innate immune system, since they do express several germline encoded receptors and the nature of their "memory" remains uncertain.

NK cells mediate their effector functions through both contact dependent and contact independent mechanisms. Upon cognate interaction NK cells can directly lyse the target by delivery of continuously produced granules containing perforin and granzymes or by inducing apoptosis through death receptor mediated pathways such as Fas-L and TRAIL (35, 36) and reviewed in (37). NK cells do not express TRAIL except for an immature NK cell population in the liver which can exert function through TRAIL and also further develop to give rise to mature NK cells (38). NK cells can recognize and kill antibody coated targets through binding via the Fc receptor in the so called ADCC reaction (27, 39). Upon activation, NK cells can secrete cytokines, chemokines and growth factors such as IFNγ, TNF-α, MIP-1α/β and GM-CSF. This can lead to direct control of infections. Alternatively, it can alter the immune response by activation and maturation other cell types (27, 40, 41). NK cells need several factors to be functional, such as IL-15, IL-12 or IL-18. There is evidence that IL-15 has to be presented by either macrophages or dendritic cells (42-44).

The last decades much more have been learned about the regulation of NK cell activity; activating and inhibitory receptors, NK cell education status, responses to and secretion of cytokines - both pro- and anti-inflammatory. One, if not the most important, regulatory mechanism of NK cell reactivity is the "missing self" recognition. The molecular mechanisms behind this reaction are partly known, but they are far from clear and still under investigation. The NK cell research field today continues to search for and characterize basic features of the cells and their interactions with the environment, but attempts to exploit NK cell manipulation for clinical purposes have also started.

## 1.3 NK CELL DEVELOPMENT

The cells of immune system are called leukocytes and are all generated from hematopoietic stem cells in the bone marrow. The immune cells are further divided into a myeloid and a lymphoid cell lineage dependent on different developmental steps further discussed below. The adaptive immune system contains the B and T lymphocytes which are generated in the lymphoid cell lineage. These cells express antigen specific receptors and are called adaptive since they can give rise to a faster and more efficient immune response upon a second encounter with the same antigen. The innate immune system contains several cell types, both of the myeloid and the lymphoid cell lineage. They express an array of germ line encoded receptors, some of which show a broader specificity. Examples of cells belonging to the innate immune system are macrophages, granulocytes, dendritic cells which are the main antigen presenting cells, with the power to activate and regulate the adaptive immune system, and NK cells. Development of hematopoietic cells occurs in the bone marrow and is divided into several steps, each phase controlled by cytokines, activation of transcription factors and interaction with stromal cells in the bone marrow. Different combinations of these factors contribute to the decision for the next differentiation step. Whether the development is completed when the cell exits the bone marrow depends on cell type, for example the NK cell is functional and ready while T cells need further development in the thymus.

### 1.3.1 Commitment to the common lymphocyte linage

The transition to go from pluripotent hematopoietic stem cells (HSC) to a committed lymphoid progenitor, ELP and CLP (early or common lymphoid progenitors respectively), is dependent on specific gene expression. Both ELP and CLP can give rise to all 3 lymphoid cell types; B, T and NK cells.(45, 46). It has been shown that several transcription factors (TF) are important in this process. TFs important in the generation of lymphoid progenitors are the Ikaros and Ets families ((47, 48) and reviewed in (49-51)). The involvement of these transcription factors is indicated by the fact that Ikaros deficient mice lack all T, B and NK cells and that the loss of Ets-1 leads to the absence of NK cells in the bone marrow, lymph node and spleen while the family member PU.1 affects all lymphoid cells, although mainly B and T cells, and also the development of some myeloid cell types (52). The transition from ELP to CLP has been shown be at least partly regulated by Helix-loop-Helix (HLH) proteins which can act as both transcription activators and inhibitors. Mutant mice lacking an inhibitory Helix-loop-Helix protein, Id2, have no NK cells (53).

### 1.3.2 Commitment to the NK cell lineage

The first identification of a committed NK cell progenitor was done in vitro: $Lin^-$ $CD3^-$ $CD122^+NK1.1^-Dx5^-$ cells were shown to give rise to $NK1.1^+$ cells only but not to T or B cells (54). These committed NK cell progenitors had transcripts for IL-15Ra chain, Ets-1, Id2 and GATA-3. GATA-3 is needed to achieve mature NK cells with Ly49r expression and proper function (55). NK cells were suggested to develop via the following stages: $CD122^+NK1.1^-Dx5^-$ - $CD122^+NK1.1^+Dx5^-$ - $CD122^+NK1.1^+Dx5^+$. Ly49 expression was acquired at the second stage and fully cytotoxic functions were achieved in the most mature population (54).

More recently an additional intermediate step on the way from CLP to NKP has been identified (pre-NKP) and the definition of the NKP was refined (rNKP) (56). Pre-NKPs lacked CD122 expression but both pre-NKP and (r)NKP developed to functional Ly49 expressing NK cells, but not to T or B cells, in vivo after transfer to an immune deficient host.

In vitro cultures and the use of different knockout (KO) mouse strains, show the importance of several critical factors such as cytokines and transcription factors in the development of NK cells from CLPs. NK cell development and differentiation in vitro to a mature and functional cytotoxic cell requires cytokine cocktails in combination with stromal cell interactions (57-59). In vitro cultures with only cytokine supplements generated small amounts of cytotoxic cells but did not lead to Ly49 receptor expression or mature NK cells (see below). Mice deficient for IL-2, -7 and -15 have shown that these cytokines are important for NK cell maintenance and function but not for development per see, at least not at the early stage (60, 61).

### 1.3.3 NK cell maturation

When a pre-NKP has become an NKP, the NK cell has a long way to go before it becomes a mature and functional cytotoxic cell. This developmental process includes several intermediate stages, differently named by different investigators. Here the steps will be referred to as stage I-IV (classification by Yokoyama) (62), (see figure 1 for additional steps and combined nomenclature). These steps are defined by expression of cell surface markers, integrins and receptors, and selective proliferation ((62) and reviewed in (63)). Some of these markers are expressed transiently while others are permanently expressed. The first step is defined by expression of CD122 and approximately 10% of these NK cells express the activating receptor NKG2D (stage I) (61). This is followed by up-regulation of NK1.1 (NKR-P1c), the inhibitory receptor CD94/NKG2A and the death receptor TRAIL (stage II). All these markers are expressed on fully mature NK cells, except for TRAIL which is mainly found at stage II and III but can be up-regulated on mature NK cell after stimulation. At stage II, the integrin Mac-1 (CD11b) and CD43 start to be expressed initially at a low level that will increase with NK cell maturation. At stage III, Ly49 receptors are expressed together with the tyrosine kinase receptor c-kit and $\alpha_v$ integrin. Stage IV is characterized by high expression of Dx5 (integrin α2), down-regulation of the integrin $\alpha_v$ and NK cell expansion by

massive proliferation. In stage V, NK cells are fully mature with high expression of Mac-1, CD43 and full cytotoxic capacity. How different transcription factors such as GATA-3, T-bet and MEF regulate these maturation steps after commitment to the NK cell linage is not fully understood and will not be discussed here.

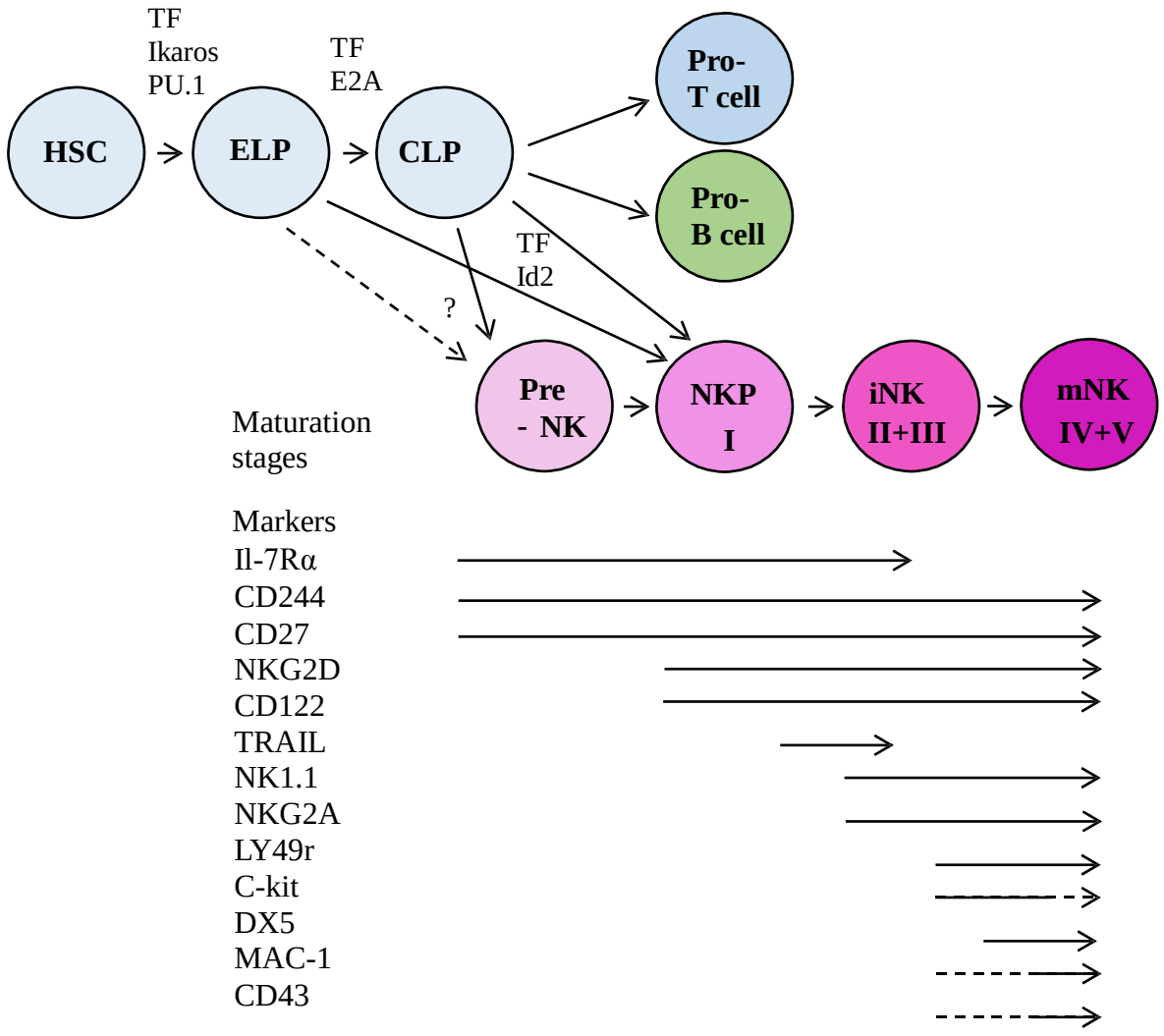


Figure 1, Described in section 1.3.1-3, nomenclature according to Yokoyama and Di Santo

### 1.3.4 Maturation based on CD27 and CD11b expression

The late maturation process and the acquisition of functional competence can be further divided into sub-steps. NK cells can be divided in to 4 different subgroups based on CD27 and Mac-1 expression (62, 64-66). CD27 is a member of the TNF super family and its expression has been shown to correlate with increased survival and maturity in T and B cells (67). Activation of NK cells through CD27 can induce cytokine production (68, 69) Macrophage-1 antigen (Mac-1, CD11b, Integrin $\alpha_M$) is an integrin expressed on several leukocytes and is important in several processes such as cell adhesion and migration (70).

Adoptive transfer together with gene expression analysis showed the following sequential maturation stages $CD27^{lo}Mac\text{-}1^{lo}$ (DN) – $CD27^{hi}Mac\text{-}1^{lo}$ – $CD27^{hi}Mac\text{-}1^{hi}$ (DP) – $CD27^{lo}Mac\text{-}1^{hi}$ (62). The ability to proliferate was highest in the DN subpopulation and decreased with every successive maturation step. The intermediate DP population is the most functionally active regarding cytotoxicity, cytokine production (IFNγ, GM-SCF), has a greater proliferating potential and is the most migratory population responding to chemokine stimulation. $Mac\text{-}1^{hi}$ but not $Mac\text{-}1^{lo}$ expressing NK cells from bone marrow, spleen and liver produce IFNγ in response to cytokine stimulation (62, 66). The most mature NK cell population, $CD27^{lo}Mac\text{-}1^{hi}$, has an increased expression level of the self-specific inhibitory receptors Ly49C/I and KLRG-1 (an inhibitory receptor expressed on fully mature NK cells, for description see below) compared to cells from the other developmental stages. These fully mature NK cells have a low functional level e g poor cytokine production capacity, almost no cytotoxic function, low or no chemotactic activity and reduced proliferation. Hayakawa et al. speculate that the most mature NK cells may represent a long lived/senescent NK cell population due to the reduced proliferation (65). NK cell subsets based on CD27Mac-1 expression also display different tissue distribution. Spleen and liver contain all maturation steps while the bone marrow and lymph nodes contain mainly immature and DP cells and the blood and lungs are mainly sources for fully mature NK cells.

### 1.3.5 Innate lymphoid cells

During the last years of research, a new branch of the innate immune system has been characterized, the innate lymphoid cells (ILCs) which earlier only comprised NK cells (reviewed in (71-73)). The innate lymphoid cells differentiate from a common lymphoid progenitor (CPL) in an IL-2 dependent manner and none of them express an antigen specific receptor. There are multiple systems suggested for how to group ILCs. According to the classification system by Diefenbach et al., there are four subgroups of ILCs; cytotoxic ILCs (NK cells) and cytokine producing ILCs (ILC1-3). ILCs are mostly located at barrier surfaces, such as in the interface of the intestine, were they protecting us from pathogen invasion. The innate lymphoid cells differentiate from a common lymphoid progenitor (CLP) (like T cell, B cells and NK cells) in an IL-2 dependent manner. It has been suggested that the ILCs develop from a common innate lymphoid progenitor (CILP), an intermediate step between CLP and pre-NKP/NKP (see fig 1). The subgroups of ILC1-3 are divided due to their developmental requirements (expression of transcription factors) and their mature effector functions. ILC1 is defined as $NKp46^{+}NK1.1^{+}$cells, produce IFNγ (enhancing response against intracellular bacteria) and are found preferentially in the small intestine and liver. ILC2 express the IL-7 receptor, the IL-2 high affinity receptor CD25 and can stimulate a $Th_2$ response by production of type 2 cytokines e g IL-4, -5, -9 and -13. ILC3 consists of many cell populations which express the transcription factor RoRγt, but differ in NKp46 expression. A population of both ILC2 and ILC3 have the possibility to regulate T cell responses, ILC2 have a direct and indirect effect on $T_H2$ responses via expression of MHC II molecules and via production of type 2 cytokine while the ILC3 can induce T cell anergy by expression of MHC II without any additional co-stimulatory receptors. In addition ILC3 can

produce several cytokines such as IL-17 and -22, and are important in immunity against extracellular bacteria. A recent study by T Kim et al. showed that the RoRγt$^{+}$ ILCs are important for creating a good stromal environment participating in the development and function (e g elimination by RMA-S in vivo) of NK cells (74).

## 1.4 NK CELL RECOGNITION: MAJOR HISTOCOMPATIBILITY COMPLEX

NK cells express activating and inhibitory receptors. Both categories include receptors that bind MHC I and receptors that bind to other ligands (75). Inhibitory NK cell receptors carry intracellular signaling motifs (immunoreceptor tyrosine based inhibitory motif, ITIMs), that transfer the inhibitory signal into the cell. The activating receptors lack intrinsic signaling motifs and depend on association with transmembrane adaptor proteins such as DAP12 and DAP10. These adaptor proteins carry different signaling motifs in their intracellular parts and transfer the activating signals into the cell. In the early 90's the first MHC I specific receptor (Ly49A) and the NK cell gene complex (NKC) located on chromosome 6 were discovered in mice by Yokoyama and colleges (8, 76). NKC codes for many NK cell receptors, including the main receptor families Ly49 and NKR-P1 (8, 76).The molecules of the largest NK cell receptor family in mice, C-type lectin-like Ly49 receptors, recognize different alleles of MHC I (77). Both of these receptor families are type II transmembrane glycoproteins that belong to the C type lectin-like family.

There is an extensive homology between the extracellular domains of the different Ly49 receptors, e g almost 90% homology between the inhibitory Ly49A and the activating Ly49D receptor (both recognizing H-2D$^{d}$; hereafter referred to as D$^{d}$). However, they still interact differently with the D$^{d}$ molecule (75). The main difference between activating and inhibitory receptors is in the intracellular signaling part where the former signal via the adaptor molecule DAP-12 (via immunoreceptor tyrosine based activating motifs, ITAM) and the latter via ITIM (see below). The receptor family mediating the corresponding function in humans is the killer immunoglobulin-like receptors (KIR) which also contain both activating and inhibitory family members. This family can also be divided in two groups of receptors which differ in their cytoplasmic tail, short or long, and for which signaling is mediated via DAP12 or ITIM respectively (75b).

To be able to understand how the NK cell function and responsiveness are influenced by MHC I interactions, the MHC will be briefly discussed with focus on parts important for this thesis. The major histocompatibility complex (MHC) is called histocompatibility-2 (H-2) in mouse and human leukocyte antigen (HLA) in humans. It contains three sets of genes; class I, II and III, and is located on chromosome 17 in the mouse and 6 in the human. Mice have two or three MHC Ia loci; K, D and some strains also express L. The equivalent genes in humans are; HLA-A, -B and –C. Examples of non-classical MHC Ib genes in the mouse are Qa-1$^{b}$ and in human HLA-G, -E and MICA and B. MHC I molecules are expressed on almost all cell types at various levels and presents mainly intracellularly derived peptides for cytotoxic T cells while MHC II is expressed on antigen presenting cells such as dendritic cells and B cells (upon activation) and their major task is to present extracellular peptides to T helper

cells. MHC I is subdivided into class Ia and Ib where Ia is called classical and Ib is called non-classical MHC I; both subclasses are important for NK cell education and proper function.

The MHC I molecule is composed of one membrane anchored α-chain that non-covalently associates with shorter $\beta_2$-microglobulin ($\beta_2$m) subunit encoded outside of the MHC complex (on chromosome 3 in mouse and 12 in human). For a MHC I molecule to be presented on the cell surface the α-chain needs to be associated with a β-chain and the complete molecules has to be loaded with a peptide. Cells lacking the β-chain express only very low levels of unstable MHC I molecules. MHC I molecules are loaded mainly with peptides from intracellular proteins which are transported from the cytoplasm into the endoplasmatic reticulum (where the MHC I molecule is assembled) by the protein transporter associated with antigen processing (TAP). Cells deficient for TAP, such as the RMA-S line used in this thesis, also express low levels of MHC I.

MHC is one of the most polymorphic chromosome regions leading to an enormous variation between individuals. This variation increases the number of antigen peptides that can be presented at the population level, resulting in a higher chance that the species will survive infections with different pathogens. Different inbreed mouse strains carry different MHC haplotypes (genotypes). This makes it possible to study influence of allelic variation with known differences. For this thesis, mice carrying different genotypes for MHC I have been used (Table 1).

Other molecules related to MHC molecules but not encoded in the in the MHC region are the CD1 molecules. The CD1 family molecules, as MHC I, associate with β-chain but present glycolipids for NKT cells, T cells with a invariant T cell receptor and expression of some NK cell markers.

**Table 1. Mouse strains, MHC I molecules and Ly49r relevant for this thesis**

| Mouse strain | Haplotype/ molecules expressed | Educating receptor |
|---|---|---|
| B6 | H-2$^b$; K$^b$ and D$^b$ | Ly49C and I |
| IMSR (B6) | H-2$^b$; K$^b$ and D$^b$ | Ly49C and I |
| $\beta_2$m$^{-/-}$ and TAP $^{-/-}$ | H-2$^b$; low levels expressed | (Ly49C and I) |
| K$^b$ single | H-2$^b$; K$^b$ | Ly49C and I |
| D$^d$ single | H-2$^b$; D$^d$ | Ly49A, and $G_2$, |
| D8 | H-2$^b$; K$^b$, D$^b$ and transgenic D$^d$ | Ly49A, $G_2$, C and I |
| 129 | H-2$^b$; K$^b$ and D$^b$ | Ly49I (V and O)* |

*Tetramer binding has been demonstrated but if they are also educating is unknown.

## 1.5 NK CELL RECOGNITION: RECEPTORS

### 1.5.1 Inhibitory NK cell receptors

#### *1.5.1.1 Ly49, KIR and NKG2 families*

As mentioned above, activating and inhibitory Ly49 receptors belong to the C type lectin-like receptor family. The NK gene complex code for 8-18 different Ly49 genes, depending on mouse strain, with extensive allelic polymorphism (8, 78, 79). Most of the genetic studies were performed in B6 and 129 mice (80, 81). The 129 and B6 mice (used as wild type, wt, in many studies) share some genes and alleles but they also possess strain specific genes. Some alleles have occurred through duplication (79). The B6 genome codes for ten Ly49 receptors, eight of which are inhibitory, Ly49A,-B, -C, -E, -F, -$G_2$, -I, and –J. Two of these genes encode for activating receptors, Ly49D and –H, which are described in the next section (82, 83). All receptors are not expressed on all NK cells, the Ly49 genes are expressed independently of each other on different subpopulations of NK cells in an overlapping fashion, creating a diversified NK cell repertoire (84, 85). The inhibitory receptor consists of two disulfide-linked homodimers, each with a cytoplasmic tail containing ITIMs that transfers the signal in to the cell (77). The best characterized inhibitory Ly49 receptors are Ly49A and Ly49$G_2$) both binding to $D^d$, and Ly49C, binding to $K^b$ (86, 87). A number of studies have addressed how Ly49r bind to MHC I and to which allele(s) each receptor binds. Hanke et al. studied Ly49r specificity using cell-cell adhesion assays in combination with MHC I/peptide tetramer staining (88). Only Ly49C and -I interacted with $H2^b$ products while five Ly49r bound to $H2^d$ products, including Ly49A and $G_2$ which is of importance in this thesis. However, there are conflicting data regarding whether Ly49A can bind also to $K^b$ (88-90).

In humans, the highly polymorphic killer cell immunoglobulin like receptor (KIR) family encodes monomeric receptors recognizing MHC I. The activating KIRs have a short (S) cytoplasmic tail while the inhibitory have a long (L) cytoplasmic tails (containing 2 ITIM motifs), which is reflected in the terminology, e g KIR2DS1 or KIR2DL1. These receptors are completely lacking in mice while only one Ly49 pseudogene has been found in humans (33, 91, 92).

Different inhibitory Ly49r and KIRs recognize different MHC I alleles, making it possible for certain NK cells to react on down-modulation of one specific MHC I molecule in missing self recognition (see discussion below) (82, 88, 93-95). In order to recognize and bind to MHC I molecules, Ly49 receptors require that a peptide is bound in the MHC I pocket inducing the correct conformational structure (96). Ly49 receptors are not peptide specific in the same way as the T cell receptor but there are data suggesting that some Ly49r can display selectivity, i e some peptides are non-permissive for $K^b$ recognition by Ly49C and I (89, 96, 97).The binding affinity of Ly49 to MHC I molecules may be influenced by carbohydrates on the MHC molecules, but this probably does not alter the binding specificity (98, 99)

CD94/NKG2A, which also belongs to the C-type lectin like family, represents a third type of receptor recognizing MHC I molecules. The CD94 and NKG2 genes are genetically linked and are expressed both in mice and humans (100-104). Both the murine and human genome contain several genes for NKG2 receptors, A, C and E in mice and A, C, E and F in humans, but only NKG2A is coding for an inhibitory receptor (101, 103-107). The CD94 molecule can be expressed as a homodimer, without the capability to bind Qa-1 or HLA-E or mediate intracellular signaling, or together with a NKG2 molecule (104, 108). CD94/NKG2A is a disulfide-linked heterodimer with allelic variation (109-111). The NKG2A receptor monitors the MHC I expression by binding non-classical MHC I molecules, Qa-1 and HLA-E (102, 108, 112, 113) in mice and humans respectively. Qa-1 and HLA-E present peptides from the leader sequence from some but not all MHC I alleles in a TAP-dependent manner (114, 115). In wild type (wt) B6 mice, Qa-1 presents peptides derived from $D^b$ but not from $K^b$. CD94/NKG2A presents a second layer of MHC I recognition. Why has evolution preserved two modes of inhibitory MHC I recognition? It could be that CD94/NKG2A can sense only a broader loss of MHC I since it can bind to leader sequences from several MHC I molecules, while Ly49r can sense a delicate change in MHC I expression where only one out of several MHC I is down-regulated. Both alternatives have been observed in different unhealthy settings, e g in certain viral infections or by tumors, indicating a requirement for both. The NKG2A system might have evolved first, but it was not enough and therefore an extra layer of surveillance was added. However, in the B6 mice the Qa-1-NKG2A system is the only system surveilling loss of the $D^b$ MHC I molecule since no Ly49r bind strongly to $D^b$ in the B6 mice (88).

NK cells also express inhibitory receptors recognizing other ligands than MHC I ligands. The NKC on chromosome 6 contains genes coding for three receptor families of C-type lectin-like transmembrane glycoproteins, Ly49r and NKG2/CD94 described above and the NKR-P1 gene family (9, 81, 116, 117) The NKR-P1 family is polymorphic and in B6 mice, it consists of NKR-P1A , -C, - B/D and –F. Only the NKR-P1A gene has been found in humans (118-123). Other mouse strains encode for additional NKRs. The B6 strain code for NKR-$P1B^{B6}$ (or sometimes referred to as NKR-P1D) (124). (120). Both NKR-P1B and-D are inhibitory and recognizes Ocil/Clr-b, which is also a C-type lectin-like transmembrane glycoprotein encoded in the NKC. It is expressed on a wide array of hematopoietic cells: myeloid cells, T, B and NK cells but not on erythrocytes (121, 122, 125). There have been speculations regarding the importance of the NKR-ligand interaction as an additional MHC independent missing self system, see section 1.9.3 .

#### *1.5.1.2 KLRG1*

KLRG1, Killer cell lectin like receptor 1, is expressed on murine and human NK cells and on activated T cells (126, 127). In the mouse, KLRG1 belongs to the family of C-type lectin like receptors encoded on the same chromosome but distal to the NK cell gene complex containing the Ly49r and NKR-P1 receptors. It is expressed as a monodimer that can use disulfide bonds to create di-, tri- and tetramers on approximately 30-50% of the NK cells

(128, 129). In NK cell biology, KLRG1 is considered a maturation marker since it is expressed on fully mature, $CD27^{lo}Mac\text{-}1^{hi}$ NK cells, which are less cytotoxic and less prone to proliferate, but respond to cytokine stimulation with e g IFNγ production. However, KLRG1 is not needed for normal NK cell development and maturation (66, 130) . KLRG1 can be acquired upon homeostatic or virus induced proliferation (131, 132). KLRG1 binds to classical cadherins (E, N and R) which are expressed on epithelial cells and are used for cell-cell contacts. Cadherins are sometimes downregulated in tumors, possibly to avoid infiltration of immune cells (133). Early studies displayed contradictory results regarding the influence of KLRG1 on NK cell function. Gründemann et al. showed that target cells expressing high levels of E-Cadherin did not inhibit the killing of the target by IL-2 activated NK cells (133). On the other hand, other groups showed that blocking of KLRG1 restores killing of targets expressing E-cadherin, and that antibody crosslinking of KLRG1 can inhibit IFNγ production, hence indicating an inhibitory function of KLRG1 (134, 135). Furthermore it has been reported that it signals via ITIMs recruiting SHIP-1 and SHP-2 upon phosphorylation (136).

#### *1.5.1.3 The SLAM family member 2B4*

The murine and human 2B4 receptors were discovered by screening of monoclonal antibodies that would activate NK cells (137, 138). The 2B4 receptor belongs to the SLAM (signaling lymphocytic activation molecule) receptor family and is composed of two Ig-like domains and a cytoplasmic tail containing four specific signaling motifs (immunoreceptor tyrosine-based switch motif, ITSM) which defines the common signaling pathway used by the SLAM-receptor family (see section 1.7.4) (139, 140). 2B4 is expressed on almost all immature and mature NK cells, both in mice and humans, and it binds to CD48 which is constitutively expressed on all hematopoietic cells (137, 138, 141).The SLAM receptor family is mainly important in recognition of hematopoietic target cells which express their ligands (142).

In mice, and maybe also in humans, 2B4 can mediate activating as well as and inhibitory signals. Some data indicate that it may be mainly inhibitory since blocking of 2B4 can lead to increased tumor control, and NK cells from 2B4 deficient mice have displayed increased function against CD48 expressing targets (137, 143).

### 1.5.2 Cell surface molecules involved in NK cell activation

One of the first discovered and most studied activating NK cell receptors is the low affinity receptor for IgG, the CD16 (FcγRIII) first described on mouse NK cells by Kumar and colleagues (39, 144). It is a glycoprotein belonging to the Ig superfamily. CD16 binds to the Fc part of antibodies and can thereby mediate ADCC. This is a function that connects the innate and adaptive immune system, since it allows the NK cells to kill target cells labeled for destruction by specific antibodies produced by B cells. This function is likely responsible for at least part of the effects when monoclonal antibodies against tumor cells are used in cancer treatment.

#### *1.5.2.1 Activating NK cell receptors*

As mentioned above, the Ly49 receptor family also includes activating members, Ly49D and –H in B6 mice. The Ly49D receptor associate with DAP12, containing ITAM signaling motifs, and is therefore capable of inducing cytotoxicity after antibody induced ligation or binding to its ligand $D^d$ (145). Other activating Ly49r (not expressed in the B6 mouse) are, Ly49P and –W, which also interact with H-$2^d$ (146, 147). The Ly49H receptor expressed in B6 mice recognize the protein m157 expressed on cells infected with the murine cytomegalovirus (MCMV) (148-150). This protein is encoded in the virus genome and resembles MHC I in structure. It is possible that it has evolved as an immune evasion strategy, since the m157 also binds to the inhibitory Ly49I in other mouse strains such as 129 mice.

The NKR-P1 family introduced above also contains activating receptors (119, 120, 123). NKR-P1C is an activating receptor with yet no known ligand, but it's recognized by the NK1.1 antibody and frequently used as a marker for NK cells in the B6 mouse strain.

There is also a group of receptors that has mainly been studied in humans, the natural cytotoxic receptors (NCR). In humans this "family" consists of four members. NKp30, NKp46 and NKp80 are expressed on resting human NK cells while the fourth member, NKp44, is expressed only on a subset of NK cells upon IL-2 activation, as reviewed in (151). It has been reported that at least NKp30, NKp46 and NKp44 can trigger cytotoxicity against both tumor and virus infected cells (152). This effect is mediated through recognition of different ligands or epitopes; NKp30, NKp44 and NKp46 recognizes viral hemagglutinins from several virus strains but intracellular proteins are also recognized, such as HLA-B transcript 3 by NKp30 (153, 154) and reviewed in (155). NKp44 has been shown to bind to ligands that are up-regulated during HIV infection leading to lysis and spreading of the virus.

Biassoni et al. discovered a new gene encoding for a murine homologue to the human NKp46 and termed it MAR-1. It has later been renamed to NKp46 (156). It is the only NKR homologue found in mice so far. At least in mice, NKp46 is expressed early in NK cell development and it is present on almost all mature NK cells (156). (29). NKp46 is an activating receptor. (156). It is important for resistance against some viral infections and tumors, through binding to Hemgglutinins and unknown ligands respectively (154, 157). The third MHC I binding C-type lectin-like receptor family mainly contains activating receptors, the heterodimers CD94/C, -E (and in humans also –F) and the homodimer NKG2D. In mice and human, NKG2C (and –E in mice) associates with the intracellular adaptor molecule DAP-12 to mediate intracellular signaling (104, 158-160). NKG2D has been extensively studied. It recognizes molecules that are up-regulated on cells by stress and it is essential for resistance against some experimental cancers, through activation of NK cell mediated cytotoxicity (19, 161, 162). This activating receptor is expressed on almost all mouse and human NK cells and it is encoded by a non-polymorphic single gene (101, 163). NKG2D is distinct from the other NKG2 family members by several criteria, for example it is expressed as a homodimer. In mice, the NKG2D receptor exists in 2 isoforms, NKG2D-L (long) and

NKG2D-S (short). NKG2L is mainly associating with the adaptor molecule DAP-10 while the NKG2D-S isoform is transcribed after activation and can associate with both DAP-10 and DAP-12 (164, 165). It is thought that DAP-10 mainly mediates cytotoxicity while NKG2D DAP-12 signaling leads to both high cytotoxicity and cytokine production (166). Human NK cells only express the NKG2D-L isotype which mediates both cytotoxicity and cytokine production by signaling through DAP-10 (167, 168).

In mice, NKG2D recognize several glycoprotein as ligands; retinoic acid early inducible-1 (Rae-1) isoforms α, β, γ, δ and ε, murine ULB-binding like transcript-1 (MULT1) and histocompatibility 60 (H60) (169-171). These ligands are induced by Toll like receptor signaling, virus infection or products generated via the heat shock and DNA damage pathway (in response to for example irradiation), leading to expression on many tumor cells, strengthening the relevance of NKG2D activation by cellular stress (172), reviewed in (173, 174).

In humans, NKG2D binds to ligands in a group of proteins called ULBP (up to six variants) and 2 ligands encoded by the MHC complex, MHC I chain-related proteins A and B, MICA and MICB (175, 176). These ligands has also been shown to be up-regulated through the DNA damage and heat shock pathway upon cellular stress and cell transformation (reviewed in (177).

NKG2D ligands, both in mice and human, have been found in a soluble form. The relevance of these is not totally clear, however recent studies in mice have shown that Rae-1g and Mult1 in soluble forms in certain situations can mediate an enhanced anti-tumor effect (171, 178).

#### *1.5.2.2 Adhesion molecules and NK cell function*

In addition to the receptors discussed above, different adhesion molecules such as DNAM-1 and LFA-1 are important in regulating NK cell functions. In mice, DNAM-1 (adhesion molecule and activation receptor DNAX accessory molecule 1) is expressed approximately on 50% of the NK cells and recognizes CD155 (Poliovirus Receptor, PVR) (386). DNAM-1 is involved in regulating NK cell cytotoxicity against tumor and virally infected cells (179, 180). In addition, the expression of DNAM-1 correlates with the NKs cell educational status, i e inhibitory input and responsiveness, implicating a role for DNAM-1 in NK cell education (181). LFA-1 (Leukocyte function-associate antigen-1) recognizes ICAM-1, -2 and -3. The latter are up-regulated on endothelial cells during the early inflammatory response, making it possible for the leukocyte to attach and "roll" as a first step for entering the infected tissue. LFA-1 is necessary for signaling through the activating co-stimulatory molecule DNAM-1. This was shown by using NK cells from patients with a deficient β1 integrin (leukocyte adhesion syndrome). These NK cells had deficient DNAM-1 mediated cytotoxicity that could be rescued by restoring the LFA-1 cell surface expression by genetic reconstitution.

#### *1.5.2.3 Activating receptors are used simultaneously in combination*

Many of the activating NK cell receptors have been suggested to be co-receptors since they need to be activated simultaneously with another type of receptor in order to lead to proper activation for cytotoxicity and cytokine production in resting cells (182). Human resting NK cells have a restricted regulation of their activation compared to IL-2 pre-activated NK cells. In experiments with IL-2 activated human NK cells all receptors tested (CD16, NKp46, NKG2D, 2B4 and DNAM-1) could induce in vitro cytotoxicity independently of other receptors. Resting NK cells on the other hand required co-signaling through pairs of activating receptors to induce cytotoxicity or cytokine secretion. It was observed that the activating receptors synergize in different constellations, i e most of them did not enhance function of all of the other receptors. (182-184). No receptor tested in the study could by itself induce NK cell response, with exception of the CD16 receptor. Bryceson et al. suggested the term co-activation receptors to describe these receptors that can only function in synergistic pairs.

## 1.6 NK CELLS AND TARGET CELL ELIMINATION

When an NK cell meets a potential target the contact surface that is formed between the two cells is called the immune synapse. This formation facilitates receptor interaction and signaling. If the NK cell decides to eliminate the target a lytic hit will occur in the synapse, mediated by release of cytotoxic granules containing perforin and granzymes. When a cytotoxic granule is released from the NK cell to the interaction interface, the vesicle and plasma membrane fuse, leading to exposition of molecules on the NK cell surface, including lysosomal- associated membrane protein-1 (LAMP-1). LAMP-1 (CD107a) is often used as a marker of NK cell mediated degranulation in different functional assays (185).

## 1.7 NK RECEPTOR SIGNALING PATHWAYS

### 1.7.1 Activating receptor signaling pathways

In this section I will present an overview of the signaling pathways mentioning some of the main players and principles, reviewed in (166, 186-189).

The majority of the activating receptors (such as Ly49r, CD16, NKR-P1C and NKp46) need to associate with adaptor molecules to be stably expressed and to be able to signal. All receptors do not associate with all adaptor molecules. The adaptor molecules DAP12, CD3ζ and FcεRI-γ contain ITAMs in their cytoplasmic tail. Upon activation and receptor crosslinking, the two tyrosines (Y) in the ITAM motif (YxxL-x6-8-YxxL) become phosphorylated by Src family kinases (Fyn, Lck, Src, Yes, Lyn and Fgr), and act as docking sites for Syk family kinases (manily Syk but also Zap70). Activation of Syk family kinases lead to triggering of downstream signaling via phosphatidylinositol-3-OH kinase (PI3K) and Vav-2/3, phospholipase C-γ PLCγ (PLCγ-1 and 2) and the adaptor protein growth factor receptor-bound protein 2 (Grb2). Activating signaling results in proliferation, cytokine and chemokine production and cytoskeleton rearrangements needed for cytotoxic granule release.

NKG2D preferentially associates with DAP10 carrying a different signaling motif, YINM (YxxM). It has been suggested that DAP-10 signaling is initiated by phosphorylation via either Src family kinases or by the kinase Jak3. After phosphorylation, the short cytoplasmic tail of DAP-10, containing one signaling motif, can recruit two signaling molecules; (Grb2) and the phosphatidylinositol-3-OH kinase (PI3K) via the binding site p85. Signaling through both molecules are needed for full activation (maximal $Ca^{+}$ influx) but since their binding sites overlap only one of them can bind at the time. Only the Grb2 pathway (via Vav-1) can by itself trigger cytotoxicity. Studies using mutations in the different binding sites for Grb2 and p85 has led to the conclusion that Grb2 signals through vav-1, SLP-76 and PLC-γ2.

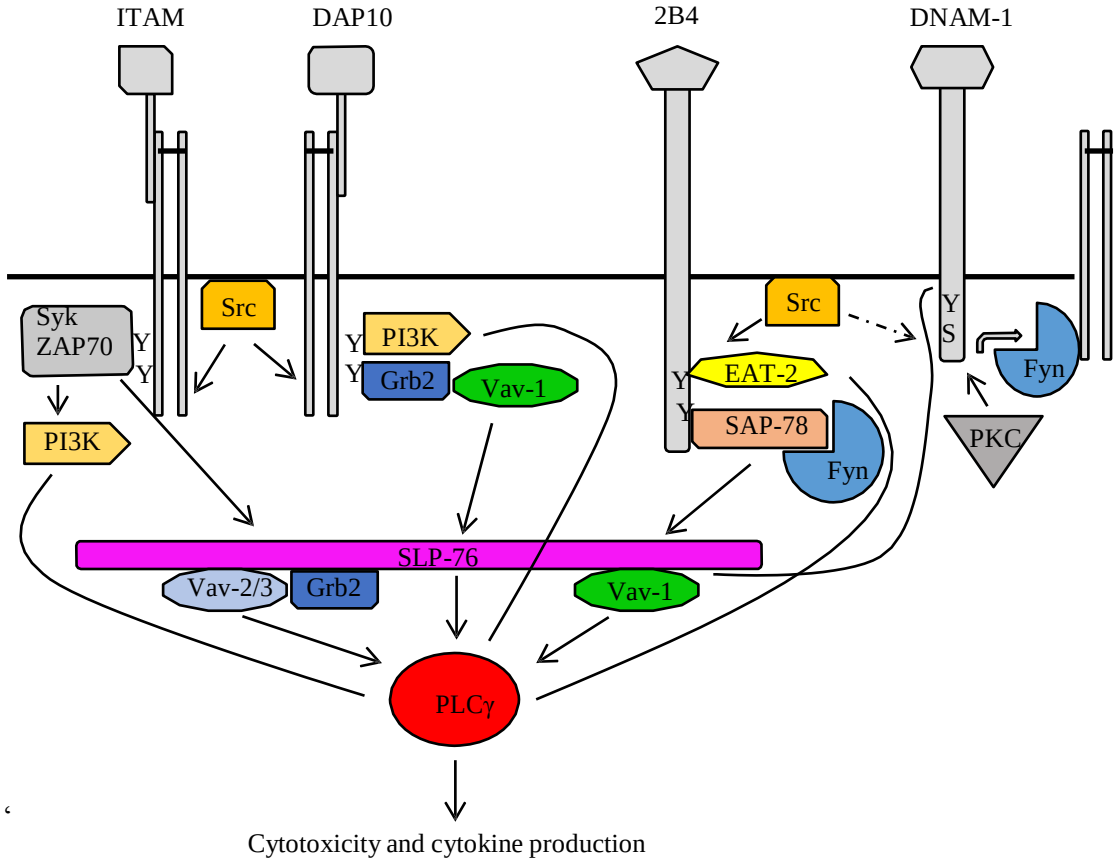


Figure 2, Activating NK cell signaling described in 1.7.1 and 2

### 1.7.2 DNAM-1 and LFA-1 signaling

An interesting signaling pathway involves the DNAM-1/LFA-1 constellation. When the DNAM-1 receptor is activated by crosslinking, it is recruited to lipid rafts and tightly bound to the cytoskeleton in the immune synapse (190). Crosslinking also induces phosphorylation of the intracellular domain by protein kinase C (PKC) which makes it possible for DNAM-1 to associate with LFA-1 (191, 192). The DNAM-1-LFA-1 association is necessary for DNAM-1 signaling; there is reduced DNAM-1 cytotoxicity in patients with leukocyte adhesion deficiency syndrome (191). After association LFA-1 recruits the Src kinase Fyn that

helps to phosphorylate another binding site on DNAM-1 leading to signaling via SLP-76, Vav-1 and PLCγ2. It has been speculated whether DNAM-1 may be involved in the synapse formation since it binds both to the cytoskeleton and the adhesion molecule LFA-1.

### 1.7.3 Inhibitory receptor signaling pathways

The largest families of inhibitory receptors in mice and humans are the Ly49 and KIR families respectively, reviewed in (166, 186, 193). Ly49 receptors mediate their inhibitory signal by being within a close distance to the activating receptor signaling molecules. Crosslinking of the inhibitory receptors themselves leads to weak phosphorylation (activation of the signaling motif) while simultaneous crosslinking of inhibitory receptors and activating receptors lead to a higher degree of phosphorylation. Phosphorylation of ITIMs (V/IxYxxL/V) is probably dependent on Src family kinases. Phosphorylated ITIMs on Ly49r serve as a docking site for two SH2 domain containing protein tyrosine phosphatases (deactivators), SHP-1 and SHP-2, (194) but can also recruit the lipid phosphatase SHIP-1(166). Ly49r preferentially associate with SHP-1/SHP-2 while KLRG1 mainly recruits SHIP-1 (136). After recruitment of phosphatases to ITIM motifs, they may inhibit NK cell effector function and proliferation via dephosphorylation of proteins important in NK cell activation. Vav-1 has been shown to be one of the main targets for dephosphorylation since it is a key component in many activating signaling pathways. Further, ITIM mediated inhibition in human NK cell results in two separate inhibitory mechanisms 1) SHP-1 targeting Vav1 for dephosphorylation and 2) tyrosine phosphorylation of Crk (195). Crk in complex with another molecule are involved in actin organization in the synapse. There are speculations regarding if this phosphorylation and loss of complex formation might lead to reduced actin synapse formation.

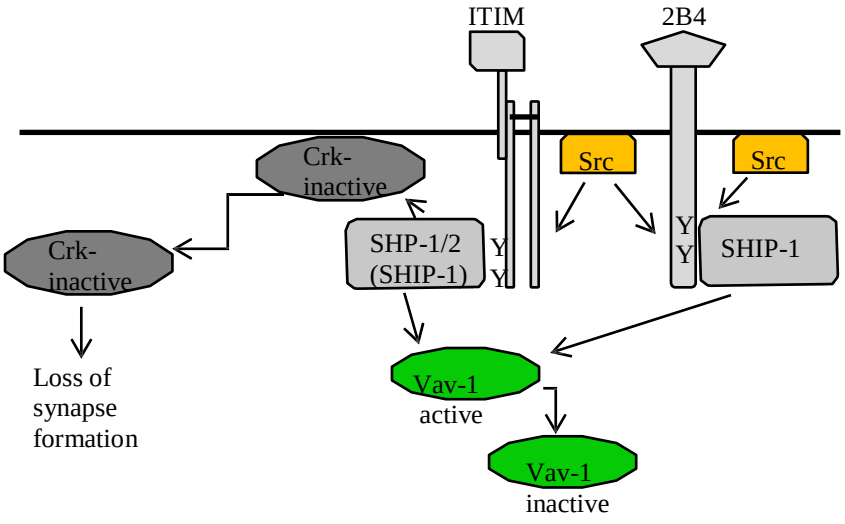


Figure 3, inhibitory NK cell receptor signaling described in 1.7. 3-4

### 1.7.4 The SLAM family member 2B4; activating or inhibitory?

2B4, like other SLAM family members, has four tyrosine-based ITSM in its cytoplasmic tail and can mediate both activating and inhibitory function. 2B4 can recruit several signaling

molecules that belong to the SLAM-associated protein family (SAP) and contain a SH2 domain. In mice, three different signaling molecule recognize the ITSM: SAP, EAT-2 (Ewing´s sarcoma-activated transcript-2) and ERT (EAT-2 related transducer), although ERT has been shown to only be expressed in cytokine activated NK cells (196, 197). There are conflicting data regarding when 2B4 has an activating or inhibitory function and how this process is regulated in mice. Further, human 2B4 is thought to be mainly activating and express only SAP and EAT-2 (183).

There are data showing that both SAP and EAT-2 can promote activation in two ways; by further activate the signaling cascade and by blocking the access for inhibitory signaling molecules (SHIP-1) to bind to the cytoplasmic tail and thereby initiating inhibition (196, 198). Upon 2B4 ligation, ITSM becomes phosphorylated probably by a Src kinase which enables recruitment of SAP and/or EAT-2. SAP recruits Src family protein tyrosine kinase Fyn (via SH3 domain binding to SAP via an arginine at position 78 (SAP-78). Activation of Fyn leads to phosphorylation of Vav-1. Activation of SAP is important for increased conjugate formation, cytotoxicity and cytokine production (142, 198). However, EAT-2 mediate activation via PLCγ2 and $Ca^{+}$ signaling to accelerate polarization and release of cytotoxic granules (196).

There are data showing both inhibitory and activating functions of 2B4. Chlewicki et al. showed that loss of 2B4 signaling, either via blocking or in KO mice, leads to increased elimination of target (RMA-S $CD48^{+}$) cells and increased IFNγ production (199). However, Dong et al. and Pérez-Quintero et al. have shown that the different functions of 2B4 depend on if the target is of hematopoietic origin or not, that lack of functional SAP or EAT-2 leads to reduced killing (of $CD48^{+}$ targets) and IFNγ response compared to wt NK cells (142, 196, 198)

Chlewicki et al. proposed a dual regulatory function of 2B4, in both mouse and men, and that the outcome is determined by several factors: receptor expression, level of crosslinking and the amount of available adaptor molecules (SAP) (199). Data indicated that SAP may be the limiting and controlling factor in the system. The authors speculated that if there is too much crosslinking of 2B4, there will not be enough SAP to bind and block 2B4 interaction with negatively signaling adaptors ( SHIP-1), mediating an inhibitory signal. This would function as a mechanism to protect healthy cells expressing CD48 from elimination. Loss of 2B4-CD48 interaction has been observed to result in NK cell dependent in vivo and in vitro killing of syngeneic NK cells (200). A recent study provided evidence for 2B4 expression on many dendritic cell (DC) subtypes and that the 2B4-CD48 interaction between DC-NK cells influences the NK cell activity. DC from 2B4 KO mice induced a higher in vitro responsiveness upon co-culture (201).

As will be discussed later in this thesis, NK cells may adapt to “abnormal” signaling input in various knockout mice by retuning of their activation threshold. Interpreting results from knockout mice may thus be very difficult. How deficiencies in some of these molecules,

important in activation or inhibitory pathways, affect the NK cell pool and functions will be further discussed in the results and discussion section in relation to the IMSR mice.

## 1.8 LY49 NK CELL RECEPTOR REPERTOIRE FORMATION

### 1.8.1 General pattern of Ly49r expression

As mentioned above, mice carry eight or more Ly49 genes (8, 78, 79). Each MHC I specific receptor, Ly49 and CD94/NKG2A, is expressed on individual NK cells in an overlapping, partly stochastic fashion. Thus, individual NK cells may express from zero to several Ly49 receptors, the common range being one to five (84, 202-204). Different Ly49r recognize different MHC I alleles, but since the genes for receptors and ligands are located on separate chromosomes (6 vs 17) they are inherited independently. This means that individual NK cells can express receptors specific for non-self MHC I allele, and sometimes only such receptors, i e they lack receptors for self MHC I. The same principle is true for the human KIR receptor system (389) and as I will discuss below this has implications for the development and function of NK cells. Regarding receptors, their expression and importance for NK cell education, I will from hereon only discuss the murine system, since that is of highest relevance for this thesis.

### 1.8.2 Ly49 receptor acquisition

NK cells start to express the CD94/NKG2A receptor at development stage II ($NK1.1^+$) and they proceed to express Ly49 inhibitory receptors at stage III ($c\text{-}kit^+$ $\alpha_v{}^+$). Once Ly49r become appear on the NK cell, the expression pattern is stable over time (62, 95, 205, 206).

Little is known about the forces that drive the process of receptor acquisition. It has been shown in vitro that CD94/NKG2A expression can be achieved by differentiating NK cell progenitors by cytokine stimulation (207, 208), while initiation of Ly49 receptor expression needs additional undefined signals from interaction with bone marrow stromal cells (209). The critical signal for induction of expression of Ly49r is not known but IL-15 has been suggested to play a role. In one study of IL15 deficient mice, both the NK cell number and the Ly49 expression could be rescued by administration of exogenous IL-15 (210). On the other hand, there are data from two groups showing that NK cells development can still proceed in the absence of IL-15. Although these studies revealed that the NK cells from these mice are of a less mature phenotype ($Mac\text{-}1^{lo}$ and $CD43^{lo}$), the NK cells had normal Ly49 expression levels (Ly49A, -G2 and D) and effector functions (cytotoxicity and cytokine production) (61).

### 1.8.3 Allelic expression of Ly49 genes

As mentioned in the beginning of this section, MHC I specific receptors are expressed in a partly stochastic pattern. All individuals possess two alleles for each gene - one on each homologous chromosome. It has been shown for both Ly49A, -C and –G2, that in almost all NK cells, only one of the two alleles is expressed (211, 212). This was first observed by Held et al. who initially termed this process “allelic exclusion” (213). They showed that the

process did not involve termination the expression of all Ly49 alleles on the same chromosome, it was rather affecting each locus independently (213). Among NK cells expressing only one Ly49A allele, the frequency of the allelic expression was divided equally between the two chromosomes. In addition, it was also seen that expression from both alleles could occur (for Ly49A and G2 but not for C), and that the frequency of the bi-allelic expressing cells was close to the frequency obtained by multiplying the mono-allelic frequency. Held et al. suggested two models for how this can occur: 1) "independent control" meaning that this process could be regulated by several "trans-acting factors", each regulating the expression of a separate Ly49 gene, and every "trans-acting factor" would be active during a limited time frame in the sequential order of receptor acquisition or 2) "competitive control", all genes are controlled by the same set of factors but some/one of these "trans-acting factors" could be in limited access leading to competition accounting for that only one allele is expressed. The ordered expression (to be further discussed below) could be due to that different Ly49 genes are only available during a limited time period due to differences in their promoters (213).

The second model would result in lower levels of the Ly49 receptors compared to unlimited access of regulatory factors. This has been shown for Ly49A, for which the level of expression depends on the transcription factor TCF-1. In mice heterozygous for TCF-1, the NK cells display approximately a 50% reduction in Ly49A expression compared to TCF-1 homozygous mice, indicating that the transcription factor is the limiting factor (214). Further, TCF-1 deficient mice have alterations in their Ly49r defined NK cell repertoire in a MHC I independent manner. TCF-1 seems to activate transcription of Ly49A and –D (reduced expression in TCF-1 $\beta_2m^{-/-}$ mice) while suppressing expression of Ly49G2 and –I, leaving Ly49C unaffected (214, 215).

It is still unknown if there is monoallelic expression and if so which mechanism(s) control it. The "competitive control" hypothesis fits best with the additional discoveries that have been made. There is an emerging field studying different promotors, bi-directional transcription and if there is any common transcriptional factor used at low level to ensure monoallelic and reduced Ly49r expression but no such TF has been found. However, it has been shown that the expression pattern is depending on multiple promoter regions of which some are bidirectional. Independent of underlying cause, epigenetic mechanisms such as DNA methylation has been shown to preserve the established monoallelic expression (216). An interesting finding is that monoallelic expression might be influenced by MHC I expression (217). It is not known if this leads to important functional consequences, however it is of interest if MHC I has a third effect on the NK cell pool.

### 1.8.4 Ordered and stable Ly49 gene expression

Regardless of the mechanisms activating the expression of Ly49r, there is evidence that the different loci become expressed in an ordered fashion. To study this, two different approaches were used with slightly different but not necessarily conflicting outcomes. Early transfer and in vitro (stroma cell culture) experiments showed that $CD3^-NK1.1^+$ NK cells could generate

Ly49G2,-C/I and -F expressing NK cells, in that order, while Ly49A expression was only observed when $CD3^{-}NK1.1^{-}$ precursors were used, indicating that Ly49A commitment occurred before NK1.1 expression (205, 209). Ly49 receptors were thus expressed in the order of Ly49A-Ly49G2-Ly49C/I. Two models were suggested to explain this; 1) either the genes are expressed individually in a sequential fashion starting with expression of only Ly49A and then proceeding as stated above or 2) initially all genes are on, but their expression is terminated in an ordered way, losing the ability to express Ly49A first and in the end genes for only Ly49C/I can be activated.

In the second approach, RT-PCR of RNA was used to identify the kinetics of Ly49 receptor expression on NK cells in in vitro culture (84). The result from this study showed that receptors could be detected in "sets". The first set is CD94, -NKG2A, -C and Ly49B, expressed in all analyzed NK cell clones. This group was followed by early Ly49r, Ly49G2 already at day 4 and Ly49C/I begun to be expressed at day 4-7. The group of Ly49A, -D, -E and –F was observed later, at day 21. So, here the expression order was Ly49G2, Ly49C/I and ending with Ly49A, different from the results reviewed above (84). Analyses of gene expression of NK cells in late cultures revealed that some clones expressed for example Ly49D but not Ly49C or –I indicating that all genes are not expressed at the same time (84). This approach is more a direct study on the actual gene expression, so it is possible that the Ly49 genes are transcribed in a different order compared to the order of cell surface expression.

In addition to the knowledge regarding the ordered pattern of inhibitory Ly49 receptor expression, Smyth et al. showed that Ly49H and -D are expressed after inhibitory Ly49r expression in a non-random fashion e g they are often co-expressed and even at a higher frequency on NKG2A negative cells (218).

Regardless of order of expression or mechanisms controlling activation of NKG2A and specific alleles of Ly49 receptors, several studies have shown that once a gene/allele for receptor expression is activated the expression of that receptor is stable over time. This has been shown both in vivo in the periphery after transfer (both NKG2D and Ly49r) and in vitro in IL-2 cultures (Ly49r) (95, 205, 206, 219). The regulation of Ly49 transcription has still not been thoroughly elucidated, but a complex regulation system is emerging, involving multiple promoter regions of which some are bidirectional, as well as epigenetic mechanisms to maintain expression (220).

## 1.9 MHC I REGULATED NK CELL EDUCATION; A PROCESS WITH AT LEAST TWO CONSEQUENCES

It is now established that MHC I molecules of the host influence the NK cell pool in at least two different ways: 1) by shaping the NK cell repertoire such that NK cell subsets expressing 1-2 self-specific inhibitory receptors become overrepresented and 2) by determining the NK cell responsiveness at the single cell level. These processes are called repertoire skewing and

"licensing" or "arming/disarming" respectively. These two outcomes together represent combined NK cell education and will be discussed below.

### 1.9.1 Skewing of the NK cell repertoire

The first real evidence for host MHC I influence on the NK cell repertoire was published by Held et al. (221). I They compared Ly49A, C and G2 either expressed separately or in combination on spleen NK cells from H-$2^d$, H-$2^b$ and MHC I deficient mice. The major alterations in Ly49 receptor expression were found among the subsets expressing two or three receptors. NK cells from MHC I deficient mice had approximately a 3 fold higher fraction of cells co-expressing Ly49A/C or Ly49A/G2 compared to NK cells from H-$2^d$ mice. A reduced number of single receptor-expressing NK cells were also observed, but it was not as pronounced. These early data suggested that there is an MHC I-dependent process specific for sharpening the self-specific inhibitory receptor repertoire which disfavors multiple receptor expressing cells (thereby decrease the average no of Ly49 receptors/cell). This phenomena is called "skewing of the NK cell repertoire", where repertoire refers to the different NK cell subsets expressing 0-5 inhibitory receptors (204, 221). It should be noted that this was at the time studied with limited antibody panels. Today, it is possible to do a more complete analysis with slightly different or at least refined conclusions, as discussed below.

This general pattern was confirmed by Salcedo et al. studying Ly49A, -C and -G2. They observed a reduction of each respective Ly49r in the presence of its cognate MHC I ligand (222). An interesting finding in this paper was that very limited amount of MHC I expressed on TAP-deficient (both MHC I deficient) was sufficient to introduce skewing of the NK cell repertoire. This was shown by a higher frequency of Ly49C expressing cells in NK cells from $\beta_2$m-deficient mice compared with TAP-deficient mice ($TAP^{-/-}$ have a slightly higher MHC I expression then $\beta_2m^{-/-}$).

To explain how repertoire skewing could appear, the Raulet group postulated 2 models; "the two step selection model" and the "sequential model". Both these models are built around the theory that each NK cell must express at least one self-specific inhibitory receptor to ensure self-tolerance. However, it is now known that this assumption is incorrect since NK cells expressing no inhibitory receptor for self MHC I have been described. Skewing may not be important for self-tolerance but both models in modified form can still explain the observed pattern. However, skewing may nevertheless be important to shape the NK cell repertoire, perhaps to ensure existence of sufficiently many NK cells that can selectively recognize lack of a specific MHC I allele.

#### *1.9.1.1 The selection model*

The selection model postulates that the Ly49 receptor expression starts with a stochastic process where each individual NK cell switches on expression of one or a number of inhibitory receptors, some being self-specific, others lacking a self MHC I ligand in the host. Each NK cell would then go through two steps of selection, similar to T cell selection, with a

positive selection step to secure that the NK cell recognize self MHC I by expressing at least one self-specific inhibitory receptor to achieve self-tolerance (for how self-tolerance is established see education section 1.9.2 ). The second selection step, negative selection, would eliminate cells with many self-inhibitory receptors, to ensure that the NK cell have a functional repertoire that can sense loss of MHC I alleles in an efficient way.

#### *1.9.1.2 The sequential model:*

The selection model suggests that the developing NK cell adds expression of one receptor at the time in a sequential, but random fashion. After each round (of new receptor expression) the NK cell tests the MHC I mediated inhibitory signal sensed on cells in the environment. If the signal is too weak or missing, the NK cell will be allowed to express additional Ly49 receptor followed by a new signal control step. When the MHC I inhibitory interaction is strong enough the process is terminated. This theory would ensure self-tolerance since the cell would continue this process until at least one self-specific receptor is expressed and the co-expression of self-specific inhibitory receptors would be minimized due to prevention from expressing additional receptors.

#### *1.9.1.3 Comparison of the two models*

Several studies have been performed to establish the mechanism that controls MHC I dependent skewing of the repertoire. Held et al. used Ly49A transgenic mice expressed on H-$2^d$, H-$2^b$ and MHC-deficient background and studied expression of Ly49A, -G2 and -C (223). The major effect of the Ly49A transgene on expression was observed in the endogenous Ly49G2/A population where the expression was significantly reduced (~3 fold) in H-$2^d$ mice, moderately reduced in H-$2^b$ and unchanged in MHC I-deficient mice. All strains still showed Ly49G2/A co-expression and Ly49C expression was almost unaffected in all strains. Further, endogenous Ly49A RNA levels were reduced in H-$2^d$ but not in MHC I deficient mice upon transgenic expression. These data are in favor of the selection model since the sequential model states that if strong inhibition is mediated all additional receptor acquisition will be stopped. The transgenic mice continued to express endogenous Ly49A and –G2 at reduced levels and Ly49C at almost normal levels which could be allowed in the selection model since Ly49C is a non-self-specific receptor in H-$2^d$ mice.

As described above, Williams et al. cultured NKPs on stromal cells to achieve Ly49 expression and analyzed the gene expression profile (via PCR) of NK cells after 4, 7 and 21 days in culture. This in vitro study showed that the Ly49 receptors were acquired in a sequential fashion and not all expressed at once.(84).

Two in vivo studies showed evidence for regulation of the Ly49 receptor repertoire according to the sequential model using either transgenic mice or several mouse strains expressing different MHC I alleles. Fahlén et al. generated Ly49A, -C and Ly49A/C transgenic mouse strains on several H-2 backgrounds. The most clear cut data were obtained in the Ly49C transgenic mice on H-$2^b$ background (224). Ly49G2, -D and A were all down-regulated on NK cells in these mice despite lack of ligands for Ly49G2 and-D and presence of only a

weak Ly49A ligand in these mice. These results are consistent with a sequential model since the total receptor expression is reduced while, according to the selection model, non-self-specific inhibitory receptors would be allowed at a normal frequency.

Hanke et al. studied co-expression of the NK cell Ly49 receptor on NK cells from eight congenic mouse strains in correlation to MHC class deficient mice (225). This study is one of the first analyzing several Ly49 receptors separately, which was possible due to the development of monoclonal antibodies specific for Ly49I and -F. The results indicated that all MHC I alleles examined, have an impact and influence the skewing of the NK cell repertoire. Co-expression was reduced even in the presence of MHC I ligand for only one of the receptors, favoring the sequential model for repertoire skewing.

More recent studies have been performed by members of our group. In the first, two in silico models were developed; one to simulate the two-step selection model and one for the sequential model. The models were designed to simulate receptor repertoire formation in four different single MHC I gene expressing mouse strains; $K^b$, $D^b$, $D^d$ and $L^d$. The results were fitted to expression of Ly49A, -C, -G2 and -I in MHC I deficient mice and modeled for three of these receptors at a time (226). The data were then compared with actual expression of receptors on NK cells from these mouse strains, as determined by antibody staining and analysis by flow cytometry. The data confirmed that Ly49AG2 co-expression was reduced in in $D^d$ and $L^d$ mice but not in $K^b$ and $D^b$ mice lacking the ligand for these receptors. Interestingly it was found that the fraction of NK cells expressing a single self-specific inhibitory receptor was increased. When the experimental data was compared with the probabilities generated in silico, the modeling according to the two-step selection model predicted the outcome with a higher score.

Brodin et al. generated single MHC I hemizygous and homozygous $D^d$ single transgenic mice where the former expressed approximately 50% of the $D^d$ levels observed in the latter. The increase in $D^d$ levels resulted in ~50% reduction of Ly49Asp (single positive, negative for Ly49C, -G2, -I and NKG2A) indicating an MHC I dose dependent regulation of the Ly49 receptor expression (204). All other receptors were expressed at the same level in both transgenic mice. Furthermore, the expression of $D^d$ skewed the NK cell repertoire by enriching for NK cell subsets expressing one to two self-specific receptors (but negative for all the other receptors) while reducing the frequency of NK cells expressing three-five self-specific inhibitory receptors in comparison to NK cells from MHC I deficient mice. The Ly49Asp NK cells showed an MHC I dose dependent reduction in apoptosis and increased sensitivity to cytokine stimulation compared to MHC I deficient mice.

The study also provided information on MHC dependent influence on NK cells expressing the activating Ly49D receptor, also recognizing the $D^d$ molecule. NK cells expressing Ly49D without any additional inhibitory MHC receptors are thus potentially autoreactive in $D^d$ mice. Ly49D single positive cells (negative for inhibitory Ly49r and NKG2D) had an increased frequency of staining positive for the apoptotic marker Annexin V compared to MHC I deficient mice. This suggested that apoptosis could be the mechanism to eliminate potentially

self-reactive NK cell subsets. In conclusion, the study confirmed and expanded the knowledge regarding MHC I dependent regulation of the NK cell repertoire by selecting against cells co-expressing self-specific inhibitory receptors. Further, data indicated that proliferation and apoptosis could be mechanisms regulating the observed skewing of the NK cell repertoire.

Taken together these data show that MHC I expression regulates the NK cells receptor repertoire, perhaps to enrich for the subsets that may be most efficient in detecting lack of a single MHC I allele. The exact mechanisms regulating the process are still not known. All studies mentioned above have been performed on mature NK cells from the spleen, except for the PCR analysis of RNA expression performed on NK cells generated from NKP cells. These studies do not reveal if the skewing phenomenon is a peripheral mechanism (due to interactions and signals in mature cells) or if it occurs in the bone marrow during NK cell development. In addition, the possible processes involved in regulation of the skewing such as proliferation and/or apoptosis, is unknown. This topic was further studied in paper IV.

### 1.9.2 Acquisition and control of NK cell responsiveness and NK cell tolerance

Early in vitro and in vivo studies show that lack of MHC I is enough to render otherwise normal cells sensitive to NK cell mediated killing. These studies also showed that NK cells from an MHC I deficient host are self-tolerant to $MHC^-$ but otherwise normal cells, although they still have the capacity to kill other types of targets cells (227-230). These findings raised questions regarding how NK cells become self-tolerant towards normal cells. A term frequently used in discussions regarding NK cell education is responsiveness. Responsiveness is defined by the NK cell's capability to respond to activating signals through degranulation or cytokine production, where low and high responders are called hyporesponsive and hyperresponsive/responsive respectively

As reviewed in the previous section, the first hypotheses explaining how NK cells achieve tolerance towards self-postulated that all NK cells express "at least one" self-specific inhibitory receptor, thus autoreactive NK cells should not exist but should have been deleted or adapted to express a self-specific receptor. With the development of techniques and antibody reagents, making it possible to co-stain for all known MHC I specific inhibitory receptors on the same NK cell, the "at least one" model could later be proven incorrect. NK cell subsets lacking expression of any MHC I self-specific inhibitory receptor were demonstrated and studied. However, already during the 90's the question was raised regarding if autoreactive NK cells actually were irreversibly deleted, since they could be detected in certain situations.

MH Johansson et al. showed, already in 1997, dynamic features for NK cell tolerance using $D^d$ transgenic B6 mice "DL6", in which the transgene was inherited as a single dominant gene, but showed a mosaic expression pattern. Between 20-70% of the cells in these mice expressed the $D^d$ transgene (231). NK cells from these mice were tolerant in vivo towards H-

$2^b$ bone marrow grafts but rejected MHC I deficient targets. In vitro tolerance for self was broken upon short separation time of $D^{d+}$ vs $D^{d-}$ NK cells in vitro, leading to killing of $D^{d-}$ cells by the $D^{d+}$ NK cell population. This indicated that the autoreactive cells are still present and that there may be a need for constant exposure to MHC ligand deficient cells to set and retain tolerance.

A new era in the research on NK cell education was initiated in parallel by the Raulet and Yokoyama laboratories using the possibility to study potentially autoreactive NK cells at the single cell level. Both laboratories published similar results showing that NK cells without a self-specific inhibitory receptor exists and that they are self-tolerant. Although the data were quite similar, two different hypotheses regarding how this self-tolerance is achieved were presented (see below).

Fernandez et al. showed by cell surface staining that there exists a subpopulation (approximately 10% of the total NK cell pool) of NK cells in B6 mice which do not express any known MHC I self-specific inhibitor receptor and do most probably not express an unknown self-specific receptor (203). This population was defined by the lack of expression of Ly49C/I/NKG2A- (CI/NKG2A-) while the population expressing self-receptors in B6 mice (Ly49C/I/NKG2A$^+$) was called CI/NKG2A$^+$. This NK cell subpopulation was self-tolerant and responded poorly (almost as bad as MHC I deficient NK cells) in cytotoxicity assays against YAC-1, and $\beta_2$m-deficent Con A blasts compared to CI/NKG2A$^+$ NK cells. It also showed a reduced IFN$\gamma$ production in response to various in vitro stimuli such as YAC-1, RMA-Rae-$\gamma$, $\beta_2m^{-/-}$-Con A blasts, as well as a weak response to antibody crosslinking of activating receptors, such as NK1.1 and Ly49D. In other words, it displayed a hyporesponsive phenotype. However, the subset could still mediate function after positive stimulation with PMA and responded to *Listeria* stimulation indicating that this subpopulation has the capability to respond at least in some situations. The CI/NKG2A$^-$ NK cell subset had a normal phenotype regarding expression of other receptors and maturation markers. A decrease in KLRG1 expression, similar to what has been observed in MHC I deficient mice, was observed. The authors put forward a hypothesis stating that NK cells, during development and inhibitory receptor acquisition, interact with cells in their environment which influence the responsiveness of the NK cells. The model was termed the disarming model. It states that an NK cell is responsive by default and to remain responsive it needs to receive an inhibitory signal via a self-specific inhibitory receptor. If the NK cell lacks such receptors and signals it will become hyporesponsive by an active process to ensure self-tolerance.

On the other hand Kim et al. suggested a hypothesis for NK cell self-tolerance called the licensing model (202). It postulated that all NK cell are non-functional /unlicensed from the beginning and upon interaction between an NK cell expressing self-specific inhibitory receptor and cognate MHC I molecules functional competence and a licensed state is conferred to the NK cell. Thus, NK cells without a self-receptor are self-tolerant by nature and those who are not potentially self-autoreactive receive the license to kill due to inhibitory

receptor interaction. This hypothesis was based on data showing that NK cells expressing self-specific inhibitory receptors, both in two different wild type mouse strains and in two transgenic mouse strains expressing only one MHC class I allele, display an increased IFNγ response after antibody crosslinking of activating receptors compared to the same NK cell population from an MHC I deficient host. Furthermore, NK cells expressing a modified Ly49A receptor with an ITIM mutation in H-$2^d$ mice failed to mediate an increased IFNγ production upon stimulation, suggesting that the licensing signal might be dependent on the inhibitory Ly49 receptor itself, in this case Ly49A. Yokoyama and colleagues suggested that licensing may occur through activating signals by Ly49 receptors in an ITIM dependent, but a SHP-1 independent, way which results in a functionally responsive NK cell.

These two pioneering papers in the field of immune cell education changed the whole NK cell research in several ways, both the view on NK cell development and the way by which investigators analyze the NK cell pool.

A third model is "the rheostat model" was postulated by Petter Höglund and colleagues (232-234). It states that responsiveness in NK cells is not a fixed and absolute state (on or off), instead it is a dynamic quantitative feature that can change over time to adapt or retune to the host environment by monitoring the balance between activating and inhibitory input. According to the rheostat model, NK cells receiving stronger inhibitory signals will achieve higher responsiveness. Strong inhibitory signals could be due to expression of an inhibitory receptor for a MHC I allele with high educating impact: some MHC I alleles are better than others at educating NK cells and tuning up NK cell responsiveness. Strong inhibitory signals could also be due to co-expression of multiple inhibitory receptors actin in synergy. This highly responsive NK cell population will be able to respond with multiple functions, degranulation (CD107a) and cytokine production (e g IFNγ), and also have a stronger response per cell (i e produce more IFNγ).

This model was suggested based on old and new findings. Early studies regarding NK cells and self-tolerance have shown that this mechanism is reversible. As mentioned above, MH Johansson showed in the 90ths that when the MHC I environment is altered the NK cell can change and reset its capability to eliminate a specific target cell type.

Further S Johansson et al. showed that NK cell education is influenced by both the quality and the quantity of inhibitory signaling in the NK cell (235). This was shown by using several mouse strains expressing a single MHC I allele and measuring the MHC I educating impact by in vivo rejection of MHC I deficient targets. Rejection, i e the reactivity of the NK cell population, was not only influenced by the impact strength of MHC I molecules but also by the frequency of NK cell expressing a receptor for the MHC I allele. Later, it was shown by the same authors that each Ly49r have a broader capacity than previously expected to bind to different MHC I alleles influencing the responsiveness of the NK cell. By studying in vitro responsiveness of specific NK cell subsets from mice expressing only one specific MHC I allele they observed that single positive NK cells, for either Ly49A,-C,-G2 or-I, in four different single MHC I expressing mouse strains, displayed a higher frequency of

degranulation after stimulation compared to MHC class I deficient mice. This indicated that all of the inhibitory receptors had interacted with all MHC I alleles leading to acquired responsiveness though licensing.

This concept was further proven by Brodin et al. by correlating the amount of inhibition to the responsiveness at the single cell level. NK cells receiving a higher degree of inhibitory input, either by expressing several inhibitory receptors or by coming from an animal expressing several MHC I educating alleles, showed increased degranulation or IFNγ response (233). Using $D^d$ hemizygous and homozygous mice the same authors also showed that NK cells expressing a single inhibitory receptor from homozygous mice with a higher expression of a strong MHC I educating allele had an increased responsiveness and capability to respond to activating stimuli with several functions, such as degranulation and cytokine production, compared to the same NK cell subset from a hemizygous animal. This indicated that the expression levels of an MHC I allele directly controls the NK cell function and responsiveness. The rheostat model states that the NK cell education depends on the quantity of inhibitory input. The net balance between the inhibiting and activating signals will determine the responsiveness i e NK cells receiving strong signals will be able to respond strongly with several functions compared to NK cell receiving a weak signal. Furthermore the system is tunable over time, dependent on how much inhibitory signals an NK cell perceives.

As previously described, MHC I influences the Ly49 inhibitory receptor repertoire by skewing the repertoire enriching for subsets expressing one or two self-specific inhibitory receptors. However, the inhibitory receptor KLRG1, not recognizing MHC I, is also influenced by MHC I expression but in a different manner than the Ly49 receptors, i e there is a higher frequency of NK cells expressing KLRG1 in the educated ($Ly49r^+$) NK cell population (128, 204). One of the first studies on murine NK cells and KLRG1 showed that the expression was up to 3 fold reduced in MHC-deficient mice ($\beta_2m^{-/-}$, $Tap^{-/-}$, $K^bD^{b-/-}$). The same study also revealed that a strong MHC I ligand increased the expression of KLRG1 further, thus NK cells from $H\text{-}2^d$ expressing mice had a higher frequency of KLRG1 expression compared to NK cells from mice expressing $H\text{-}2^b$. Although KLRG1 does not directly bind to MHC I, the expression may be dependent on signaling generated from MHC I-Ly49r interactions since loss of the signaling molecule SHP-1 reduced the KLRG1 expression (128). KLRG1 expression by NK cells has thus been found to correlate with education. However, KLRG1 expression is influenced by other processes as well, such as cell proliferation and activation (131, 132). The importance of KLRG1 function in NK cell biology in general (and education in particular) is not fully understood. There are indications that KLRG1 binds to monomeric E-cadherin and by multiple interactions inhibits NK cell function (236, 237). KLRG1 binds to a conserved region, allowing it to monitor expression of several cadherins and may thus mediate MHC I independent missing self recognition important for tumor surveillance (237).

### 1.9.3 MHC I independent missing self recognition and education

Another receptor binding to a self-ligand is the receptor 2B4 binding to CD48 expressed on cells of hematopoietic origin. Lee et al. studied NK cells from 2B4 deficient mice using RMA ($CD48^+$) and RMA-S ($CD48^+$) tumor cells in vitro and in vivo. They found that 2B4 deficient mice eliminated the two targets with the same pattern as wt mice (increased killing of RMA-S due to missing self), however the total responses were increased against both targets i e 2B4 mediated inhibition occurs independently of the MHC I system (238). However, upon introduction of Rae-$1_\gamma$, both the 2B4 and MHC I dependent inhibition were overriden leading to an almost identical efficient elimination of both RMA-Rae-$1_\gamma$ and RMA-S- Rae-$1_\gamma$ by either 2B4 or wt NK cells. Further, in vivo subcutaneous tumor outgrowth assay showed that NKG2D ligand expression could override the influence of both inhibitory systems, although RMA-Rae-$1_\gamma$ tumors grow out at later time points. In addition, at an early tumor stage, either missing self recognition or activating ligands per se were enough to induce tumor elimination. However, both systems were needed to induce complete regression. The authors conclusion was that 2B4 is an MHC I independent inhibitory pathway sensing self via CD48 and that stressed induced ligands can bypass both inhibitory systems.

The accepted concept regarding NK cell education is that NK cells via Ly49 receptors are educated on MHC I ligands and therefore receive responsiveness and the capability to perform missing self rejection of MHC ligand deficient, but otherwise normal host cells. However, other self ligands have been shown to influence this function. Ly49A, but not other Ly49r, has been shown to bind to a non-classical MHC I molecule, H2-M3, which is mainly expressed on B cells (239). H2-M3 influences the education and responsiveness of the $Ly49A^+$ population since mice deficient in H2-M3 show a reduced NK cell responsiveness and tolerance to H2-M3-deficient bone marrow compared to wild type mice which reject such cells.

Further, there are data indicating the importance of MHC I independent missing self rejection via the inhibitory receptor NKR-P1B using Clrb-deficient mice (125, 240). $Clrb^{-/-}$ NK cells have a higher expression of NKR-P1B compared to wild type NK cells, similar to increased Ly49r expression on NK cells from MHC I-deficient animals. Interestingly, $Clrb^{-/-}$ bone marrow was rejected in wild type mice in a NKR-$P1B^{hi}$ NK cell dependent manner, indicating a missing self function of the NKR-P1B-Clrb system. In vitro stimulation with NK1.1 or IL-12 showed differences in IFNγ release between $Clrb^{-/-}$ and wild type NK cells but not between NKR-$P1B^{hi}$ and NKR-$P1B^{lo}$ from $Clrb^{-/-}$ mice. However the differences observed regarding NK1.1 treatment could be due to reduced NK1.1 expression by the $Clrb^{-/-}$ NK cells. Rahim et al. also studied the NKR-P1B-Clrb system, with focus on NKR-P1B receptor deficient mice (241). These mice had a normal phenotypic expression, although a reduced KLRG1 expression similar to NK cells from MHC I-deficient mice was observed. The NKR-P1B-deficient mice showed a normal IFNγ response towards in vitro stimulation and among the wild type NK cells a higher responsiveness was observed when both NKR-P1B and Ly49C/I was expressed, compared to expressing them separately or not at all. In

vivo elimination studies revealed that in vivo pre-activated wild type NK cell could reject $Clrb^{-/-}$ spleen cells while this capacity was eliminated in the NKR-P1B-deficient mice. However, the NK cells from the NKR-P1B-deficient mice had an increased rejection of MHC I-deficient spleen cells compared to wild type mice, while both could reject double $Clrb^{-/-}$ MHC I- target cells with a high efficiency. Taken together these two studies using the NKR-P1B-Clrb system indicates that the expression of the NKR-P1B receptor can contribute to the NK cell education, total responsiveness and that the receptor may have a parallel (to Ly49r-MHC I) missing self recognition function regulating for example rejection of bone marrow transplants.

In conclusion these data indicate that there are several missing self recognition systems influencing the NK cell overall responsiveness and decisions regarding if a potential target cell should be eliminated or not. This sheds some light on how complicated and tightly regulated the NK cell system is. Although the Ly49r-MHC I interactions may have the strongest influence, all pieces will be weighed and summed up shifting the balance towards activation or inhibition.

### 1.9.4 NK cell education and the influence of Ly49-MHC I cis-interactions

Ly49 receptors on NK cells can interact with MHC I ligands in either trans (on another cell in the environment) or in cis (on the NK cell itself). There are conflicting data and interpretations regarding how much each interaction contribute to the functional and educational status of the NK cell.

Early studies by our group and others showed that cell surface levels of Ly49A as detected by antibody staining in flow cytometry were lower in MHC I ligand expressing mice compared to ligand-negative (242, 243). Furthermore, in studies of NK cell from mosaic $D^d$ transgenic mice, all NK cells ($D^{d+}$ and $D^{d-)}$ down modulated Ly49A levels in vivo, while only the $D^{d+}$ NK cells kept Ly49A levels low upon in vitro culture. This suggested that $D^d$ ligands on the NK cell itself could interact with Ly49A and modulate expression levels (244).

In addition NK cells were shown to take up MHC I alleles via trogocytosis from surrounding cells, influencing the NK cell function (245, 246). Sjöström et al. showed that B6 NK cells expressing Ly49A could upon transfer to a Balb/c ($H\text{-}2^d$) mouse acquire $D^d$ molecules on their cell surface already 20 min after transfer. This phenomenon was also seen for $K^b$ and the Ly49C receptor in transfers of MHC-class I-deficient NK cells to a $K^b$ expressing environment. Transfer of $D^d$ to $Ly49A^+$ cells in vitro reduced Ly49A mediated NK cell lysis of sensitive targets but the $Ly49A^+$ cells that acquired $D^d$ molecule could still be inhibited by targets expressing the $D^d$ molecules.

One of the first papers demonstrating cis interactions shows that Ly49A binds to $D^d$ using the same binding site in cis and in trans interactions, indicating that cis interaction can prevent the NK cell from binding in trans and thereby become inhibited (247). Moreover, $Ly49A^+$ NK cells from a $D^d$ expressing animal were less inhibited by target cells, both in IFNγ

response and target cell lysis, than NK cells from a $D^{d-}$ mouse indicating that cis interaction can influence NK responses by limiting inhibition from trans interactions.

Andersson et al. showed using a GFP-$D^d$ molecule that $D^d$ is specifically acquired by $Ly49A^+$ but not by $Ly49A^-$ NK cells from a B6 mouse while acquisition by NK cells expressing an endogenous $D^d$ ligand was reduced by approximately 90% due to occupancy (248). Acquisition could only partly be restored by acidic treatment (removing the endogenous cis-bound $D^d$), suggesting a 60% reduction in Ly49A cell surface receptors on NK cells from a $D^d$ expressing mouse compared to those from a B6 mouse and that 75% of the Ly49A receptors expressed are bound in cis. Thus, only 25% of the expressed molecules are available for trans interactions and inhibition via MHC I ligands on potential target cells. These data explain the reduced inhibition of $D^{d+}$ $Ly49A^+$ cells described above.

In later studies the role of cis vs trans interaction in NK cell education has been investigated. The data are conflicting. Kim et al. showed that the educational process, licensing, of $Ly49A^+$ NK cells depended on a functional Ly49A receptor since the responsiveness was decreased when the inhibitory signal was lost (202). Chalifour et al. took this a step further and analyzed if Ly49A dependent education was mediated through a cis or trans interaction or both (249). NK cells expressing an Ly49A receptor with a mutation in the stalk region making it incapable to bind to MHC I in cis, hence only binding to MHC I in trans, was still fully capable to be inhibited via $D^d$. However, NK cells from a $D^{d+}$ host expressing the mutant Ly49A receptor displayed a reduced killing of H-$2^b$ lymphoblasts in vitro compared to wt$Ly49A^+$ NK cells from a $D^{d+}$ host, suggesting that cis interactions were important for education. In addition, expression of Ly49A on NK cells in an H-$2^b$ animal reduced the responsiveness, both missing self killing and IFNγ production, by the NK cells. This was presumably due to the presence of a non-educating receptor (Ly49A) since it could be restored by introduction of the $D^d$ molecule. However, introduction of $D^d$ molecules could not restore reduced responsiveness in NK cells expressing the mutant Ly49A, indicating a role for cis interaction in education.

Further, NK cells from an H-$2^b$ animal (B6; educated mostly via Ly49C and I) co-expressing Ly49C and -A had a reduced IFNγ production compared to $Ly49C^+A^-$ NK cells. The responsiveness could be restored upon $D^d$ expression or by artificially creating Ly49A-cis interaction in an H-$2^b$ animal by Ly49A antibody binding, indicating a negative effect of the unengaged inhibitory receptor, thus dampening the NK cell responsiveness. These data indicate that Ly49r-MHC I cis interactions, in the case of Ly49A, may be involved in tuning the NK cell responsiveness both positively in response to cis ligand interactions but also by reducing the function of NK cells in absence of a cis-bound ligand.

At the end of 2013 two papers were published at approximately the same time, one stating that only Ly49-MHC I interactions in trans are of importance for NK cell education while the other argued that both cis and trans interactions are needed for education and maintenance of responsiveness.

Ebihara et al used an inducible MHC I single-chain-trimer: $K^b$-$\beta_2$m-peptide ($K^b$-tg) on MHC I-deficient background and observed that $Ly49C^+$ (vs $Ly49C^-$) NK cells became responsive already at day one after MHC I up-regulation while responsiveness of $Ly49A^+$ (vs $Ly49A^-$) cells was not changed (250). This system was used to analyze the influence of cis vs trans interactions on NK cell education. $K^b$ negative NK cells transferred into a $K^b$-tg environment, followed by $K^b$ induction, gained responsiveness in the $Ly49C^+$ subset while $K^b$-tg NK cells transferred into a $K^b$ insufficient host (followed by induction) remained low responders. This indicated that Ly49-MHC I interactions in trans (occurring only in the former situation) play a major role in NK cell education. Trogocytosis, i e transfer of $K^b$ molecules from host to donor $K^b$ negative NK cells occurred but it was unspecific since both $Ly49C^+$ and $Ly49C^-$ NK cells gained $K^b$ expression. This could result in cis-interactions on donor $Ly49C^+$ NK cells. However, since donor $K^b$-tg NK cells did not get licensed in $K^b$-negative host (despite cis interactions), cis interactions may not be sufficient for licensing. In addition, regardless of if NK cells expressed $K^b$ or not, they only became licensed upon interaction with $K^b$ on hematopoietic cells while they remained unlicensed if $K^b$ was only expressed on the stromal cells. In conclusion this study suggests that the NK cell is educated via MHC I interactions in a trans and depends on MHC expression on hematopoietic cells. Further, the process can be independent from development and there are no clear alterations in the NK cell phenotype after licensing has occurred. It should be noted that the results do not exclude a role for cis interactions in education. It cannot be excluded that $K^b$-negative NK cells were influenced by ligands acquired by trogocytosis.

However, Bessoles et al. used two different models to study the loss of MHC I interactions in either cis or trans (251). In the first model the $D^d$ molecule was depleted from the system either in cis (from NK cells) or in trans (from T cells). In the second model Ly49A was altered so that it could only interact with MHC I either in cis or trans. Both systems showed that cis and trans interactions are both important to gain fully functional NK cells and that mainly cis interactions influence the MHC I receptor repertoire. Loss of either cis or trans interaction led to impaired in vivo missing self rejection and reduced CD107a responses after in vitro stimulation. Similarly, NK cells educated only via cis interactions or only via trans interactions did not display $D^d$ dependent increase in IFNγ production. These results indicate that the NK cell require interactions to MHC I both in cis and in trans to become fully functional i e perform missing self rejection and to fully respond to in vitro stimulation.

In conclusion there are different opinions regarding the role of cis vs trans interactions in education. However, the cis and trans interactions may be responsible for different parts of the education. The data suggests that cis interaction may be more important to ensure skewing of the repertoire. Bessoles et al. showed that both cis and trans interactions were needed to gain full NK cell responsiveness while Ebihara et al. suggested that only the trans interaction was needed, however a role for cis interactions could not be completely excluded by their data. Thus data from both groups are compatible with a model where both cis and trans interactions are involved in education.

## 1.10 NK CELLS AND IMMUNOLOGICAL MEMORY

NK cells are innate cells with a lymphoid origin but the classification as an innate cell and the notion that previous antigen encounters are irrelevant for NK cell responses has been questioned. NK cells are similar to B and T cells in several aspects. They go through several maturation steps and an educational process to acquire fully functional competence (described above) which raises the question if they should be classified as belonging to the adaptive immune system. This question has become emphasized by several groups who have found indications of immunological memory with long lived NK cells and an increased response upon a second pathogenic challenge.

One of the first studies regarding if NK cells could generate an antigen specific response involved contact hypersensitivity after administration of a chemical hapten (252). This was done in mice lacking T and B cells, where it was observed that the contact hypersensitivity to certain haptens was NK cells mediated; NK cell depletion eradicated the response and transfer of liver-derived Ly49C/I$^+$ NK cells from hapten-exposed mice to naïve mice could induce the higher response. It was antigen specific since an enhanced response was observed by a second injection with the same chemical, with persistence up to a month after the injection, while injection of a different hapten did not induce an increased response. Memory was only transferred via the liver NK cell population expressing inhibitory receptors for self MHC I e g Ly49C/I and in a NKG2D dependent fashion. These data may imply that licensing via the inhibitory receptor is a prerequisite for acquisition of NK cell memory although the receptor responsible for the hapten recognition is unknown.

One of the most used virus infection models in NK cell research is murine cytomegalovirus (MCMV), where the NK response has been characterized in detail. Major breakthroughs in this field were made by the groups of Welsh, Vidal and Yokoyama by showing that the recognition of MCMV infected cells is dependent on the Ly49H$^+$ receptor (expressed by approximately 40-50% of naïve NK cells from B6 mice) and that it binds to the virus encoded protein m157 on infected cells. (150, 253, 254). The Ly49H-m157 interaction leads to clearance and virus control. In addition, Sjölin et al. strengthened the evidence by showing that the DAP12 signaling molecule coupled to Ly49H is crucial to gain resistance to the virus (255). During an MCMV infection, the Ly49H$^+$ population undergo antigen specific proliferation and expansion; the lack of m157 during the MCMV infection does not result in proliferation of the Ly49H$^+$ subset (256).

In more recent studies Lanier and colleagues used the MCMV model to analyze NK cell memory (257). When Ly49H expressing NK cells were transferred to MCMV infected mice lacking expression of a functional Ly49H receptor the transferred NK cells expanded 100-1000 fold and mediated virus clearance. These NK cells were more responsive upon a second virus challenge, as measured by degranulation and IFNγ production. The transferred NK cells could be detected in several organs such as liver and spleen and

persisted up to 2 months after the infection was cleared. The transferred NK cells could even be transferred to a second and a third host and continued to proliferate and expand. Further, transfer of a Ly49H$^+$ NK cells from a virus experienced host could protect neonatal mice from deadly infection. In correlation to education, it is the uneducated hyporesponsive NK cells, Ly49C/I negative, within the Ly49H population, that are the most proliferative and important in protection against MCMV infections (258). These data show that NK cells can be more long lived than the originally determined lifespan with a half-life of 17 days (259). It also implies the existence of some sort of memory since the competence can be transferred to a second host with the ability to give a more pronounced response compared to naïve mice.

Yokoyama's lab searched for possible memory-like effects with a different approach (260). Spleen cells were activated in vitro with IL-12, IL-18 and a low dose of IL-15, fluorescently labeled and transferred into naïve hosts. The cytokine activated NK cells produced high amounts of IFNγ during the first week after transfer but after that returned to background levels. When activated and transferred NK cells were in vitro re-stimulated with either cytokines (IL-12 and -18) or crosslinking of activating receptors, they responded by IFNγ production. These results were observed up to 4w after transfer and indicate that an NK cell intrinsic effect accomplished by cytokine activation can create memory-like NK cells with increased life span and responsiveness upon a second challenge (261).

In conclusion these studies implies the existence of a memory-like feature since the competence can be transferred to a second host with the ability to give a more pronounced response compared to naïve mice. However, if this is memory, as defined for T and B cells, is still unclear.

As mentioned above, MCMV infection leads to expansion of the NK cell population expressing the virus specific activating receptor Ly49H. Similar to this expansion, it has been shown in humans that HCMV seropositivity can drive long lasting expansions of NK cell populations expressing certain activating receptors, the most dominant is NKG2C but also some of the activating KIRs are expressed. They represent a "memory pool" with enhanced function such as IFNγ production (262-265). Schlums et al. recently showed that among healthy adults there was a correlation between seropositivity and expansion of NK cell populations lacking the intracellular signaling molecules FcεRγ, SYK and EAT-2 (266). Further, reactivation of HCMV infection after a hematopoietic cell transplant could specifically lead to expansion of NK cell populations lacking those signaling molecules while seronegative patients or patients with no reactivation of their CMV infection had no alterations in their expression pattern, indicating that the expansion is caused by the HCMV infection. The NK cells negative for FcεRγ, SYK and EAT-2 displayed an adaptive phenotype (with a distinct expression pattern of receptors), altered response pattern and increased proliferation and survival as a response towards infected cells and ITAM-dependent signaling. Together Schlums et al. and Lee et al. has shown that the lack of the

EAT-2 and SYK signaling molecules is probably due epigenetic alterations (hypermethylations) (159, 266).

## 1.11 NK CELLS AND CANCER

Cancer is one of the most growing health problems today. Standard therapeutic procedures such as surgery, irradiation and chemotherapy have been proven to be efficient against the primary tumor but less successful against aggressive metastatic diseases. New types of therapies are on their way including more specific targeting approaches directly aiming at the inhibition of tumor-specific pathways and proteins. Another more recent approach is immunotherapy focusing on using the destructive properties of the immune system to induce anti-cancer activity. This is done by 1) by boosting an existing anti-tumor immune response or increasing the tumor recognition by peptide (for example a tumor antigen) or cell-based (DC or whole tumor cell) vaccines to produce cellular memory via B and T cells or 2) by generally activating an anti-tumor effect via administration of cytokines, such as IL-2, to enhance proliferation and function or via effector molecules (antibodies) and immune cells, such as NK cells, without generating immunologic memory.

### 1.11.1 Immune surveillance

In the 1950s Burnet proposed a theory of immune surveillance against cancer and this was further developed during the late sixties when the T cells were discovered (267, 268). In its original form the theory states that effector cells, T cells, of the immune system are constantly searching for and eliminating tumor cells and thereby guarding us from tumors. However, in the mid-seventies it was found that athymic nude mice, lacking T cells, did not develop more spontaneous cancer than control mice, although they were extremely susceptible to tumor formation after polyoma virus infection. In the late seventies, in the beginning of the NK cell era, many investigators took an interest in these newly discovered cells as possible actors in immune surveillance. To test the involvement of NK cells and to learn if NK cells had evolved to protect us from cancer, scientists asked questions such as; do mice completely lacking or carrying deficient NK cells develop more spontaneous tumors than wild type mice?

Early studies were comparing homozygote C57BL (B6) mutant beige (bg) mice bearing a partial NK cell deficiency to heterozygote or normal B6 mice in different settings to evaluate the importance of NK cells in tumor development. In summary it was shown that NK cell deficient mice had an increased tumor growth and faster death after administration of syngeneic leukemia cell lines or by spontaneously developing tumors and had a reduced in vitro cytotoxicity. Further, the mutant mice had increased frequency of metastases in the lung when using an NK cell sensitive target but had the same amount of tumors as the controls when NK cell insensitive target was used (269-271).

Using the B6 beige mice as a model for selective, total NK cell deficiency has been questioned since the NK cells are not completely deficient and these mice also have a

cytotoxic T cell, granulocyte and platelet deficiency. So other mice and approaches or techniques were used to prove the involvement of NK cells in immune surveillance.

Perforin-deficient mice were used to show that NK cells are needed for in vitro and in vivo elimination of RMA-S and YAC-1 tumor cells and could protect against lung metastasis in two different tumor models. Perforin dependent mechanisms were the major contributors to tumor protection (272, 273).

It took many years and technology improvement to be able to generate a mouse that is only deficient in NK cells and no other parts of the immune system. Kim et al. used a transgenic mouse which lacks almost all NK cells but still have a normal frequency of B and T cells and almost equivalent frequency of NKT cells to wild type (274). These mice had a reduced in vitro and in vivo elimination of the NK cell sensitive targets YAC-1 and RMA-S tumor cells. In addition they had a 60-fold increase of lung metastases caused by injection of B16 melanoma compared to wild type mice.

Other ways of studying the role of NK cells in immune surveillance and tumor growth has been to deplete NK cells by antibody administration or to target e g activating receptor genes. As above, many groups have investigated this and it is today an accepted method and used as a standard control in almost all in vivo assays involving NK cells. One of the first studies was performed by Okumura et al. using anti-asialoGM1 serum to deplete NK cells in BALB/c nude mice and then challenging them with syngeneic tumor cells or YAC-1 cells (275). Depletion of NK cells resulted in a much higher frequency of tumors and increased tumor size. In addition they found that carcinogen induced tumors grew out faster when the mice had been NK cell depleted. NKG2D-deficient mice were generated and had normal NK cell functions except for the NKG2D signaling. The mice showed an increased and faster incidence of both malignant prostate adenocarcinomas and B cell lymphoma but had no effect on the development of carcinogen induced sarcomas compared to the controls (161).

The general conclusion from these studies is that NK cells can participate in immune surveillance but the effect of NK cells depend largely on the tumor itself. Tu et al. used $NKC^{KD}$ mice (mice lacking Ly49r on 80% of their NK cells) to test the influence of MHC I specific inhibitory receptors in immune surveillance (276). These mice had an increased sensitivity to various MHC I-deficient tumors while MHC I sufficient tumors grew equally well in both $NKC^{KD}$ and wild type mice. Introduction of Ly49I on $NKC^{KD}$ background restored the tumor elimination capacity in the $NKC^{KD}$ mice illustrating the influence of Ly49r and education in immune surveillance against MHC I-deficient tumors. Moreover in a spontaneous B cell lymphoma model there was an earlier onset in $NKC^{KD}$ mice and isolated tumors from $NKC^{KD}$ mice had a decreased MHC I expression, suggesting that tumor editing to escape T cells occurred in absence of missing self reactivity by NK cells.

As mentioned in the beginning of this thesis, NK cells were discovered as cells which had a natural capacity to kill and eliminate tumor cells. So why cannot our NK cells protect us from cancer? First, NK cells are quite few, so even if they able to kill and eliminate they are simply

in many cases not efficient enough to eliminate the cancer cells as fast as the cancer cells are growing. Another problem is that NK cells are often located in the periphery of the tumor and do not have access to all tumor cells. However, even if they are in the right location the tumor itself or the tumor milieu can dampen the NK cell function.

### 1.11.2 Therapies based on transplantation or adoptive transfer of cells

In early clinical studies, NK cells were used as a tool to treat cancer based on cytokines such as IL-2 administered systemically. The idea was to improve the NK cell count by proliferation and to boost their function, with or without prior adoptive transfer of ex vivo IL-2 activated NK cells (lymphokine activated killer, LAK cells). This early type of treatments failed since the high dose of IL-2 caused systemic toxic effects, e g in the form of vascular leakage and cardiopulmonary complications; furthermore it could be lethal to the NK cells which died by exhaustion (277). Today the field has developed and NK cells can be either efficiently expanded ex vivo or generated from hematopoietic stem cells from the bone marrow or from umbilical cord blood. In vitro development of NK cells is performed via standardized protocols including cytokines only or in combination with feeder stroma cells (278, 279).

Another up-coming source of NK cells that can be infused into patients are malignant NK cell lines for example NK-92 which is derived from an NK cell lymphoma. (280). Two phase 1 clinical trials have shown that infusion of pre-irradiated NK-92 cells into patients with different solid (such as sarcomas, blastomas, malignant melanomas) tumors is a safe procedure and that the cells can persist in the circulation at least 48h after administration. Some of the patients had a stable disease after the infusion (281, 282).

#### *1.11.2.1 Therapy based on transplantation of hematopoietic (stem) cells*

Allogeneic hematopoietic stem cell transplantation is perhaps the best treatment for blood cancers such as leukemia and lymphoma. The donor T cells are known to promote engraftment and elimination of cancer cells via graft-versus-leukemia effects. The drawback is that mature donor T cells sometimes also recognize the recipient's healthy cells, resulting in graft-versus- host disease, a serious and sometimes lethal complication. Donor T cells can be depleted before the transplantation but this increases the risk of opportunistic infections and can reduce graft-versus-leukemia effects. NK cells are among the first cells to be regenerated and show functional capacity, e g cytotoxicity, after transplantation.

In the setting of transplantation or adoptive transfer, the KIR/ligand mismatch (in the graft-versus-host direction) is used to achieve alloreactivity. This means that the recipient lacks at least one KIR-ligand present in the donor (KIR incompatibility), so that the educated NK cells can perform missing self reactivity against the tumor cells (279). This genetic combination is sometimes achieved in haploidentical stem cell transplantations were the recipient and the donor are matched for only one HLA haplotype. Ruggeri, Velardi et al. are pioneers in this field (21, 22). In the first report they studied 60 leukemia patients where 20 were transplanted with a KIR-HLA epitope mismatch. They found that the donor derived NK

cells of these patients killed the recipient's leukemia cells without causing graft-versus- host disease (21). In the second extended study 34 out of 92 acute myeloid leukemia patients were predicted to have a graft versus host NK alloreactivity. There was a clear correlation between such KIR-HLA class I ligand mismatch and reduced risk for leukemia relapse, the probability of relapse free survival in 5 years was 60% in the patients receiving alloreactive NK cells compared to 5% in the group receiving NK cell without a mismatch (22). They also used NOD/ SCID mice, tolerating human cells since they lack lymphocytes, to infuse either AML cells alone or in combination with either non-alloreactive or alloreactive NK cells. The mice only survived when they had been given alloreactive NK cells. Furthermore, they found that alloreactive NK cells can prevent graft-versus-host disease by eliminating recipient APC which can prime T cells. In a further extended study adding up to a total of 112 acute myeloid leukemia patients the same pattern was observed, only the patients receiving alloreactive donor NK cells showed a decrease in relapse and an increased overall survival (283).

Stern et al. observed increased survival after 5 years following a haploidentical stem cell transplantations, particularly if the alloreactive NK cells came from the mother rather then from the father (284).

More refined and detailed studies focusing on the specificity and phenotype of the allogeneic NK cell populations after haploidentical HSCT have shown that not only the inhibitory KIR-ligand mismatch can influence the outcome after treatment; also the activating KIRs can play a role, both for preventing leukemia relapse and infections (285, 286). This effect was only observed in settings with NK cell alloreactivity and might be mediated by the capacity of the activating KIR to override NKG2A mediated inhibition (287). These results may help in selection of the most "optimal donor" in the future.

The effect of KIR ligand incompatibility has also been studied in hematopoietic stem cell transplantations conducted on partially HLA matched unrelated donors rather than haploidentical donors. Giebel et al. studied patients with myeloid malignancies(288). Patients receiving transplants with KIR ligand incompatibility had an increased probability for both disease free and overall survival at 4.5 years after transplantation. However, several other studies have reported different outcomes. Davies et al. tested the impact of HLA allele mismatch for at least one allele on 175 patients with acute myeloid or chronic myeloid leukemia who received unrelated donor bone marrow transplantations (289). They found a trend towards reduced severe graft versus host disease and an increased survival in patients without KIR mismatch. Farag et al. studied 1571 patients treated with unrelated donor transplantation for myeloid malignancies (290). The KIR ligand matched group had the lowest risk of treatment failure and increased survival but there was no difference in leukemia relapse between the matched or mismatched groups. The different outcomes in these studies may reflect the different conditioning used and/or the preparation of the bone marrow/cell graft; for example Giebel et al. used protocols involving T cell depletion while Davies did not.

Other recent studies have focused on alternative sources of grafts such as umbilical cord blood, which carry an NK cell population that is most active after transplantation and additional receptors contributing to NK cell activation and anti-tumor effects. There are conflicting data regarding the effect of KIR ligand mismatch also in these studies. Willemze et al. showed increased survival and reduced relapse occurrence after KIR ligand mismatch in umbilical cord blood transplantation (291). However, Brunstein found negative effects associated with KIR ligand mismatch, such as increased risk for death and graft-versus-host disease after transplantation of umbilical cord blood with reduced conditioning (292). The differences observed could be due to different conditioning protocols and/or to whether one or two units of cord blood were used.

According to the missing self model, NK cell mediated graft-versus-leukemia effects can be achieved when an educated NK cell recognize a leukemia cell in the recipient lacking the cognate HLA ligand. However, some studies observe graft-versus-leukemia effects in an HLA-matched or KIR ligand matched setting. Yu et al. showed that the hyporesponsive NK cells expressing KIR for a non-self HLA class I ligand from a matched donor could become activated and produce more IFNγ and become more cytotoxic against tumor targets lacking cognate HLA ligand (293). Both effects were transient and gone by 200 days after transplantation. However, other studies have shown disparate results. Björklund et al. made a retrospective analysis on patients with myeloid malignancies treated with HLA matched stem cell transplantation from a sibling (294). In contrast to Yu, Björklund et al. observed that NK cells which were $NKG2A^{-}KIR^{+}$ for a non-self HLA class I ligand were very similar to $NKG2A^{-}KIR^{-}$ hyporesponsive immature NK cells both in early and late phase (up to 6 moths) after transplantation. Furthermore, Haas et al. used a cohort of 60 patients receiving either KIR-ligand matched or mismatched HSCT transplants from either unrelated bone marrow, peripheral blood or umbilical cord blood grafts to study long term effects on NK cell education (295). Transplanted NK cells displayed a reduced responsiveness during the first period (at least 100 days) after transplantation but normalized within a year and remained stable for three years thereafter. Using donor HLA ligands they observed a rapid and stable educational pattern after HLA-mismatched transplantation which seemed to be determined by donor cells i e hematopoietic cells.

It has also been shown that other factors than mismatch of inhibitory HLA specific receptors contribute to NK cell mediated graft-versus-leukemia effects, which might contribute to why increased NK cell activation and tumor elimination is observed in HLA matched transplantations. Pende et al. tested the expression of activating ligands on both myeloid and lymphoblastic leukemia cells and their relevance for tumor lysis (296). Antibody blocking of the activating receptors NKp30, NKp46 and DNAM-1 effectively inhibited lysis of myeloid derived leukemia while only partly inhibiting lysis of lymphoblastic leukemia. Blocking of NKG2D had a mild effect on leukemia cell lysis independently of origin. The results from the antibody blockade were explained by a generally high expression level of ligands for DNAM-1 (poliovirus receptor, PVR, and Nectin-2) but low levels of ligands for NKG2D (ULBP) or 2B4 (CD48) by several myeloid leukemia subtypes, while lymphoblastic

leukemia showed variable expression. The latter tended to co-express activating ligands in variable combinations In conclusion, the results suggest that the outcome of the treatment depend on many factors, such as responsiveness of the NK cell (educational status), expression of activating receptors including KIRs and not least, the variability of the cancer cells.

#### *1.11.2.2 Therapy based on transfer of NK-cells*

Another way of increasing NK cell number or gain NK cell cytotoxicity is to adoptively transfer NK cells alone or together with stem cells/bone marrow in a hematopoietic cell transplant. In the initial NK cell adoptive transfer studies, autologous (from same individual) ex vivo expanded and activated NK cells were used in combinations with low doses of IL-2. The upside of the studies was that the treatment could be performed without any severe side effects and that low doses of IL-2 could be administrated to achieve an increase in the NK cell number. However, the big drawback was that the NK cells displayed reduced cytotoxicity in vivo and that the treatment had a limited effect in the cancer patients (297). The lack of anti-cancer reactivity by the autologous NK cells was mainly due to KIR-HLA class I interactions between the NK cell and the tumor cell, i e limited missing self recognition (298, 299).

More recently, adoptive transfer of alloreactive mature haploidentical NK cells has been investigated, such cells should perform missing self recognition on recipients tumor cells. Benefits with this method are that the patient receives mature functional NK cells and that less immune suppression is needed, leading to reduced occurrence of opportunistic virus infection. Miller et al. as well as Curti et al. have shown that adoptive transfer of haploidentical mature NK cells can increase survival in patients (total 56 patients tested), most suffering from acute myeloid leukemia but also other cancer types (300, 301). However, this field needs to be further explored.

### 1.11.3 Improving NK cell infiltration and cytotoxicity against tumors in vivo

The ability to generate, expand and culture NK cells for adoptive transfer in an efficient way opens up more possibilities. As stated above, it appears that some cancers may be resistant to immune attack due to lack of infiltrating NK cells or due to reduced responsiveness of the NK cells caused by the tumor milieu. NK cell activity and infiltration in the tumor can be improved by manipulation of the NK cell (homing capacity) or the tumor (by upregulation of activating ligands, homing receptors or secretion of chemo-attractants). NK cell activity and function can be enhanced by administration of cytokines, such as IL-2, IL-12, IL-15, IL-18 and IL-21, either separately or combinations (302). Since high doses of IL-2 can lead to systemic toxicity, IL-2 dependent NK cell proliferation and activation may instead be achieved by autocrine secretion of IL-2 using genetically modified NK cell line NK-92 (303). Further, NK cell function (NK cell line or human NK cells) can also be improved via transduction with a chimeric receptor with specificity for a certain cell type or a certain tumor antigen to increase cytotoxicity (304, 305).

It is also possible to make the tumor cells more sensitive to NK cell mediated killing for example via inducing up-regulation of death receptors or activating ligands. Doxorubicin is a chemotherapeutic drug that can make tumors more sensitive to NK and T cell mediated killing via increased expression of the death receptor TRAIL-R (TRAIL- tumor necrosis factor-related apoptosis-inducing ligand) (306).

Another way of making tumor cells more NK cell sensitive is to induce expression of activating ligands, shifting the balance towards activation and target lysis. For many years anti-cancer agents has been used as treatment, mainly because of their direct cytotoxic effect on tumor cells. Many of these agents target DNA and induce DNA damage leading to cell cycle arrest; and activation of the DNA repair system. If the damage is too severe the cell will activate the apoptotic pathway and commit suicide. A problem is that these agents can induce DNA damage to all highly proliferating cells, targeting for example cells from the bone marrow, hair follicles and epithelial cells in the gastrointestinal track, inducing side effects. Other complications are that many tumors are deficient in their activation of the apoptotic pathway, by mutations in key genes such as p53.

A more newly discovered function by some of the anticancer agents is that they also activate the host immune system, for example the NK cells. One way of activating NK cells is by upregulation of activating ligands on tumor cells. NKG2D is one major receptors used by NK cell to eliminate tumor cells. As elaborated in section 1.5.2.1 the expression of NKG2D ligands (in human MICA, MICB and ULBP and in mouse Rae-1γ, H60 and Mult1) are in general very low at the normal state (reviewed in (307)). But they are often induced by stress and the DNA damage pathway in tumors and virus infected cells (172, 173). Several anti-cancer compounds are known to induce up-regulation of NKG2D ligands either in a defined or an undefined way. For example, melanoma patients are often treated with Dacarbazine (DTIC) which has been shown to have a antitumor effect both in human and mice and by an undefined way enhance expression of NKG2D ligands on melanoma cells(308). The NK cell recognizes the ligand on the tumor cell and becomes activated, kills the melanoma cell and produce IFNγ. Production of IFNγ increases the level of MHC I on the tumor cell, making it a potential target for cytotoxic T cells (CTL). Khallouf et al. used a mouse model for pancreatic cancer and showed that the anticancer effect by the combination of 5-flourouracil (5-Fu) and IFNα was due to enhanced NKG2D ligand and MHC I expression resulting in activation of NK cells, CTL and DCs (309).

Another way to make NK cells more reactive against tumor cells is to remove components in the tumor microenvironment that can cause immunosuppression (reviewed in (310). It has been shown that myeloid-derived suppressor cells (MDSCs) have a negative effect on lymphocytes by generating reactive oxygen species (ROS). High levels of reactive oxygen species in the extracellular space can induce cell and tissue damage. T cells and NK cells are sensitive to reactive oxygen species which can induce reduced cytotoxicity by alterations in activation pathways. Hellstrand et al. have developed the use of the drug histamine dihydrochloride in combination with a low dose of IL-2 as a treatment for AML patients in

their first complete remission after chemotherapy (310, 311). This drug/cytokine combination has been shown to increase T and NK cell activity in cancer patients and reduce the frequency of relapses in acute myeloid leukemia patients (311-314). The suggested mechanism behind this effect is that histamine reduces the production of reactive oxygen species by myeloid cells, resulting in restored cytotoxicity and sensitivity to IL-2 in T and NK cells (314, 315).

### 1.11.4 Activation of NK cell via antibody treatment

Antibodies can be used to activate or guide NK cells to attack tumor cells. There are two general types of activation 1) the antibody targets a structure on the tumor cell, thereby inducing ADCC upon (CD16/FcγIIIA) receptor recognition of the Fc part of the antibody 2) antibody blockade of self-specific inhibitory receptors on the NK cells, inducing missing self recognition and killing. The ADCC reactivity may contribute to the anti-tumor effects observed in patients treated with monoclonal antibodies.

Trastuzumab (Herceptin®) is a humanized antibody binding to the growth factor receptor Her2/neu expressed by breast carcinoma cells. Retuximab (Rituxan®), is a monoclonal antibody binding to CD20 expressed on B cells and B cell lymphomas (316, 317). For both of both these antibodies, the evidence in the literature suggests that the anti-tumor effect may be due both to ADCC and direct effects on the tumor cells (receptor-ligand blockade or a direct apoptotic effect).

Unfortunately, in the case of rituximab, many of the patients respond poorly to the treatment. Failure of this type of treatment can be due to neutralizing antibodies that block the Fc interaction, or polymorphisms in the gene encoding the Fc receptor leading to variation in its binding affinity to antibody. It can also be due to that the Fc part of the antibody binds to an inhibitory Fc receptor ($Fc\gamma IIB_2$) expressed on monocytes and macrophages, thereby reducing the total anti-tumor activity (320).

Ravetch et al. have studied the role of activating Fc receptors and ADCC in treatment with antibodies against tumor antigens, by generating mutant mice lacking the γ chain necessary for assembly of various Fc receptors expressed on murine innate immune cells such as NK cells (318). They showed that Fcγ receptor deficient mice were normal with respect to in vitro NK mediated killing of YAC-1 cells while they had lost the ability to kill antibody coated targets via ADCC. To test if the Fcγ receptors and ADCC are important in tumor immunity in vivo, the mutant mice were used to study development of syngeneic melanoma metastasis after passive or active immunization (319). In both set-ups, the wild type mice treated with passive or active immunization against a melanoma antigen showed significantly reduced metastasis compared to untreated wild type mice. However, the Fcγ-deficient animals did not acquire any immune protective effect after any of the treatments despite that the active immunization induced both B and cytotoxic T cell responses. These data indicate that the Fcγ receptor mediating ADCC is important for antibody mediated tumor immunity in vivo.

In a follow up study the same group showed that the efficiency of any given treatment depends on the net effect on many pathways. The inhibitory FcγRIIB receptor is expressed on myeloid effector cells, such as monocytes and macrophages, and on B cells to negatively regulate antibody production.

FcγRIIB-deficient mice were generated to test if this receptor influences the outcome of antibody mediated anti-tumor activity in vivo. Without treatment, the deficient mice developed melanoma metastasis in a manner comparable to wild type mice. However, when treated with the antibody against melanoma antigen, the FcγRIIB-deficient mice gained significantly more protection by the treatment; they developed less lung metastases than antibody treated wild type mice. Furthermore they tested how both the activating Fcγ and the inhibitory receptors contribute to function and end-result of two known pharmaceutically approved antibody treatments, trastuzumab and rituximab described above. The anti-tumor effects of trastuzumab and rituximab were studied using xenograft models based on human breast carcinoma and B cell lymphoma respectively. They intercrossed the activating Fcγ and the inhibitory FcγRIIB-deficient mice to athymic nude mice (320). In both tumor models, the tumors grow and spread equally well in non-treated mice. Treatment with trastuzumab or rituximab inhibited tumor growth and development almost completely in control mice while in the Fcγ-deficient mice very little protection was observed. However, treating FcγRIIB receptor deficient mice lacking the inhibitory Fc receptor with the mouse antibody equivalent to trastuzumab resulted in less tumor burden, indicating that other pathways and other cells than NK cells are important for tumor eradiation and treatment success. To further prove the role for Fc receptor mediated ADCC, the mouse trastuzumab was modified via point mutations. These mutations lead to reduced binding to Fc receptors on the effector cell, resulting in reduced tumor rejection.

Other groups have studied the how the efficacy of the ab treatment can be optimized although the patients have Fcγ receptor polymorphisms. Busfield et al. developed a humanized antibody with improved ADCC capacity targeting the IL-3 receptor expressed in on many tumor cells and blasts from acute myeloid leukemia patients (321). This antibody had been engineered to achieve increased affinity to CD16, which augmented both in vitro ADCC mediated killing and in vivo elimination of leukemic cells. In addition, treatment with the antibody increased in vitro elimination of leukemia cell by NK cells from acute myeloid leukemia patients. (321).

Taken together these studies show the importance of considering the whole picture, in this case the whole immune system, and how it is affected, and not a particular cell type. They also address the importance of designing the antibody for optimal effect, either by high affinity to the Fcγ receptor and low binding capacity for the inhibitory FcγRIIB receptor, or by combining improved efficacy with a specific target increasing the overall function.

#### *1.11.4.1 Increased NK cell activity induced by self-specific inhibitory receptor blockade*

The second type of antibody treatment used to activate anticancer effects by NK cells is based on blockade MHC I binding inhibitory receptors. This is a more specific way of activating NK cells, and at least in theory, only the NK cells that have been educated for missing self recognition, the most responsive NK cells. In addition, this type of treatment can be used when the tumor expresses HLA/MHC I, when other approaches may fail due to the strong inhibition overriding the desired NK cell activation. The effect of blocking inhibitory receptors on NK cells has been studied by many groups, both in mice and in humans. Koh et al. were the first to block self-specific inhibitory receptors for MHC I in the mouse, aiming to induce missing self recognition of tumor targets expressing MHC I. They used $F(ab')_2$ fragments of the antibody 5E6, blocking the murine inhibitory receptor Ly49C and I. They observed increased killing of syngeneic MHC I expressing cells (e g C1498 tumor cells) in vitro and in vivo in a NK cell dependent manner (322-324). This concept had not really been developed further at the time the studies in this thesis were initiated. It has now applied in several settings and explored mechanistically, and the concept of NK cell inhibitory receptor blockade is currently in clinical trials. The topic will be further discussed in result/discussion section 3.5.

#### *1.11.4.2 Checkpoint blockade*

Removal of inhibition, as described above, is used to tilt the balance towards NK cell activation and target elimination. This concept is quite similar to what has been termed immune checkpoint blockade, a term that is mainly if not exclusively used for T-cell regulation. Indeed, immune checkpoint blockade was developed to activate antitumor activity by T cells. Checkpoints are inhibitory pathways controlling the immune system to ensure self-tolerance, and minimize the risk for tissue damage caused by a too strong or sustained immune response. Immune checkpoints are characterized by a receptor-ligand interaction which can be targeted by either antibody blockade or by introducing recombinant receptor or ligand. Antibodies can be used to target receptors or ligands on lymphocytes to increase antitumor activity instead of targeting the tumor cells directly. T cell activation is regulated via two signals, binding of a specific Ag to the T cell receptor and a co-stimulatory signal (CD28 binding to CD80 or CD86 on the antigen presenting cell). The activation is inhibited by CTLA4 (mediating inhibitory signals) on T cells also binding to CD80 and CD86 on the antigen presenting cell, dampening the activation. CTLA4 was the first immune checkpoint regulating antibody to be approved for clinical use, in patients with metastatic melanoma. There is a positive effect on overall survival and a significant number of dramatic, durable responses, which for this patient group represents a remarkable progress (325). Blocking the inhibitory pathway is associated with a potential risk of toxicity due to a sustained immune response, and severe adverse effects of autoimmune or inflammatory nature develop in approximately 20% of patients. In the last year, drugs for blockade of another checkpoint; PD-1/PD-L1 has been approved for melanoma and lung cancer (326).

## 1.12 IMMUNODEFICIENCIES

### 1.12.1 Primary immunodeficiencies

This thesis is mainly about murine NK cells with some comparative comments on human NK cells in relevant sections, such as the one on clinical application above. The following section on immunodeficiencies will deal exclusively with the human system, since the development of clinical observations and basic immunology has provided a great opportunity to learn more about NK cells and how important they are for our wellness. What we can learn from the mouse system will be presented in the discussion together with experimental results obtained during the thesis studies.

Primary immunodeficiencies (PIDs) represent a collective name for disorders that is caused by the inborn lack or a dysfunctional component of the immune system. This weakens the individual's immune system and host defense against unwelcome intruders. Many of these patients are diagnosed as babies or in young life due to recurring and persistent infections such as bacterial or opportunistic virus infections (i e herpes simplex virus or Epstein bar virus). Milder defects can be diagnosed later in life. To be defined as a primary immunodeficiency the cause of the disease must be direct and persistent and not induced by circumstances such as medication or infection. Primary immunodeficiencies are caused by a genetic defect; most of these affect more than one function or cell type of the immune system and quite many of the genetic alterations have been identified and explored at the biochemical level. Some defects cause a developmental arrest, resulting in lack of one or more subsets of cells; others do not influence development as much as effector functions in mature cells. Different defects generate different symptoms. Other defects can cause autoimmunity or even cancer. To treat the patient hematopoietic stem cell transplantation is needed. NK cells can be affected by immunodeficiency syndromes. For example, in some form of severe combined immunodeficiency (SCID) the developmental of NK cells is arrested in parallel with impaired T cell and/or B cell development (327).

There are also defects that affect a distinct function in mature lymphocytes. Hemophagocytic lymphohistocytosis is a lymphoproliferative disorder which can be subdivided into primary or secondary HLH (reviewed in (328)). Primary HLH or Familiar hemophagocytic lymphohistocytosis (FHL) is a group of diseases, FHL1-5, each caused by different mutations with an autosomal recessive inheritance. These patients are often infants with life threatening symptoms, which is due to a defective T and NK cell cytotoxicity. The common denominator between the mutations is that they disturb either the granule content or delivery in cytotoxic lymphocytes. This results in an overstimulated immune system causing massive cytokine production and infiltration. Familiar hemophagocytic lymphohistocytosis type 2 can be caused by different mutations in the perforin gene PRF1 resulting in low or absent levels of perforin (constitutively expressed by NK cells, memory CD8 T cells) in cytotoxic T and NK cells (329, 330). Common manifestations are reoccurring severe infections and malignancies in both children and adults (331-334). The only cure is hematopoietic stem cell transplantation. For secondary or acquired HLH which can occur later in life there have been

associations to triggering via virus infections. HLH is not a single disease, it is rather several syndromes caused by an uncontrolled immune system with massive inflammation.

Other primary immunodeficiencies result in severely impaired development of only one of the three lymphocyte lineages. The DiGeorge syndrome involving a thymic defect leads to selective T cell deficiency. Brutons X-linked agammaglobulinemia involving a defective signaling molecule leads to B cell deficiency. There is also a special branch of primary immunodeficiencies that involves a selective NK cell deficiency (NKD). As in the case of primary immune deficiencies affecting T and B cells, NK cell deficiency is defined as a persistent abnormality in the patient that is stable over time and not induced by as environmental alternation as mentioned above. To be defined as a selective NK cell deficiency the defect must only affect the NK cell population by size, function or both; it must not affect other cell types of the immune system. For example perforin deficiency, mention above, is a primary immunodeficiency but not a NK cell deficiency since it also affects cytotoxic T cells (335). In fact many primary immunodeficiencies affect the NK cell population in several ways such as impaired development, survival or cytotoxic function but they also affect other parts of the immune system reviewed in (336-338).

Two types of selective NK cell deficiency have been observed, for which the terms classical natural killer cell deficiency (CNKD) and functional natural killer cell deficiency (FNKD) have been proposed (336). Classical natural killer cell deficiency is currently defined by the complete lack of NK cells in peripheral blood or that NK cells constitute less than 1% of peripheral blood lymphocytes. In functional natural killer cell deficiency on the other hand there is an almost normal NK cell count in the peripheral blood, but the NK cells are non-functional. Both types of NK cell deficiency lead to decreased host defense and increased occurrence of virus infections. Both classical and functional natural killer cell deficiency is further subdivided by the genetic mechanisms causing the deficiency. The disorders discovered so far are termed CNKD1, CNKD2 or FNKD1.

### 1.12.2 Classical NK cell deficiencies

The first patient with classical NK cell deficiency was documented in 1989.Today at least 19 patients have been identified, all presenting with the absence or severely reduced number of NK cells in the peripheral blood. Furthermore the few NK cells that were found lacked cytotoxic capacity. Many of these patients died prematurely and more than 50% developed severe infections with a virus in the Herpes virus family. Three patients have been treated with hematopoietic stem cell transplantations, two with a great success (339). There are two known genetic mechanisms causing classical natural killer cell deficiency, CNKD1 and CNKD2. The first (CDNKD1) case was caused by haploinsufficiency in the transcription factor GATA2. GATA2 is important for correct NK cell development (379). CNKD2 is caused by a mutation in the gene *minichromosome maintenance 4* (MCM4) which is important for correct DNA replication (380). Patients with CNKD2 have a reduced total number of NK cells but a higher frequency of immature NK cells) in the small NK cell population found. The total NK cells population from these patients shows a low cytotoxicity

against known targets, which could be due to lack of mature cells (381). It could be due to that the perforin containing mature NK cells are simply too few or that a mutation in MCM4 impairs NK cell function.

### 1.12.3 Functional NK cell deficiency

When a patient has no other deficiencies in the immune system except dysfunctional NK cells, they are categorized as functionally NK cell deficient (382, 383). These patients have a normal total NK cell count but the NK cells lack the cytotoxic capacity. Today only one single gene defect is known to cause functional NK cell deficiency, FNKD1, which is a mutation in the gene encoding for the receptor CD16, known to be the most important receptor for NK cell mediated ADCC. This mutation is in the extra cellular part of the molecule but does not affect the CD16-antibody binding, and paradoxically these patients show a normal ADCC capacity (384). At least three patients have been diagnosed with FNKD1, all of them presenting with recurrent virus infections and with reduced NK mediated cytotoxicity against the K562 cells in the clinical immunology testing (382, 385 ). The lack of cytotoxicity could be due to that CD16 normally can bind to CD2, a member of the SLAM receptor family that can induce spontaneous cytotoxicity, but the mutated CD16 lack this capability and can therefore not act as a costimulatory receptor and cytotoxicity will be abrogated (as discussed above, most NK cells receptors except CD16 needs to function in pairs to be able to give fully activation).

# 2 AIM

The overall aim of thesis has been to study missing self recognition by NK cells and how they are educated by host MHC I molecules to perform this function; in particular, whether these processes can be manipulated in an experimental therapeutic setting, as well as their relation to a new type of immune defect in the mouse.

Paper I) To characterize the deficient missing self recognition displayed by the Impaired Missing Self Recognition (IMSR) mice.

- Is the observed NK cell defect due to the targeted gene in these mice?
- Is the IMSR NK cell deficiency due to lack of any activating or inhibitory receptor?
- Are the inhibitory receptors functional i e can they mediate inhibition?
- Is the observed missing self deficiency intrinsic to the NK cell or/and is it due to lack of education?

Paper II) To evaluate if it is possible to manipulate missing self recognition by inhibitory receptor blockade in vivo to enhance elimination of syngeneic MHC $I^+$ tumor cells

- Is it possible to mimic missing self recognition and thereby enhance elimination of MHC $I^+$ sufficient tumor cells in vivo without breaking tolerance towards self?
- Can self-tolerance be secured during a longer treatment period and is the anti-tumor sustained (important issues for clinical application)?
- Can the anti-tumor effect be further enhanced by already establised treatments such as cytokine stimulation?

Paper III) To study if NK cells can retune their responsiveness upon/due to altered MHC I expression

- Is it possible to retune the NK cell responsiveness by altering the inhibitory input, either via inhibitory receptor blockade or via transfer of mature NK cells to an altered host?
- If the responsiveness is increased (up-tuned) are NK cells still self-tolerant?
- Do the retuned NK cells display any phenotypical changes?
- Is the reduced responsiveness in inhibitory receptor blockade caused by crosslinking of receptors leading to direct induction of inhibitory signals in the effector function?

Paper IV) To investigate if the observed skewing of the inhibitory receptor repertoire occurs already in the bone marrow during NK cell development

- At which NK cell developmental stage does the skewing of inhibitory receptor repertoire occur?
- Which mechanistic processes are involved in creating the enrichment for certain populations, increased proliferation or reduced proliferation?

# 3 RESULTS AND DISCUSSION

In this section of the thesis I will focus on those of my discoveries that I think are most interesting, and also on some data that have not been published. I will not follow the order of the papers, instead I will discuss them in an integrated way, under six different themes, sometimes referring to more than one of them under each theme. For all results and details you are welcome to read the papers (I-IV).

## 3.1 GENETIC DEFECTS AFFECTING NK CELLS

As mentioned in the introduction, selective NK cell defects in humans are classified by either lack of NK cells or impaired function of NK cells (with normal numbers). These defects are caused by a few known mutations. However, the murine system can be manipulated both specifically, by targeting a certain gene. This has been used to show the importance of different molecules in NK cell function. NK cells from an MHC I-deficient host are hyporesponsive due to lack of licensing/arming via the MHC I self specific inhibitory receptors. Although many defects are intentionally induced, others have arisen spontaneously and some of them are of unknown basis.

In paper I we characterized a defect observed in a mouse strain, termed IMSR - Impaired missing self recognition. As the name implies, this mouse strain shows impaired missing self recognition; it lacks the ability to eliminate target cells lacking MHC I.

The IMSR defect was discovered in a gene targeted "KO" mouse strain deficient for a non-classical MHC I like gene, CD1, but with otherwise normal MHC I expression. We unexpectedly observed that the mice had severely impaired capacity for NK cell mediated rejection of MHC I-deficient cells. Control experiments with independently derived KO strains for the same gene, as well as $F_1$ and $F_2$ intercrosses with B6 mice indicated that the functional NK cell defect was not caused by the targeted gene, that the trait was recessive and that the two phenotypes, IMSR and deficiency in CD1 expression, segregated independently.

IMSR mice were similar to wt B6 mice in terms of NK cell number as well as NK cell surface phenotype regarding activating receptors and maturation markers. All receptors expressed in B6 mice are also expressed/present in the IMSR mice. However, there was a significantly altered inhibitory receptor repertoire, as evaluated by a large panel of antibodies to activating and inhibitory receptors (see section 3.4.2). In vivo, the NK cells showed a severely impaired capability for missing self rejection of normal spleen cells ($\beta_2m^{-/-}$) as well as of tumor cells (RMA-S). Kinetic studies and tumor outgrowth experiments revealed a profound defect rather than just a delay in missing self rejection. Experiments with mixed bone marrow chimeras indicated that the defect is NK cell intrinsic and not due to the host environment. In vitro assays also showed a prominent defect in missing self recognition, which in most settings could not be overcome with cytokine stimulation. In contrast, there was a normal or only partly impaired capacity with

respect to other NK functions, such as ADCC and NKG2D dependent cytotoxicity. Furthermore, positive allorecognition by NK cells in vivo was not affected: the mice showed a normal capacity for DAP-12 dependent rejection of MHC disparate ($D^d$) cells. However, NKG2D dependent elimination of tumor targets in vivo was severely impaired. For discussion regarding NKG2D and Ly49D see section 3.1.2.

Our results indicate that this mouse strain has a genetically based, NK cell intrinsic and almost selective deficiency in the killing pathway used to sense missing self. Our data argued against possible causes like a complete lack of inhibitory receptors or lack of one or several known activating receptors. As discussed in paper I, lack of inhibitory receptors, lack of functional signaling molecules or other cell types has been shown to influence NK cell function in different ways, but none of the known characterized defects is really consistent with the one observed in the IMSR mice.

The defect may be possible to identify by whole genome sequencing after backcrossing the IMSR defect appropriately on the B6 background. At this stage, one can only speculate regarding the cause of the observed NK cell phenotype in the IMSR mouse. First, the inhibitory receptors or their signaling pathway may be non-functional, which could disturb missing self recognition while leaving other NK functions relatively intact. Second, the dysfunction may involve an unknown activating receptor or signaling molecule, critical for missing self recognition but no other types of activation. Third, at steady state, the NK cell may receive too much activating or inhibitory signals inducing hyporesponsiveness either via "cross-tolerance" or a too high activation threshold to overcome. I will discuss these possibilities below.

### 3.1.1 Are abnormal inhibitory receptors causing the NK cell deficiency?

Even if NK cells from IMSR mice expressed inhibitory Ly49 receptors, it was possible that these cannot signal, and the lack of missing self recognition would thus due to absence of MHC I dependent education similar to what is observed in an MHC I (or Ly49r) deficient mouse. (203, 340-342) NK cells from MHC I-deficient mice are tolerant towards "self", i e normal cells lacking MHC I expression. The lack of inhibitory signals from educating interactions results in hyporesponsiveness, an inability to respond sufficiently when triggered via activating receptors, due to an increased activation threshold. Although MHC I-deficient NK cells are hyporesponsive they still have functional inhibitory receptors and the ability to use them and interact with MHC I when the opportunity occurs, see below and paper III. Further, NK cells in mice lacking SHP-1 and hence the capability to signal via inhibitory receptors, are also hyporesponisve, unable to perform missing self reactivity and control tumors in vivo (342, 343).

We compared missing self recognition in IMSR and $\beta 2m^{-/-}$ mice by studying the animals´ capacity to eliminate MHC I-deficient tumor cells, RMA-S, in vivo. Both IMSR and MHC I-deficient NK cells were capable of an increased elimination of RMA-S cells compared to the MHC I expressing counterpart RMA, although with a significantly reduced efficiency

compared to wild type mice (data not shown). This indicates that NK cells from IMSR and MHC I-deficient mice both can be inhibited via their inhibitory receptors.

In addition to what was presented in paper I, we later studied in vitro killing of Con A-activated T cell blasts (Con A blasts) by IL-2 activated IMSR and B6 NK cells. In this setting we actually observed a significant increase in killing of $\beta_2m^{-/-}$ Con A blasts compared to killing of B6 Con-A blasts (data not shown). This was only observed in the highest effector target ratio (1:100) and killing was not as efficient as by B6 NK cells. In this set-up the cytokine pre-activation could partly override the deficiency normally displayed by the IMSR NK cells. In another set of experiments, we used IL-2 activated $Ly49A^+$ NK cells, isolated by FACS sorting, in a cytotoxic assay against tumor targets with or without expression of the $D^d$ ligand. The IMSR NK cells were inhibited via the Ly49A-$D^d$ interaction to the same extent as NK cells from wild type mice. Another important evidence that the inhibitory receptors of IMSR NK cells are functional is that they display the pattern of skewing of the NK cell repertoire consistent with education and inhibitory input. This will be further discussed in section 3.4.2 and 3.4.3. ). Taken together these data indicate that when we push the system either by increasing activating ligands on target cells or by cytokine activating the NK cells we can detect reactivity regulated by MHC inhibitory receptors, showing that IMSR NK cells have the capability to signal and discriminate via these receptors (344).

The IMSR NK cells showed a partly impaired ADDC against antibody coated targets. This is interesting, for at least two reasons 1) the defect in the IMSR mice affects different parts of NK cell function with different degrees of severity and 2) in patients with functional NK cell deficiency type I, which carry a mutation in the CD16 locus, the NK cells have normal or almost normal ADCC but are deficient in killing of a NK cell sensitive tumor K562 (K562 is the prototype for human NK cell target cells equivalent to YAC-1 in mouse). In the case of the FNKD1 patients the mutation in the CD16 locus alters the co-stimulatory effect of CD16. Here we observed a reduction in CD16 dependent function per se but it was not as severely affected as the missing self recognition. An additional similarity to an NK cell deficiency observed in humans is NK cells with a mutation in the Wiskott-Aldrich Syndrome protein coding gene which are defective in natural cytotoxicity and in ADCC. However, these NK cell regain their function upon IL-2 activation. (345).

### 3.1.2 Is NK cell deficiency caused by alteration(s) in activating receptor pathways?

The observed deficiency in the IMSR NK cells could be due to a mutation influencing one or several of the activation pathways. The most obvious would be a loss of function leading to lack of activation. Many such mutations affecting signaling pathways downstream of activating receptors have been described. As discussed in paper I, none of them fits the IMSR profile completely. However, some other specific deficiencies are quite similar in their observed phenotype e g Fyn or loss of total activating receptor(s) signaling. Another option is that the deficiency is due to a mutation resulting in a gain of function mutation,

resulting in hyper-signaling in an activation pathway. How this could fit our results will be discussed below.

Ly49D and NKG2D are two activating receptors which mediate their signal through DAP12 or DAP10 respectively. We tested these two signaling pathways in different settings. IMSR NK cells could eliminate Ly49D sensitive target in vivo as well as produce IFNγ and degranulate in vitro after Ly49D antibody stimulation. In contrast, IMSR mice were not capable of eliminating RMA-Rae-1γ tumor cells (expressing high levels of NKG2D ligand) in vivo while both B6 and $\beta_2$m-deficient mice did (figure 7 and data not shown). Interestingly, Cerwenka et al. showed that injection of RMA-Rae-1γ tumor cells into the same CD1d1 KO mouse strain as ours resulted in prolonged survival of the mice compared to mice injected with the parental RMA tumors (346). This is not in total conflict with our data. The deficiency in NKG2D-mediated rejection observed by us may not be total but rather a severe defect observed in short term experiments which can be overcome during a longer time period. In addition, it has been shown that elimination of RMA Rae-1 is dependent on both NK cells and $CD8^+$ T cells (35, 169). It is possible that the $CD8^+$ T cells mediated tumor elimination was observed in the long term experiments but not in our short in vivo assay. Taken together these results indicate that the IMSR mice have a fully functional signaling via Ly49D and DAP12 but not via NKG2D, DAP10 or downstream of DAP10. These data are further supported by the fact that DAP12-deficient mice show an intact missing self recognition (347, 348). Raulet generated an NKG2D-deficient mouse which lacks the capability to eliminate NKG2D ligand expressing tumors both in vitro and in vivo (161).These mice showed a normal missing self rejection. Even if the NKG2D receptor in the IMSR mice is mutated or the signal is severely impaired, the IMSR mice must have an additional or a different defect explaining the impaired missing self reactivity.

As mentioned above, no known genetic NK cell defect in mice or humans is really consistent with the phenotype observed in the IMSR mice. However, there is at least one study of dysfunctional NK cells sharing many similarities with the IMSR NK cells. This "NK cell deficiency" is not genetic, but caused by chronic exposure to ligand for the activating receptor NKG2D in vitro (349). B6 NK cells were co-cultured with RMA-H60 (expressing high levels of NKG2D ligand H60) for 3 days. Interestingly, this chronic stimulation did not only abrogate the NKG2D-DAP10 mediated killing, it also impaired ADCC (via CD16-FcRγ) and missing self killing of MHC I-deficient tumor cells (RMA-S). Ly49H-DAP12-mediated killing remained intact. In addition, the NK cells had a reduced in vitro IFNγ response to RMA-S cells or plate bound antibody stimulation via NK1.1, while Ly49D stimulation gave a significantly increased IFNγ response. The functional phenotype observed after chronic ligand exposure is almost identical to the phenotype observed in the IMSR NK cells. As may be noted in figure 6 in paper I, NK cell from the IMSR mice without antibody stimulation displayed an increased spontaneous release of IFNγ and the same was true for the NK cells which had been chronically activated, indicating some sort of increased basal activation state (paper I figure 6, my unpublished data and (349). In addition, the NK cells chronically exposed to the NKG2D ligand had an impaired $Ca^{++}$

influx (needed for NK cell activation) after stimulation via NKG2D, CD16, NK1.1 and NKp46, while Ly49D and Ly49H $Ca^{++}$ influx remained almost intact. The induced NK cell defect affecting multiple activation pathways was only observed when NK cells were exposed to targets expressing NKG2D or Ly49D ligands (using DAP10/12 or DAP12 respectively) but not to targets triggering CD16 or inhibitory Ly49r. So, continuous stimulation through either DAP10 or DAP12 can induce, as termed by the authors, "cross-tolerance" while the other receptors/adaptor proteins tested cannot.

Chronic exposure to activating ligands for NKG2D has also been studied in vivo. Oppenheim et al. generated several transgenic mouse strains with different range of normal cells expressing the NKG2D activating ligand Rae-1 (350). Upon constant exposure to its ligand, NKG2D was downregulated both in vitro and in vivo. The NK cells from Rae-1 transgenic mice could not kill targets in vivo expressing the Rae-1 ligand or eliminate MHC I-deficient spleen cells. This impairment could be restored by treating the mice with poly (I:C) which activates NK cells via Toll like receptor 3 expressed on myeloid cells, resulting in type I interferon production. In contrast to NK cells from Rae-1 transgenic mice, those from IMSR mice cannot regain ability to kill MHC I-deficient tumors by cytokine pre-activation in vitro or gain ability to respond to plate-bound antibody stimulation in vitro after in vivo pre-activation with a the type I interferon inducer tilorone.

One can speculate that the defect in the IMSR mice could be due to increased interaction with NKG2D ligands in vivo inducing "cross-tolerance". But if this was the case, IMSR NK cells from mixed bone marrow chimeras in wt mice should not display the defect while wt NK cells from mixed bone marrow chimeras in IMSR mice should. In contrast, the defect was intrinsic to IMSR NK cells (see section 3.1 above). In addition, NK cells in the IMSR mouse express NKG2D with a slightly but not significantly reduced MFI (mean fluorescense intensity). The reduction in expression levels for NKG2D is most likely not responsible for the reduced function since NK cells from MHC I-deficient mice also expressed lower levels of NKG2D (paper III and unpublished data) but were still capable of rejection of tumor cells expressing NKG2D ligands. Altogether these observations argue against that the IMSR NK cells are chronically exposed to NKG2D or Ly49D ligands mediating sustained DAP10/12 signaling.

However, the studies by Coudert and Oppenheim et al. support my idea that the cause behind the IMSR phenotype could be overstimulation of NK cells, e g that activating signaling is constitutively on, even in the absence of cognate receptor-ligand interactions. This could potentially be caused by an overexpressed or altered signaling molecule or by the lack of a phosphatase so that the signaling is not abrogated. If this would be the case it could give the same functional defect as observed in "cross-tolerance" caused by chronic exposure to ligand.

Despite the similarities between the IMSR functional phenotype and the effect of chronic NKG2D ligand exposure, NK cells with a total lack of NKG2D also share features with the IMSR NK cells. Removal of both NKG2DL and -S expression leads to a reduction of the

spleen size due to a reduction in the B, T and NK cell compartments (351). This is partly true also for the IMSR mice which show a reduction in spleen size and total lymphocyte number, have normal proportion of NK cells and T cells but reduced frequency of follicular B cells and almost no marginal zone B cells (351) (Mikael Karlsson's unpublished data). Mice lacking NKG2D expression display a normal NK cell maturation pattern in the spleen but a reduction of more mature and KLRG1 expressing NK cells in the bone marrow. Interestingly, NK cells from NKG2D-deficient mice display the opposite responsiveness pattern to IMSR NK cells i e they have an increased cytokine production in response to certain types of stimulation and the mice display an increased survival to cytomegalovirus infection. In addition, the NK cells from these mice have a faster cell division rate but are also more susceptible to apoptosis in both IL-2 and IL-15 in vitro cultures. In summary, we cannot exclude that NKG2D is involved in the IMSR deficiency, further investigation is needed.

Another strong activating receptor expressed by a considerable proportion of NK cells in at least some mouse strains is Ly49H, recognizing the MCMV encoded protein m157. Bolanos et al. used a transgenic mouse chronically displaying m157 to the NK cells (352). This exposure induced reduced function of $Ly49H^+$ NK cells, not only in response to m157 but also to Ly49H independent stimulation in vitro via NK1.1 and YAC-1. The induction of this reduced responsiveness was DAP12 dependent, and was observed in mature NK cells but was rapidly reversible if ligand exposure was removed. These results corroborate that increased signaling from an activating receptor can lead to impaired general NK cell function.

Similar to the NKG2D-deficient mice discussed above (351), mice lacking NKp46 also display a hyperresponsive phenotype. Narni-Mancinelli et al. showed that absence of NKp46 function, either in mutant mice or by antibody blockade in wild type mice, generated hyperresponsive NK cells with a stronger cytokine response after in vitro activation and a prolonged mouse survival after MCMV infection (353). The endogenous ligand for NKp46 is unknown, but these observations indicate that it may play a role in tuning the NK cell responsiveness. These results may thus be interpreted in the context of education but unfortunately the authors did not investigate if there were any differences in responsiveness between NK cells expressing inhibitory receptors for self MHC I or not.

However, there are conflicting data regarding if loss of NKp46 induce hyperresponsiveness. Sheppard et al. generated NKG2D/NKp46 double knockout (DKO) mice to study the influence of activating receptors on NK cell phenotype and function (354). The NK cells from the DKO and the NKG2D single KO mouse showed altered phenotypes, increased number of mature cells and changed receptor expression. In line with the other NKG2D KO mice presented above, these NKG2D KO and DKO mice responded more efficiently by IFNγ production to some activating stimuli. However, the NKG2D KO studied did not alter resistance to MCMV infection. Further, the NKp46 KO mice showed no alteration in either phenotype or functional responses of NK cells which is in contrast to the results presented

by Narni-Mancinelli et al.. However, the influence of NKp46 in resistance to MCMV was not evaluated.

To summarize, it is hard to draw any conclusion regarding our defect from studies based on different KO mice since there are inconsistent data.. In any case, the IMSR mice illustrate nicely that something else than the targeted gene can cause functional changes when a KO mouse is generated and backcrossed. It cannot be excluded that the critical change affects an activating receptor or pathway these mice, this possibility should be further investigated.

### 3.1.3 Signaling pathways and molecules potentially involved in the IMSR NK cell deficiency

Many alterations in signaling pathways downstream of either activating or inhibitory NK cell receptors result in similar dysfunction as the defect observed by the IMSR NK cells. In this section I will go through some findings in this literature that appear to be of particular interest in relation to the IMSR NK cells.

The SLAM receptor 2B4 and its downstream signaling pathway may influence NK cell responsiveness. As elaborated on in the introduction (section 1.7.4) the regulation of murine 2B4 function is dependent on the SAP-family (SAP, EAT-2 and ERT) where all three members synergize to mediate activation (142). The ligand of 2B4 (CD48) is expressed on all hematopoietic cells, explaining why mice deficient for the SAP family members displayed a reduced NK cell killing of hematopoietic cells, such as RMA-S and YAC-1, as well as a reduced in vivo rejection of normal MHC class I-deficient spleen cells, while they showed an increased killing of non-hematopoietic target cells. Further, in the absence of the SAP-family, the 2B4-CD48 interaction inhibits activation through NKG2D and CD16 in a SHIP-1 dependent manner (196, 198). These data are in line with the functional phenotype of the IMSR NK cells even if reactivity to non-hematopoietic cells has not been tested yet. It would be of interest to study the general function of 2B4 in the IMSR mice and to test reactivity against a non-hematopoietic target cells. Although western blot analysis indicates that the Fyn protein is expressed at normal levels in cells from the IMSR mice, it could still be non-functional. It would also be of interest to study the SAP-Fyn pathway and to block the EAT-2 pathway in the IMSR mice.

Other important signaling molecules participating in activation of NK cells are phospholipase Cγ-1 and -2 (PLC-γ1 and PLC-γ2) and mice mainly express and use PLC-γ2 (355). Mice lacking PLC-γ2 are perturbed in NK cell maturation, in expression of Ly49 inhibitory receptors and in NK cell function. Overexpression of PLC-γ1 can restore the maturation and Ly49 expression but only partly rescue the function (356). Furthermore, PLC-γ2-deficient NK cells lack the ability to respond to almost any activation. They show defective killing of targets via NKG2D, Ly49D, missing self recognition, and lack the capability to produce cytokines after NK1.1 stimulation, but hyporesponsiveness was not due to lack of total function since the NK cells could fully respond to PMA stimulation (356, 357). However, an interesting similarity to the IMSR mice is that PLC-γ2 mice lack a

large population of follicular B cells and marginal zone B cells in the spleen (358-360) and Mikael Karlsson's unpublished data). In line with the NK cell deficiency, the marginal zone B cell deficiency in IMSR mice was also cell intrinsic.; transplantation of bone marrow from an unrelated $CD1d^{-/-}$ KO mice with normal marginal zone B cell numbers grow out in the IMSR host (Mikael Karlsson unpublished data). Further studies are needed to evaluate if both deficiencies in the IMSR mice have the same genetic basis. In addition to the mechanism behind the defect(s) per se it would be of interest is to study how the IMSR mice and their NK cells can handle a virus or bacterial infection since the PLC-γ2-deficient mice cannot control CMV infection although they can handle a Listeria infection (357). NKG2D-DAP12 can signal via both Grb2 resulting in the usage of PLC-γ2 but also via PI3K (via p85) which is PLC-γ independent. However, as described above, lack of PLC-γ2 resulted in severely impaired NK cell function in general but also through NKG2D, indicating non-complete redundancy in the system.

## 3.2 HYPORESPONSIVENESS DUE TO RETUNING AND OTHER PROCESSES

### 3.2.1 Altered responsiveness upon antibody blockade

Interactions between MHC I specific inhibitory receptors and their ligands during education regulate NK cell responsiveness in encounters with target cells. As noted in the introduction, there were initially two postulated models for how this occurs, via licensing or disarming. A third model, the rheostat model, incorporates features from both of the other models, and emphasizes that responsiveness is reversible and the NK cell can be retuned upon altered MHC I expression or inhibitory receptor input. In addition, a "sequential arming/disarming model" stating that NK cells need to interact with MHC I in both cis and trans to become fully responsive has recently been proposed (361). This model suggests that education is a two-step process; the first step is dependent on Ly49-MHC I cis interactions mediating arming and the second step maintains responsiveness via trans interactions. It will be further discussed below (section 3.2.2 and 3.4.3).

Studies in adoptive transfer models have yielded results supporting that NK cells can adapt their responsiveness after transfer to a host with an altered MHC I expression (362, 363). In two separate studies, NK cells were transferred to a host with a different MHC I phenotype and the change in NK cell responsiveness was analyzed in vitro. Mature NK cells from a MHC I-deficient environment can gain responsiveness, e g aquire the ability to produce IFNγ and respond via degranulation, after transfer to a MHC I sufficient host. Conversely, mature NK cell from a MHC I sufficient donor can lose their responsiveness upon transfer to a MHC I-deficient host. NK cell adaptation was observed to be Ly49 receptor dependent; NK cell populations expressing a self-specific inhibitory receptor adjusted their responsiveness, while NK cell populations lacking an educating receptor did not adapt the responsiveness to altered MHC I expression.

In paper III we investigated the rheostat model and retuning, particularly in relation to NK-cell reactivity to tumors. In one part of the study, we used transfer models similar to the

ones mentioned above, where we could confirm and extend the observations regarding tumor cell reactivity. These results are discussed in section 3.5.2. Here, the discussion will focus on the first part of paper III, where we addressed whether it is possible to induce retuning by antibody blockade of self-specific inhibitory receptors in vivo. This represents another approach to alter the NK cells' sensing of MHC I expression in vivo which is less invasive, avoiding irradiation and high numbers of transferred cells. The studies of possible retuning induced by inhibitory receptor blockade were initiated by some unexpected results.

Before this study, we had we demonstrated in paper II that reactivity against syngeneic lymphoma cells can be induced in mice by Ab-mediated blockade of self-specific inhibitory receptors on NK cells without breaking tolerance to normal cells. In vivo blockade of Ly49C/I inhibitory receptors on B6 (H-$2^b$) NK cells with F(ab')$_2$ fragments of the mAb 5E6 (binding to Ly49C and I) caused increased rejection of syngeneic MHC class I-expressing lymphoma cells (RMA) but not of syngeneic spleen cells, BM cells or lymphoblasts (see section 3.5). Self-tolerance was thus very robust, and somewhat surprisingly, killing of normal cells could not be induced by releasing NK cells from inhibitory blockade. However, an unexpected and interesting finding in the course of these studies was that mice treated with the 5E6 antibody showed decreased rejection of splenocytes lacking MHC I. This seemed puzzling, but this "disturbing" observation was very reproducible.

It occurred to us that the results could make sense when considered from the perspective of the rheostat model. A possible explanation for reduced elimination of MHC I-deficient spleen cells could thus be that antibody blockade was not only affecting missing self reactivity during the effector-target interaction, it was also influencing the NK cell responsiveness prior to this event by blocking inhibitory input important for the constant tuning of NK cells, thus inducing hyporesponsiveness in the targeted NK cell population. We reasoned that this effect had not been visible in the studies of MHC class I expressing tumor cells and in paper II, because the effect on retuning would be masked by the opposite effect of the inhibitory receptor blockade in the effector-target interaction phase. The hyporesponsiveness would only emerge when using MHC I-deficient spleen cells as targets. To test the hypothesis that inhibitory receptor blockade would retune the NK cells, we used the in vitro hyporesponsiveness assay, measuring CD107a expression and IFNγ production by the NK cell subsets targeted by the blockade. The Ly49I single as well as the Ly49C and I double positive NK cell subset (expressing no other inhibitory receptors) demonstrated a significant decrease in total IFNγ production and CD107a expression after stimulation with anti NKp46 antibodies. A phenotypic characterization of NK cells after in vivo Ly49C/I blockade showed no major alterations in the expression of activating receptors, maturation markers and inhibitory receptor repertoire. Interestingly, expression of KLRG1 was significantly reduced on Ly49I single and Ly49C/I double positive NK cells after Ly49C/I blockade.

According to the rheostat model, responsiveness in NK cells is not a fixed state, it changes over time according to the input from inhibitory receptors to adapt or retune to the

environment. Our results are compatible with this model and may indicate that blockade of self-specific inhibitory receptors over time retunes mature NK cells to adjust to the lower inhibitory input. Furthermore, our observations may even imply that the hyporesponsiveness can explain the robust tolerance to normal MHC class I expressing cells in spite of inhibitory receptor blockade in paper II: retuning in response to reduced inhibitory input would act to preserve tolerance. Regarding the antibody blockade of self-specific inhibitory receptors, it has never to my knowledge been shown that antibody treatment can induce retuning of NK cell responsiveness.

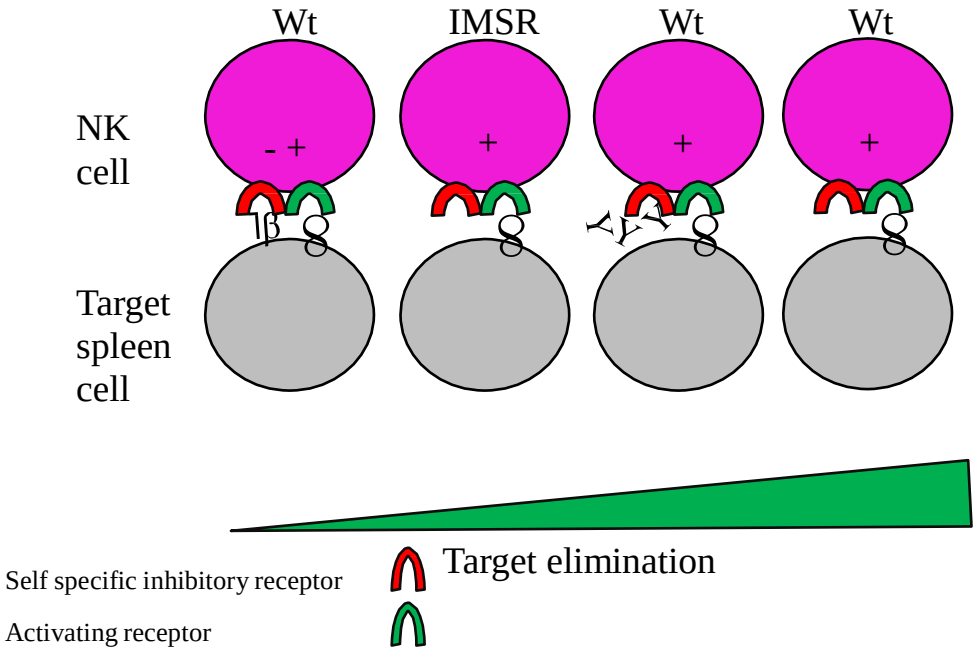


Figure 4. Four examples of NK target interactions studied in this thesis. NK cells are educated via MHC I Ly49r interactions to be able to respond against MHC I-deficient targets. NK cells sense the balance between activating and inhibitory signals and the sum of the input will determine the decision, elimination of the target cell or not. Expression of MHC I will inhibit the NK cell elimination of the target (Wt left) while lack of inhibitory input will activate the NK cell to perform missing self rejection of the target (Wt right). By altering the NK cells inhibitory input e g via antibody blockade NK cell responsiveness can be retuned to a reduced capability to perform missing self rejection (3$^{rd}$ from the left). Finally, the IMSR NK cells express inhibitory receptors and are at least partly educated, but are impaired in their missing self recognition and will therefore respond poorly towards MHC I-deficient targets.

### 3.2.2 Hyporesponsiveness induced in Ly49I single positive cells but not in Ly49C single positive NK cells

Ly49C/I blocking antibody induced hyporesponsiveness inLy49I single positive and in Ly49C/I double positive (negative for Ly49A, -G2 and NKG2A) NK cells. This was observed by reduced total responsiveness (CD107a expression and IFNγ production) after NKp46 antibody stimulation. Stimulation with PMA and ionomycin resulted in full degranulation and cytokine production, arguing against exhaustion of the NK cells. This is the first data generated in wt mice showing that Ly49I by itself can act as an educating receptor and that targeting this specific population affects responses in the mouse. Previous studies have only shown evidence for education and retuning of either the total NK cell population expressing self-specific inhibitory receptors, (Ly49C/I/NKG2A), have compared Ly49C positive versus negative populations or have studied Ly49I introduced as a transgene.

But why was retuning observed only in the Ly49I single positive population and not in the Ly49C single positive NK cell population? It might be that the binding of 5E6 $F(ab')_2$ fragment to the two different receptors, Ly49C and -I, have a different influence on their function or that the fragment binds stronger to Ly49I and therefore can alter the responsiveness of this population.

Another possibility is that Ly49I is more accessible and therefore influenced by the blocking to a higher extent. The majority of Ly49C receptors are bound by $K^b$ in cis, reducing the number of free receptors available for interaction with other cells or blocking with 5E6 $F(ab')_2$ fragments (90, 248) . In contrast, an artificial system based on transgenic expression of Ly49I on tumor cells was needed to detect Ly49I bound in cis (90). It is still possible that Ly49I may be interacting with MHC I in cis at the normal state but with a frequency below the detection limit. Ly49I is more selective in its MHC I/peptide interaction than Ly49C, so if the correct peptides are limited, the cis interactions per Ly49I expressing cell will be reduced (88-90). This could affect the accessibility of 5E6 $F(ab')_2$ as well as the effect of blockade on inhibitory input and retuning differently for Ly49C and I.

Importantly, Ly49I was also the main receptor mediating the anti-tumor effect of 5E6 treatment. This was shown by Gustaf Vahlne and Katja Lindholm in unpublished experiments done during the study for paper II. B6 mice (expressing Ly49C and –I) and Balb/B mice, (expressing Ly49C but lacking Ly49I) were treated with 5E6 $F(ab')_2$ fragments and then challenged with MHC I expressing tumors. The 5E6 $F(ab')_2$ treatment failed to induce an increased elimination of tumor cells in the Balb/B mice while a significant increase was observed in the B6 mice. Regardless of mechanism resulting in induced hyporesponsiveness in the Ly49I sp and Ly49C/I dp NK cell population the results are of importance for the understanding of NK cell education in two ways; 1) as mentioned above, this is the first time it has been showed in wt mice that Ly49I by itself is educating and can influence the outcome of the total NK cell responsiveness and 2) at least in the case

of Ly49I the education is mostly, if not completely, dependent on MHC I interactions in trans.

Our data can be interpreted in the context of the rheostat model i e reduced inhibitory input will alter the balance between activating and inhibitory signals leading to recalibration of the responsiveness. As stated in paper III, other explanations are not excluded. For example, our data could also be explained by the sequential arming/disarming model (361). The model is based on observations regarding that Ly49-MHC I cis interactions are needed to gain both functional competence and skewing of the inhibitory receptor repertoire, hence giving the licensing/arming signal. However, MHC I interaction is needed in trans for educated NK cells to remain responsive and to supply the disarming signal. This is discussed more thoroughly described below.

### 3.2.3 Hyporesponsiveness in relation to education

One possible explanation for the deficiency in missing self reactivity of the IMSR mouse could be an impaired MHC class I dependent education of NK cells expressing inhibitory receptor(s) for self. We tested this using NKp46 stimulation in the single cell responsiveness assay and found that the IMSR NK cells responded in a pattern characteristic for education in an H2b expressing mouse, i e higher response by NK cells expressing Ly49C/I/NKG2A compared to Ly49C/I/NKG2A negative cells. However, the total response of both populations was significantly reduced compared to the response by wild type NK cells (paper I). This general hyporesponsivness was also observed when the whole NK cell population was analyzed with NK1.1 antibody stimulation. However, when IMSR NK cells were triggered via LY49D, a response comparable to wild type was observed.

In addition, when responsiveness from wild type and IMSR NK cells were compared in mixed bone marrow chimera, the IMSR NK cells preserved their deficiency and remained poor responders independently of the host environment, wild type or IMSR. These data show that the deficiency in the IMSR NK cells is not simply due to a lack of educating signals from the environment and that the defect is NK cell intrinsic. However, we observed that NK cells from MHC I-deficient hosts, which are defined as hyporesponsive due to lack of inhibitory signaling (education), responded stronger to unspecific stimulation with PMA/ionomycin. This was also observed for the IMSR NK cells. The pattern was very consistent (my unpublished data and paper I). One can only speculate why this occurs. It may be that the NK cells from MHC I-deficient and IMSR mice do not get triggered to respond as frequently to other cells in vivo, and therefore have more stored granules to respond with when they stimulated in the in vitro assay.

A similar unexpected finding was that the NK cells from IMSR mice displayed a higher background release of CD107 in single cell responsiveness assays i e they responded even without added antibodies as the experimental stimulus (paper I fig 6 and unpublished data). This is interesting in relation to the hypothesis that the NK cells from the IMSR mice may

be continuously stimulated via NKG2D. Perhaps these hyporesponsive NK cells accumulate lytic granules and interferons which "leak out". Another possible explanation for the increased spontaneous release could be that the IMSR NK cells have a dysregulated, increased production of functional artillery. Coudert et al observed that continuous activation via NKG2D-L in NK cells induces a higher spontaneous production and release (364). If the IMSR NK cells suffer from chronic overstimulation, it could potentially lead to signals for production and accumulation of lytic granules, especially if they were rarely activated enough to trigger the effector machinery. It would therefore be of interest to study if wild type, MHC I-deficient and IMSR NK cells produce and store equal amount of lytic granules, containing perforin and granzymes, and cytokines in their cytosol.

Further, as mentioned in the introduction, Bessoles et al. used $D^{d+}$ mice either deficient in cis or trans interaction or lacking $D^{d}$ on NK cells or T cells respectively (251). In vitro responsiveness showed that $Ly49A^{+}CIN^{-}$ NK cells from both mouse strains were hyporesponsive upon RMA stimulation (reduced IFNγ and CD107a production). However, stimulation via NK1.1 induced increased IFNγ production by $Ly49A^{+}CIN^{-}$ NK cells only educated in cis (trans deficient) while the same NK cell population educated via trans (cis deficient) responded poorly.

In conclusion, the NK cell from the IMSR NK cells are hyporesponsive, although the data indicates that it is not due to total lack of MHC I dependent education of NK cells with self receptors. It might be caused by another mechanism influencing the general responsiveness of all NK subsets. The IMSR NK cells do not appear to suffer from generally reduced supplies of granules or the machinery used to release them.

## 3.3 NK CELL MATURATION AND CELL SURFACE MOLECULES INVOLVED IN MATURATION AND EDUCATION PROCESSES

### 3.3.1 NK cell maturation; influence of inhibitory and activating receptors

The NK cell maturation influences the responsiveness to a large extent, since mainly the NK cells with high Mac-1 expression are responsive in a normal state (62)(128, 131, 365). NK cells from MHC I-deficient mice are hyporesponsive, and they also show an altered maturation pattern, detected via altered CD27 and Mac-1 expression (365). We investigated the possibility that the hyporesponsiveness observed by the NK cells in IMSR mice and by the Ly49I sp and Ly49C/I dp NK cell population after 5E6 $F(ab')_2$ blockade could be due to an altered maturation pattern. In both situations, the relevant NK cell subpopulations showed no major changes in the maturation pattern. However, the IMSR NK cell pattern displayed similar trend, with an increase of the $CD27^{hi}Mac\text{-}1^{hi}$ subpobulation, as both MHC I-deficient and NKG2D ligand transgenic mice (365). This is rather interesting since this population, $CD27^{hi}Mac\text{-}1^{hi}$, has been shown to be the most migratory and the most responsive population, both regarding cytotoxicity and cytokine production (66).

So, could this increase of the most "potent" NK cell population be due to a compensation trying to achieve stronger responsiveness? If this is correct, it might be that the IMSR NK

cells have reached a sufficient level of function so that the full compensatory mechanism has not been elicited. In addition, it could be of interest to compare maturation and developmental stages in the bone marrow in the IMSR mice. Zafirova et al. showed that mice deficient in NKG2D have an altered maturation in the bone marrow with fewer fully mature NK cells, although the pattern in the spleen was not changed (351). However, Sheppard et al. using a different NKG2D-deficient mouse observed an enlarged mature NK cell population in the spleen (354). In conclusion, both gain of activating receptor ligand and lack of either inhibitory receptor ligand and activating receptor may potentially influence NK cell maturation status while antibody blockade does not, at least not the in the time frame used in paper III. Future studies may reveal if such a compensatory mechanism exists and what role it may play.

### 3.3.2 KLRG1 expression in relation to education and retuning of NK cells

Approximately 50% of the mature NK cells express the inhibitory receptor KLRG1(131). KLRG1 is the only marker that has shown some association with education; it is up-regulated on mature educated NK cells expressing inhibitory receptors for self (128, 204, 365). The IMSR NK cells have the same KLRG1 expression levels and frequency as NK cells from wild type mice. This is a further sign supporting that the IMSR NK cells have gone through the education process, since NK cells from MHC I-deficient mice have a significantly reduced KLRG1 expression (128). The normal KLRG1 expression observed on IMSR NK cells also suggest that the inhibitory signaling pathway is functional in these NK cells since NK cells from SHP-1-deficient mice displays a reduced KLRG1 expression, although not as reduced as in MHC I-deficient NK cells(128, 343).

Early studies showed that KLRG1 expression could be induced on homeostatically proliferating cells and also in response to strong stimulations such as a virus infection or in vitro cytokine stimulation (130, 131). In addition, Fogel et al. showed that KLRG1 is up-regulated during an early MCMV infection, although during the infection the high KLRG1 expression is maintained on Ly49H$^+$ virus specific NK cells for a longer time period (in a Ly49H interaction dependent manner) than on the Ly49H- NK cell population. (132).

However, KLRG1 is rarely discussed as a direct functional determinant for “education” i e acquired responsiveness, it has rather been perceived as a downstream consequence of the educated state. It was therefore of interest to investigate how this marker would behave upon altered responsiveness, i e when “the license” is suspended, could the KLRG1 expression be reversed? Interestingly, we found in paper III that retuning via antibody blockade as well as transfer of wild type NK cells to a MHC I-deficient host, could reverse a high established KLRG1 expression on previously educated NK cells. Similar results have been reported by Joncker et al. using the transfer model, however it has never been studied after inhibitory antibody blockade (363). This strong correlation with “educational” and status suggests that KLRG1 may be more closely associated with education then preciously described, and may be a potential marker for induced responsiveness.

The actual function of KLRG1 in NK cell function is not so well understood. This molecule may be upregulated on mature and activated NK cells to increase their possibility to migrate out in the peripheral tissue and/or to improve biding to target cells. However, the increased expression of KLRG1 on educated, highly responsive NK cells expression may reflect a regulatory function in protection against elimination of healthy cells in the surrounding (66). One can speculate that when an NK cell has a high Ly49r expression, optimal maturation (CD27hiMac-1hi) and gain maximal responsiveness a counter balance is needed, hence there is an up-regulation of an additional inhibitory receptor (KLRG1).

Ebihara et al. studying the role trans vs cis interactions for MHC I dependent education using mice and NK cells with inducible MHC I expression, saw no alterations of KLRG1 after induced education. However, this could be due to that they studied the total NK cell population and not only the NK cells who acquired responsiveness e g NK cell expressing inhibitory receptors for self (250).

## 3.4 SKEWING OF THE NK CELL REPERTOIRE AND THE INFLUENCE OF MHC I

### 3.4.1 Skewing of the NK cell repertoire and the influence of MHC I in the bone marrow

When I started my Ph.D. studies it was known from earlier studies (221, 224) and ongoing studies from our lab (204, 226) that splenic NK cells display a clear MHC dependent skewing of the inhibitory Ly49 receptor repertoire. This was observed as an increase of NK cell subsets expressing one or two different receptors (and in particular, only one self-specific inhibitory receptor) and a reduction of NK cell subsets expressing three to five receptors in a MHC I sufficient compared to a MHC I-deficient host. Little was known about where or when during NK cell development the skewing was induced. We decided to investigate this further (paper IV). By monitoring five inhibitory Ly49r individually, 32 NK cell subsets from the bone marrow were analyzed in $D^d$ single, $K^b$ single, D8 and MHC I-deficient mice (see table 1 for additional information). We found that NK cells expressing one or two inhibitory receptors for MHC I were overrepresented while NK cells expressing three to five inhibitory receptors for MHC I were underrepresented in all three MHC I expressing mouse strains compared to MHC I-deficient mice already in bone marrow NK cells. In addition, we found that the NK cells expressing only one self-specific Ly49 receptor showed the most prominent increase in frequency. By studying NK cell development from the developmental stage when Ly49r are first expressed we observed that in $D^d$ single mice the skewing of the NK cell population expressing single Ly49A or -G2 occurred already in stage III. This is the first stage where NK cells express Ly49r, but the skewing became more pronounced in stage IV, in which considerable NK cell proliferation/expansion occurs (62). In the case of Ly49A single positive NK cells the skewing was correlated to increased proliferation, as measured in vivo by BrdU incorporation. The same trend was observed for Ly49G2 single positive NK cells but the difference was not statistically significant. Regarding the reduction in the population

expressing several receptors it seemed to be a combination of both proliferation and increased apoptosis where the latter was already observed in stage III.

This is interesting in relation to observations on skewing of the Ly49r repertoire in the spleen by Brodin et al. (204). They used $D^d$ hemizygous or homozygous mice and observed not only an increase of Ly49A single positive NK cells in $D^{d+/+}$ vs $D^{d+/-}$ mice but also that the Ly49A single NK cells ex vivo had an increased IL-15 induced proliferation and a tendency to reduced apoptosis, measured by Annexin V and the pro-apoptotic marker Bim. It may be that in both the bone marrow and in the spleen, the MHCI dependent skewing of the NK cell repertoire is due to two processes taking place at the same time; increased proliferation of certain subsets and apoptosis of others, which may contribute to this pattern. These processes may act to create the most responsive, yet self-tolerant population.

In paper III we studied if the NK cell receptor repertoire can be adjusted in the short term in response to an altered MHC I environment. Transfer of splenic NK cells from a MHC I deficient to a MHC sufficient host or vice versa did not alter the expression pattern of activating or inhibitory receptors. This has also been observed by Hayakawa et al.; who transferred splenic NK cells from either a MHC I sufficient or deficient donor into RAG-$2cg^{-/-}$ mice (MHC I sufficient (365). Independently of which mechanism that is responsible for the skewing, proliferation or apoptosis, it may be so that the "settings" for the repertoire are determined at an early stage of development after which it cannot be altered. However the studies so far do not address if NK cells can alter their receptor repertoire expression if the MHC I expression is altered during development from an earlier NK cell precursor in the bone marrow. These questions are of high interest and needs further investigation.

Regarding NK cell function during development, it has previously been shown that only the mature Mac-$1^{hi}$ bone marrow NK cells are the potent IFNγ producers and that in the spleen the cytotoxicity is mediated by the $CD27^{hi}$Mac-$1^{hi}$ NK cell population(62, 65, 66). In addition, Rosmaraki et al. showed that the $NK1.1^+Dx5^+$ NK cells in the bone marrow are the most efficient killers of YAC-1 cells, although the $NK1.1^+Dx5^-$ also showed a low cytotoxicity (54). It would be of interest to study if the bone marrow NK cell needs to be educated via an Ly49 inhibitory receptor for self in order to aquire responsiveness, or if early expressed receptors such as NKG2A and NKG2D can play a major role in the early ontogeny NK of cell function. This could be investigated by analyzing the responsiveness of the 10% of the NKP cells that express NKG2D (61).

### 3.4.2 Skewing of the NK cell repertoire in missing self deficient mice and after altered MHC I recognition

The skewing of the NK cell repertoire can be used as a marker for that MHC I dependent education has occurred. This was used in both paper I and III where we studied either a mouse strain with impaired missing self recognition, IMSR, discussed in sections above, or retuning of NK cell responsiveness to altered MHC I expression. When analyzing the expression pattern of Ly49r after inhibitory receptor blockade, there was no difference in

repertoire formation. However, the inhibitory receptor repertoire of IMSR NK cells, for Ly49C, -I, -A, -G2 and NKG2A, we found that total frequency of NK cells expressing any given inhibitory receptor (independently of expression of other receptors), self-specific or not, was significantly reduced in the IMSR mice compared to B6 wild type mice. On the other hand, when analyzing single Ly49r positive NK cell populations (expressing no other inhibitory receptor) from the IMSR mice, we observed a significant increase in the populations expressing self-specific, Ly49C, -I and NKG2A, compared to B6 NK cells. The skewing of the repertoire was thus even more pronounced in the IMSR mice than in the B6 mice. This is an additional indication that the IMSR NK cells can sense MHC I via the inhibitory receptors. Further, SHP-1-deficient NK cells lack the skewed receptor repertoire pattern, NK cell population, and are more similar to MHC I-deficient NK cells(342, 343). This indicates that also the initial steps in the inhibitory receptor chain is functional in the IMSR NK cells and that at least SHP-1 is, a shared factor necessary for both NK cell inhibition and for the skewing of the NK cell repertoire (342, 343).

Even though the mechanism(s) behind skewing and NK cell education still remain unknown, these data, in combination with the results showing a relative increase in responsiveness of $CIN^+$ NK cells in IMSR mice, are indicating that the NK cells from the IMSR mice go through at least on part of MHC I dependent the education process. In addition, reduced MHC I inhibitory input on mature splenic NK cell was not enough to alter the inhibitory receptor repertoire in the short term (within 4-7 days).

### 3.4.3 Why does skewing of the NK cell repertoire occur in the presence of MHC I?

We do not know the answer to this question. There are many speculations. It could be a mechanism to enrich for the NK cell populations with the most favorable offsetting of activation thresholds in interactions involving self MHC I. If the largest NK cell populations in an MHC I sufficient environment would express several inhibitory receptors for self, all of these NK cells might receive too much inhibition in the effector target interaction (221). Another more qualitative aspect is that NK cells expressing only one inhibitory receptor for self may be more sensitive to alterations in the environment. If a virus causes down regulation of one MHC I molecule the NK cells expressing only the inhibitory receptor for that specific molecule will react but if all NK cells express several inhibitory receptors there is still the possibility to be inhibited via another receptor (366). In this way skewing of the NK cell receptor repertoire may tune the system and make it more sensitive to changes in the MHC I expression. So, why is there an even more skewed NK cell inhibitor receptor repertoire observed in the IMSR mice compared to B6 mice?

Our functional data indicate that the IMSR NK cells are incapable of performing missing self responses, regulated via inhibitory receptor signaling which could be explained by lack or dysfunction of any signaling molecule in the inhibitory signaling pathway. However, the phenotypic data indicate that the inhibitory pathway of the IMSR NK cells is at least partly functional and capable of mediating the signals needed for the skewing of the repertoire.

So, one speculative idea of why an increased skewing is observed is that the inhibitory pathway signals constantly. This would result in an increased activation threshold, and to be able to respond at all, subsets expressing one self-specific inhibitory receptor are enriched for to a higher extent than in the wild type B6 strain. This can thus be seen as a "selection" for the NK cell with lowest possible amount of inhibition to still achieve responsiveness. This could also explain why there is a reduced or impaired function through other activating receptors. If there is always a strong inhibitory signal the activation signal, e g by NKG2D, may not be sufficient to achieve a response.

In line with this hypothesis and any other explanatory model for the IMSR defect, it has previously been shown that human IL-2 activated NK cells are easier to trigger/stimulate activate compared to resting NK cells which have a more restricted regulation. To be able to respond with cytotoxicity or cytokine secretion, resting NK cells require co-signaling through pairs of activating receptors, each receptor contributing. Only CD16 was able to induce a NK cell response by itself, this was not observed by any other natural cytotoxicity receptor (182, 184). The term co-activation receptors were suggested to describe these receptors that can only function in synergistic pairs. This reasoning could be used to explain why some receptors are less affected and some not (e g CD16) in the IMSR mice.

If the "hyperinhibition" hypothesis is correct, the data could be interpreted in the following way: the activating Ly49D receptor and NKG2D could represent opposite poles. Ly49D as a strong activating receptor induces enough stimuli to overcome the inhibition while NKG2D could be mediating a too week signal to overcome the higher inhibition. It may be argued that NKG2D signaling in mice is strong enough to overcome inhibitory receptor signaling since RMA-Rae1γ (NKG2D ligand transfected) cells are rejected while the RMA mock transfected cells are not (paper I and (346). However, on RMA there are probably additional unknown activating ligands mediating co-stimulation, tilting the balance towards activation by NK cells in wild type mice. The theory regarding different stimuli needed to activate resting or pre-activated NK cells might also explain the increased in vitro killing of $\beta_2m^{-/-}$ Con A blasts observed by the IL-2 activated IMSR NK cells. In this case the cytokine pre-activation alters the triggering signals needed to shift the balance by reducing the required co-stimulation.

Another way by which MHC I influences the NK cell and its receptors is by regulating the expression level of the cognate inhibitory receptors. When a MHC I ligand is present there is a reduction in the expression levels of the corresponding self-specific inhibitory receptor on the NK cells. This can be due to at least two different mechanisms 1) internalization of the receptor-ligand complex when they are interacting in trans or 2) via cis interaction on the same cell blocking the antibody staining. There is no difference in expression level of educating receptors on NK cells from the IMSR mouse compared to B6 NK cells. This indicates that it is not a defect causing problem to perform a cis interaction by the Ly49C NK cell subpopulation.

Bessoles et al. disrupted the Ly49r-MHCI interactions, either the ones in cis or the ones in trans, in two different ways, and showed in both systems that cis interactions are needed to induce skewing of the repertoire while trans interactions seemed less important (251). Based on this and functional data showing that lack of cis induced NK cell hyporesponsiveness, the same group proposed a new model for NK cell education; the sequential arming and disarming model for NK cell education (361). The authors state that Ly49r-MHC I interactions are needed to occur in both cis and trans to achieve a long term functional NK cell population. The principle is that NK cells become armed via Ly49r cis interactions, achieve responsiveness, and as a consequence skewing of the receptor repertoire is induced. However, to avoid chronic or overstimulation (inducing hyperresponsivness) NK cells interact with surrounding cells in trans to gain inhibition/disarming. However, if only trans interactions occurs, there will be no skewing of the repertoire and no gain of responsiveness. This model can be used to interpret the IMSR defect in the following way: IMSR mice have a functional interaction to MHC I in cis, inducing responsiveness and skewing of the repertoire, however the interaction in trans is defective i e the NK IMSR NK cells are render hyporesponsive due chronic overstimulation as implied in section 3.1.2.

In conclusion, there are at least two theories which can explain the reduced Ly49r expression and increased skewing of the NK repertoire in IMSR mice; too much or too low signaling via an inhibitory or an activating receptor respectively. In the case of too much signaling, it could be either constant signaling or excess of a signaling molecule, for example SHIP-1. One could speculate that over expression of SHIP-1 would generate too much inhibitory signaling via inhibitory Ly49r and therefore alter both activating threshold, skewing and increased termination of Vav-1 signaling (observed as reduced NKG2D function). In addition, it could possibly out-compete SAP and EAT-2, making 2B4 signaling only inhibitory and tilting the balance through other activating receptors, which could explain the function of IMSR NK cells. However, if the phenotype is altered due to reduced activating signaling, as for Fyn, this would also affect the function via several receptors, although not NKG2D or Ly49D which are Fyn independent, which is partly seen in IMSR. It would however be of interest to eliminate the function of either SHIP-1 or Fyn in the IMSR and see if this alters the receptor repertoire or the NK cell function.

## 3.5 MANIPULATION OF MISSING SELF RECOGNITION IN CANCER TREATMENT

### 3.5.1 Increased tumor elimination by mimicking missing self recognition via Ly49C and I receptor blockade

Can our knowledge regarding NK cells and missing self recognition be applied in immunotherapy against cancer, i e to induce missing self recognition by NK cells against tumor cells expressing MHC I molecules? Koh et al. were the first to study this in an in vivo model for inhibitory receptor blockade. After blocking self-specific inhibitory receptors by treatment of mice with Ly49C and I with antibody $F(ab')_2$ fragments against

Ly49C and I, they demonstrated increased in vitro cytotoxicity towards MHC $I^+$ malignant cells, and reduced vivo outgrowth of transplanted tumor cells (322-324). However these studies did not address whether the inhibitory receptor blockade affected reactivity towards healthy normal cells, how it affected the NK cell population over time and whether it actually directly increased NK cell mediated elimination of tumor cells.

The study resulting in paper II was collaboration with the companies Novo Nordisk (Copenhagen) and Innate Pharma (Marseille) where we investigated the biological function and safety of blocking self-specific inhibitory receptors as a preclinical evaluation for development towards a clinical trial and future treatment of patients. We could demonstrate that blocking of the inhibitory receptors Ly49C and I in both short and long term treatment (3 days to 9w) induced increased in vivo rejection of fluorescence labeled syngeneic lymphoma cells, while there was no effect on in vivo killing of healthy syngeneic spleen, bone marrow cells or lymphoblasts. Nor was there was any tissue damage, changes in hematological parameters or other detrimental effects after long term treatment. This indicated that the administration of antibody fragments inducing increased NK cell reactivity, which potentially might induce autoreactivity, was actually safe. Part of these results, and other additional data were used in the preclinical data file submitted for approval of the first phase I clinical trial based on NK cell inhibitory receptor blockade (with KIR antibody).

As already discussed, we now realize that the robust NK cell tolerance against normal cells despite inhibitory receptor blockade may be due to the induced hyporesponsiveness observed in paper II. NK cells were however, still capable of efficient rejection of MHC I-deficient tumor cells. In addition, the effect on lymphoma cell killing effect was even more efficient when the treatment was administrated in combination with IL-2. This combined therapy also significantly reduced outgrowth of syngeneic melanoma transplants.

In parallel with and following the studies in paper II, our contact at Novo Nordisk, Nicolai Wagtmann was responsible for the first studies conducted on human cells using the same principle, blocking MHC class I specific inhibitory KIR.(367, 368).The anti KIR antibody 1 7F9 (later called IPH2101 or lirilumab as a pharmaceutical generic name) recognizes and binds to the common inhibitory KIR2DL-1, -2 and -3 receptors, thereby blocking interaction to all HLA-C haplotypes and preventing inhibition (hereafter, the term anti-KIR antibody blockade I refers to such blockade of inhibitory KIRs) (368).

The anti-KIR antibody increased cytotoxicity of IL-2 activated human NK cells from a HLA-C matched donor against patient derived acute myeloid leukemia blasts. In vivo, pre-treatment with 1-7F9 in KIR2DL3 transgenic mice increased rejection of injected CFSE labeled human PBMC (peripheral blood mononuclear cell) or spleen cells from a HLA-C transgenic mouse expressing the inhibitory ligand. NOD-SCID mice inoculated with human NK cells and acute myeloid leukemia blasts showed a high mortality rate within 4 weeks due to leukemia, but if they were also treated with 1-7F9 they survived 3 months (367). In conclusion, the first study blocking with an anti-KIR antibody increased cytotoxicity in

vitro as well as rejection of HLA expressing tumor cells in vivo. The next step was a phase I trial performed on 23 elderly patients with acute myeloid leukemia in remission. This trial showed that the dose of antibody required to give full saturation of KIR on NK cells was safe, and could be administered to induce inhibitory KIR blockade for a longer period with minimal side effects (369). NK cells from patients treated with higher doses of anti-KIR antibody expressed markers for activation such as increased expression of CD69 and elevated levels of TNF-α and MIP-1β, but there were no signs of changes in the NK cell population defined by number in peripheral blood, expression of activating and inhibitory receptors and no change in the capability to degranulate.

Treatment with anti-KIR antibody has also been tested against other types of cancer expressing HLA molecules (thereby possibly escaping NK cell mediated lysis) and in combination with other substances to enhance the efficacy. Benson et al. conducted studies on multiple myeloma using both the human setting with anti-KIR antibody and the equivalent murine system blocking the inhibitory receptors Ly49C and I with 5E6 $F(ab')_2$ fragments. In the murine setup, treatment with 5E6 fab2 fragment in combination with a NK cell stimulatory substance lenalidomide increased in vivo rejection of syngenic tumor cells (RMA) in the liver, but there was no significant effect when each treatment was given by itself. (370). These data are somewhat conflicting to our data since we observed a significantly increased rejection of RMA cells in vivo after 5E6 $F(ab')_2$ treatment alone. These are most likely to the big differences in t doses of 5E6 $F(ab')_2$ (we used at least a 20 fold increased dose) or/and different assays for NK cell mediated rejection used. When the equivalent anti-KIR was used, blocking in combination with lenalidomide in vitro enhanced NK cell function, immune complex formation, cytotoxicity and IFNγ production against autologous myeloma cells but there was no effect on NK cell activity against healthy cells. The positive effect of lenalidomide was due to up-regulation of activating molecules on the myeloma cell line. In a phase I trial conducted on 32 patients with refractory multiple myeloma, administration of the IPH2101 anti-KIR antibody in doses resulting in total occupancy, was tolerated without any toxicity (371). Comparing PBMC (peripheral blood mononuclear cell) samples before and after administration of anti-KIR showed that the patients NK cells cytotoxicity against myeloma cells had increased in vitro. The treatment led to NK cell activation e g a higher expression of CD69, but no significant changes on IFNγ response or other cytokine vivo as opposed to their first in vitro study.

In the most recent paper on the subject of increasing NK cell anti-cancer activity by combined treatment, anti-CD20 antibody (rituximab) was used in combination with inhibitory receptor blockade (372) in a murine model. Rituximab is used as a standard treatment for B cell lymphoma; it is considered to act directly on lymphoma cells by inducing apoptosis, and via ADCC by NK cells. However, some patients respond poorly. The authors used a murine lymphoma cell line transfected with the human CD20 to co-analyze the response of mice both to anti-CD20 antibody and anti-Ly49C and I fab2 fragments. They found that the combinatorial treatment, compared to anti-CD20 alone, increased CD107a expression, IFNγ production and ADCC upon co culture with the

lymphoma cells. In addition, the combined treatment increased anti-lymphoma activity and prolonged survival in mice with already established tumors compared to the treatments with each of the agents separately. Anti-KIR antibody therapy using a recombinant variant of 1-7F9 with a stabilized hinge, lirilumab, was tested in combination with anti-CD20 using the KIR transgenic mice. Mice injected with HLA-C expressing tumor cells and treated with the combination had a 2-2.5 fold increased NK cell dependent survival compared to mice given the substances separately. In conclusion, both removing the inhibitory signal from the most potent killers in combination with inducing a strong activation pathway via CD16 increases NK cell activity and leads to prolonged survival of treated mice.

Neuroblastoma (NB) is the most common extracranial solid tumor among children with a poor long term survival prognosis despite treatment such as surgery, radiation or chemotherapy. A reduced recurrence risk and a longer overall survival has been seen for high risk neuroblastoma patients treated with a monoclonal antibody recognizing the surface antigen disialoganglioside GD2. The antibody acts by activating complement to directly kill neuroblastoma cells but also by inducing NK cell mediated ADCC (3-5). An interesting finding made by Tarek et al. is that the unlicensed NK cell population (NK cells expressing inhibitory KIRs for which cognate HLA class I ligand is absent) is the most effective population mediating the antitumor ADCC effect (373). Patients missing at least one HLA ligand for their inhibitory KIRs were considered unlicensed while patients expressing HLA ligands for all their inhibitory KIRs were termed licensed. Functional in vitro tests showed that anti-GD2 treatment induced increased ADCC by both licensed and unlicensed NK cells, but the unlicensed cells responded more efficiently while the licensed ones were inhibited by the KIR-HLA interaction. As described more thoroughly above, NK cells that has been educated (KIR-HLA interaction) gain increased responsiveness. Both the human study by Tarek et al. and the murine study by Orr et al. showing that unlicensed NK cells drive the response against cytomegalovirus (258), demonstrates that the gained responsiveness through education cannot override the inhibition mediated by the HLA-KIR interaction. So, NK cell education is enough to ensure tolerance at steady state during normal conditions but can be overcome during certain conditions, such as a strong activation via either mAb treatment or viral infections.

The most efficient way harness NK cells for immunotherapy against cancer will most probably be based on combining several treatment strategies to increase the elimination efficacy by the NK cell, as mentioned in the beginning of this section. Benson et al. showed one way to do that using an anti-programmed death receptor-1 (PD-1) antibody in combination with lenalidomide against multiple myeloma (374). They showed that resting NK cells from healthy individuals express very low levels of the inhibitory PD-1 molecule but the expression was significantly elevated on both NK cells from healthy donors who had been incubated in IL-2 for 48h and on NK cells from a multiple melanoma patient. Treating NK cells with anti-PD-1 antibody enhanced both elimination capacity, by blocking the inhibitory signal and increasing granzyme B and IFNγ production via an unknown mechanism, and the migration towards multiple melanoma cells. In addition the

lenalidomide contributed by decreasing the expression of PD-1 ligand on the target cells and boost the NK cell response by inducing IL-2 production by the T cells (375).

### 3.5.2 A complicating factor: retuning of NK cell responsiveness via altered MHC I perception

As discussed above, inhibitory receptor blockade shows promising therapeutic potential. How can those results be reconciled with our results in paper III, where we found that blocking of inhibitory Ly49C/I receptors in a B6 mouse or transfer of B6 spleen cells or mature NK cells to a MHC I-deficient host resulted in retuning i e reduced rejection of MHC I-deficient spleen cells in vivo? Importantly, we found that in both systems the NK cells were still capable of efficient rejection of MHC I-deficient tumor cells

In summary, we speculate that the induced tolerance towards MHC I-deficient normal cells after removal of the inhibitory signals (in paper III), via antibody blockade or transfer to an MHC I-deficient host, is induced by an increased activation threshold towards normal cells in the environment. It is a consequence of retuning, resulting in tolerance towards normal cells but preserving function towards diseased targets expressing more activating ligands; the latter can still overcome the increased activation threshold and will be eliminated. This hypothesis could further be investigated if the activating ligands on normal spleen cells and the activating ligands triggering elimination of RMA-S were known. A relevant phenomenon can actually be observed if a strong activating ligand is ectopically expressed in RMA, the NKG2D ligand Rae1γ. Both B6 and $\beta_2$m-deficient mice eliminate RMA-Rae1γ tumor cells in vivo with a similar efficiency (data not shown). This proves that the increased activating threshold inducing hyporesponsiveness in the $\beta_2$m-deficient NK cells can be overcome when the activation is strong enough, as in the case of elimination of tumor cells but not of healthy cells after retuning. In addition, when such a strong activating ligand is present in the system, one would not expect any additional effect of inhibitory receptor blockade since the "window" to observe an altered effect is too narrow.

Using the transfer approach we also observed that NK cells can retune to gain function e g when an NK cell from a MHC I-deficient mouse is transplanted into a MHC I sufficient host: The NK cell responsiveness was altered to gain the capacity to eliminate MHC I-deficient tumor cells but still remained tolerant towards MHC I-deficient spleen cell in vivo. Why and how the NK cell remains tolerant towards MHC I-deficient spleen cells is uncertain. It could be due to education on neighboring transferred NK cells, although they are quite few in respect to the total cell count, or it might be some sort of imprinted baseline setting that cannot be altered to always ensure tolerance towards self. Independent of the mechanism, these data indicate that the interaction needed to become educated and gain responsiveness is presented, perhaps not only but at least also, in trans. However, there is also the possibility that the NK cell acquire MHC I from surrounding cells via trogocytosis and become educated via a cis interaction (245). The reduce responsiveness achieved after transfer of cells from B6 to $\beta_2m^{-/-}$ mice cannot be explained entirely by cis interactions since if that would be the only way needed for ligand presentation, the B6 NK cells would

remain efficient killers of MHC I-deficient spleen cells. However, these data could be explained by the sequential arming/disarming model. After transplantation, the B6 NK cell still engage in cis while they lack the trans interaction on surrounding cells (except the graft) leading to chronic "anergy", hence they become tolerant towards the new environment. However this model does not explain how MHC I-deficient NK cells remain tolerant towards MHCI- deficient spleen cells while gain function against tumors both lacking MHC I expression.

These data show that the NK cell responsiveness can be tuned in both directions in vivo, either to preserve tolerance towards the normal tissue (B6 to $\beta_2m^{-/-}$ transfer) or to achieve wild type responsiveness towards tumor targets ($\beta_2m^{-/-}$ to $\beta_2m^{-/-}$ transfer), which has never been shown before. Evidence that NK cells can retune their responsiveness as adaptation to changed MHC I expression has been shown previously. However, those studies were on rejection of MHC I-deficient spleen cells and did not include tumor cells; furthermore, in the case of for gain of responsiveness they addressed this only with in vitro assays for in NK cell responsiveness. The retuning impact on tumor elimination or that retuning can be induced by mimicking missing self via antibody blockade has never been shown before (362, 363) In addition, we observed that independently of transplantation direction, B6 to $\beta_2m^{-/-}$ or $\beta_2m^{-/-}$ to B6, two functions are always present 1) the strict self-tolerance is ensured and 2) the ability to eliminate MHC I-deficient tumors is either induced or preserved dependent on the direction.

Our results may be of importance for understanding and optimizing usage of NK cells as immunotherapy in a haploidentical setting and for activation via inhibitory receptor blockade. Transfer of NK cells from a non-HLA matched donor could theoretically contain both responsive and hyporesponsive NK cells. The responsive donor NK cells can potentially mediate graft vs host reactivity. Our data indicate that the NK cells educated on one type of MHC I allele in the donor would retune and become self-tolerant toward healthy recipient cells expressing another MHC class I allele, while preserving effective elimination of leukemia cells due to missing self recognition of the recipients MHC I-deficient tumor cells. However, the hyporesponsive NK cells (lacking a ligand in the donor), would display the opposite pattern. These NK cells would remain self-tolerant but at the same time gain a strong anti-leukemia effect mainly against MHC I negative tumors due to induced education by the "right MHC I allele" expressed by the recipient. In addition, the newly educated NK cell subset could also possibly have an effect, at least in the early phase, against MHC I expressing tumor cells with additional activating ligands even if the recipients educated NK cells are inactive, since the recipient NK could be non-functional due to influences from factors in the tumor microenvironment. It would be of great interest to study elimination of haploidentical tumors that are not completely lacking MHC I, which could be observed by analyzing elimination of RMA tumor cells after transfer of D8 NK cells to a B6 host. This was partly done by Kijima et al.

Kijima et al. transduced bone marrow cells with an oncogene and transplanted them to irradiated mice with different haplotypes to study the influence of NK cell functions against primary chronic myeloid leukemia (CML) (376). NK cell response against CML was dependent on missing self recognition and not due to upregulation of activating ligands (NKG2D) or alloreactive activating receptors (Ly49D). MHC I matched recipients developed disease after transplantation of chimeric grafts (cancer bone marrow cells and MHC I matched bone marrow) while transplantation to full mismatched recipients remained disease free. In a haploidentical situation (loss of one MHC I allele), missing self recognition delayed the onset of the disease but was not enough to mediate full anti-cancer protection in all recipients. Further, the NK cell influenced the disease by elimination of leukemia initiating stem cells. These results demonstrate that a functional missing self recognition is enough to render cancer cells sensitive to NK cell mediated killing, in line with the early results on hybrid resistance and MHC I transgene induced NK cell rejection of tumors discussed in the beginning of this thesis.

In addition, in the setup using $F(ab')_2$ fragments, the elimination of MHC I expressing tumors, RMA, was not as robust and strong as we had expected. This could be explained by that two individual mechanisms opposing each other were affecting the outcome. The "early" effect of the Ly49C/I blockade was observed by an increased elimination of the tumor cell induced by blocking the effector-target interaction mimicking missing self recognition. However, this effect might be much stronger if it would not have been counteracted by induced hyporesponsiveness caused by retuning, the second effect of the inhibitory receptor blockade. Evidence that the tumor killing is actually also negatively influenced to a certain extent is provided by the tumor outgrowth experiments with RMA and RMA-S (paper III, Table I). When the system was challenged to the limit with a very large tumor transplant, inhibitory receptor blockade reduced the rejection of MHC-deficient tumor cells (RMA-S). To improve this system to gain the maximum effect and a stronger elimination of MHC I sufficient tumor cells one could consider to 1) reduce antibody saturation and perhaps avoid retuning and induced hyporesponsiveness or 2) reduce the stability of the $F(ab')_2$ fragments and therefore block the inhibitory receptors for a shorter time period and therefore avoid retuning but treat repeatedly to gain the anti-leukemia effect.

NK cell education and responsiveness are influenced by other parameters then MHC I. In the model that we use to interpret our results, retuning alters the activation threshold ensuring tolerance towards healthy cells while they still maintain or gain function towards tumor cells. However, not only retuning by altered inhibitory input can change the NK cell responsiveness, virus infections and "cytokine storms" also influence the responsiveness. The NK cell pool consists of both fully responsive and hyporesponsive NK cells. The MHC I-deficient NK cells are at steady state tolerant towards self. However, Salcedo et al. showed that NK cells from both $\beta_2m^{-/-}$ and $TAP1^{-/-}$ mice could be induced to break tolerance and kill autologous lymphoblasts but remained tolerant towards MHC I sufficient targets in vitro after 4 days of IL-2 activation (344). In addition, mixed B6/$\beta_2m^{-/-}$ bone

marrow chimeras are also tolerant towards MHC I-deficient cells although this tolerance is broken upon infection with cytomegalovirus. These results indicate that the missing self system and tolerance can be calibrated and induced when the NK cell develop in an environment containing MHC I-deficient cells although strong stimuli, for example cytokines, can break the achieved tolerance (377).

Regarding NK cell education, retuning and responsiveness, it has been shown in mice that paradoxically the uneducated hyporesponsive NK cells are the most important in protection against Cytomegalovirus infections (258). This is probably due to lack of inhibitory signals in the interaction with infected cells and illustrates that the hyporesponsive state does not represent complete anergy, but rather a changed activation threshold so that NK cells cannot respond to the activation signals presented by normal cells, even if they lack inhibitory receptors for their MHC I molecules. This does not exclude that they can respond in other contexts where a qualitatively or quantitatively altered set of activation signals are presented. Further, Fernandez et al. showed that both the educated and uneducated NK cell pool responded equally well with IFNγ to a Listeria infection, indicating that the unresponsive NK cell pool has the capability to respond when it is stimulated enough (203).

In addition, Ardolino et al. showed recently that MHC I-deficient tumors induce an "anergic state" in the tumor infiltrating NK cell population leading to a reduced responsiveness and survival while MHC I sufficient tumors did not (378). However, cytokine treatment could reverse the induced anergy. This strengthen our and other´s conclusion that a combinatory treatment of inhibitory receptor blockade and cytokine treatment may be the best strategy to to activate NK cells against MHC I sufficient tumors. Only blockade induces retuning or "anergy" but according to our study (paper II) and Ardolino's data this effect may be reversed or kept in balance by cytokine administration.

NK cell responsiveness and tolerance is tightly controlled by Ly49r-MHC I interactions and the balancing effect of activating receptors. During special circumstances the well-controlled system can be bypassed. This may reflect different levels of function by the NK cell population. Maybe the educated NK cell pool is there to patrol the tissues for stressed, infected or transformed cells. They are tightly controlled to not become autoreactive but they have the ability to respond strongly when it is justified. To complement this regulated responsive NK cell pool, there are NK cell subsets expressing no inhibitory receptor for self which are a less controlled NK cell population (if activated), but they are in a hyporresponisve state during healthy conditions to ensure tolerance. This less controlled population is able to rapidly expand and give an even stronger response (seen by for example IFNγ production and CD107a expression, after PMA/Ionomycin, as discussed in section 3.2.3) by MHC I-deficient NK cells) when the activation is strong enough, since they are less inhibited. These NK cells might only be used when the danger is acute and rapid as in the case of infection.

In conclusion, the rheostat model takes all the interactions (both inhibitory and activating) and the strength of the interaction into account and the net sum will regulate the decision

and responsiveness. Brodin et al. showed that NK cells with more inhibitory signals has increased responsiveness and also respond in a more diversified way (e g several cytokines) (233). This should theoretically mean that if the NK cell receives a lot of inhibitory signals, it becomes educated and responsive, but it also needs an increased amount of activation to get triggered if inhibitory ligands are present. However, too much activation as in the case of chronic stimulation via an activating receptor leads to an overall hyporesponsiveness. To my knowledge, it has not been specifically studied how the educated NK cells respond under conditions of chronic stimulation, which would be of great interest. In addition, removal of activating receptors such as NKp46 and NKG2D results in hyperactivated NK cells clearly shows an influence of activating receptors in control of NK cell response. Further, as described above (see section 1.9.2-3), there are both MHC I dependent and MHC I independent molecules participating in the education, regulating total responsiveness observed by the NK cells. When more of the ligands (for example the endogenous ligands) are identified we will have a better understanding for how the system works and how it can be used for immunotherapy.

# 4 CONCLUDING REMARKS

I see a common thread throughout my thesis: investigation of NK education and missing self recognition, even though these themes have been approached with different questions and methods.

During my doctoral studies my colleagues and I have used IMSR NK cells as a tool to investigate how missing self recognition is related to other NK cell functions (paper I). The dogma of the field is that MHC I-deficient NK cells are hyporesponsive due to lack of inhibitory signals needed to achieve licensing/disarming. The IMSR NK cells are exposed to inhibitory ligands and can be inhibited by them upon interaction. Further, they are phenotypically mature and show signs that they have gone through education, implying inhibitory receptor mediated licensing/disarming. However, they are still missing self deficient and overall hyporesponsive in most settings. We know that the defect is not due to the targeted CD1d1 gene in these KO mice. The next step should be to identify the gene(s) responsible for the defect.

Further, we used missing self recognition to demonstrate that Ab-mediated blockade of self-specific inhibitory receptors on NK cells can induce reactivity against syngeneic lymphoma cells without breaking tolerance to normal cells. In vivo blockade with 5E6 $F(ab')_2$ fragments binding to Ly49C/I inhibitory receptors on NK cells caused increased rejection of syngeneic MHC class I-expressing tumor cells (RMA) but not of syngeneic spleen cells, bone marrow cells or lymphoblasts, thus the self-tolerance was thus very robust. The anti-tumor effect was sustained over long time and could be enhanced by co-treatment with IL-2. These results are interesting for development of therapy in the in clinical setting. However, an unexpected but very reproducible an interesting finding during this study was that mice treated with the 5E6 5E6 $F(ab')_2$ fragments showed *decreased* rejection of splenocytes lacking MHC I. This was

the starting point for the studies of inhibitory receptor blockade in paper III, were we addressed if it is possible to use this procedure to quench the inhibitory input for NK cells and as a consequence induce retuning, as predicted by the rheostat model for NK cell education. This approach to alter the NK cell sensing of MHC I expression in vivo is minimally invasive, and was therefore an important complement to the second model used in paper III based on irradiated recipients receiving high numbers of transferred cells. We could demonstrate retuning of NK cell responsiveness in both models. In this study we aimed for an almost complete (100%) receptor blockade. However, our results show that for use in treatment of cancer, the blockade needs to be optimized, by titration of dose and dosage intervals, with the goal to increase effector function in the interaction with target cells without inducing hyporesponsivness via the interactions with educating cells.

In the last manuscript of my thesis we studied one effect of MHC I education, skewing of the inhibitory receptor repertoire. We established that skewing of the NK cell repertoire occurs in the bone marrow at the developmental stage where Ly49 receptors start to be expressed. In addition, we observed a possible influence of proliferation and apoptosis as possible mechanisms regulating the frequency of different NK cell subpopulations.

# 5 ACKNOWLEDGEMENTS

First I would like to take the opportunity to thank all the people that have in some way been involved in my studies or as dear supporting friends needed during these, sometimes challenging, years. So, even if all of you are not mentioned in this section, you are still remembered.

I would like to thank my two main supervisors **Mia** and **Klas**. Even though only one of you is officially stated as my main supervisor I think you have contributed equally to this thesis. If there were such thing as shared main supervisor title it would have fitted just perfectly! **Mia**, thank you for taking me in as your young super scared master student, teaching me in the best possible way and for being a supervisor in a character of a friend. In addition, I would like to thank you for always believing in me, even when I doubted. **Klas**, with all the answers, many times I have felt that I could ask you anything (independently of subject) and you would come up with a suitable (always diplomatic) answer. Further, it is always very inspiring to discuss data with you since you always can see something good in every experiment, even though I have felt it is a total failure, encouraging me to try it again or in a different way.

**Hanna S**, thank you for being a nice and there when I needed support when it has been extra hard, especially in the beginning of my PhD, and for showing me that when it comes to science, sometimes we cannot predict what will happen but what is more important is how we react on it. In addition, it was nice for me to be the bravest, in respect to i.p injections =)

My mentor, **Tina Trollmo**, for being very warm and kind person, for sharing you experience regarding PhD studies and family-life and for inviting me to Kungshatt! =)

I have met so many friendly and nice people that have become as a "lab-family" over the years. I would like to thank you all! **Louise**, for being a lab-mum which I could always ask and you always replied with laugh and a smile. **Katja** the one and only Katja for me! Thanks for all, small and big, talks in the cell-lab making it so much more fun to split cell forever. **Kakis** for being so positive, energetic and always having a little spare time for fika. **Arnika**, for being an excellent colleague, dear friend and a fun traveling mate. You will always have a friend in me. **Sadia**, for helping out when needed and for teaching me a lot regarding different religions**. Jonas**, for being the computer guy… No, just kidding. Thank you for always giving me a minute even though you are super stressed and for being the designated driver in San Fran =). **Elin** and **Karolin**, for being very nice fika/ lunch companies and for always smiling. **Björn**, for always being friendly and a very nice Christmas party company, I will never forget the "drunk -forgotten key" incident we shared.

Further, I would like to thank both former and present members of the Klas Kärre/Petter Höglund/Björn Örnfelt/Sofia Johansson groups. **Marjet**, **Gustaf**, **Alexander**, **Rutger**, **Bruno**, **Susanne** and **Danika** for making me feel welcome when I started. **Andre** for being a super nice guy. **Petter** and **Petter** for nice looong discussions. **Linda**, **Rossanna**, **Kanth** and **Ennio** for being very nice collaborators. **Per**, **Stephan**, **Sridharan**, **Luu** and **Nadir** for sharing nice Christmas dinners. **Sofia**, **Elina**, **Sunitha**, **Arie** and **Rosa** for making the office friendly**. Benedict**, for being you and for eating my chocolate! **Stephanie**, for discussing tattoos and for being the best night working company anyone could wish for.

**Jens** for being who you are: friendly, easy to talk, honest and always speak your mind! **Arwen**, even though we met in a bit accord way, I am very glad that I got to know you.

**Michael Uhlin** the best "falutomte" in the world, for being a person that I can always count on, without you as my "cheerleader" and "trash bin" this time would have been so much more difficult. Thank you! ♥

**Carin**, I was so happy when you moved to MTC, loved it as long as it lasted, miss you a lot! Thanks for being the warm and open hearted person you are. **Joanna**, for bringing my favorite project up to speed while I was on maternity leave and it was really nice to shop and party with you in Heidelberg. **Dhifaf** and **Andreas** for being very nice conference and party companies in Heidelberg.

**Birgitta**, for all the help and nice talks in the facs-room and **Margareta, Mabbe**, **Anna-Karin** and especially **Kenth** for all help in the animal facility, without you there would have been significantly fewer experiments performed! **Barbro**, for answering all my administration/economy questions and for asking me how I am doing. In addition, I would like to thank all the personal at the administration/economy department and in the animal facility (both in the research and breeding unit), for nice assistance over the years. Co-authors from Novo Nordisk and Innate-Pharma for nice collaborations.

Dear ones outside of KI:
I would also like to thank **Jennie** for listening to my crap and for being there even when I don't have time. **Malin**, **Stefan** with family, for always being so good friends and for many relaxed dinners. **Gunilla**, **Peter**, **Johanna**, **David** and **Markus**, I would like to thank you all for treating me and my family as "family" and for that you are there for us no matter what, love you.

Till min familj, jag skulle först vilja tacka min **mormor** och **morfar** för att ni alltid har funnits där och stöttat mig oavsett orsak. Ni är de bästa morföräldrarna man kan önska sig, jag älskar er! **Farmor** och **Edvin** för att alltid visat intresse för vad jag gör och visat hur viktigt ni tycker det att det är med utbildning. Jag skulle även vilja tacka min **moster** med familj för att det aldrig är någon stress eller press, det kommer ju en ny dag…..

Till **Linus**, världens knasigaste men bästa lillebror, stort tack för att du är du och för att du drog in fina **Viva** i mitt liv.

**Mamma** och **Pappa**, tack för att ni alltid finns där helt villkorslöst, alltid ställer upp och tror på mig, jag älskar er och det här hade aldrig gott utan er. ♥

**Robin**, **Ronja** och **Emil**; TACK för att ni står ut med en ibland allt för stressad mamma, för att ni tålmodigt väntar medan "mamma jobbar" och sedan drar mig tillbaka till verkligheten och visar vad som egentligen är meningen med livet. ÄLSKAR ER! ♥

# 6 REFERENCES


1. Kiessling, R., E. Klein, and H. Wigzell. 1975. „Natural" I killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *European Journal of Immunology* 5: 112-117.
2. Kiessling, R., E. Klein, H. Pross, and H. Wigzell. 1975. „Natural"II killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *European Journal of Immunology* 5: 117-121.
3. Herberman, R. B., M. E. Nunn, and D. H. Lavrin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. *International Journal of Cancer* 16: 216-229.
4. Herberman, R. B., M. E. Nunn, H. T. Holden, and D. H. Lavrin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *International Journal of Cancer* 16: 230-239.

4a Pross, H. F and Jondal M. 1975. CYTOTOXIC LYMPHOCYTES FROM NORMAL DONORS, A FUNCTIONAL MARKER OF HUMAN NON-T LYMPHOCYTES. *Clinical and expremental medicine*; 21:226-235.

5. Kiessling, R., P. S. Hochman, O. Haller, G. M. Shearer, H. Wigzell, and G. Cudkowicz. 1977. Evidence for a similar or common mechanism for natural killer cell activity and resistance to hemopoietic grafts. *European Journal of Immunology* 7: 655-663.
6. Karre, K., H. G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 319: 675-678.
7. Ljunggren, H. G., and K. Kärre. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. *The Journal of Experimental Medicine* 162: 1745-1759.
8. Yokoyama, W. M., P. J. Kehn, D. I. Cohen, and E. M. Shevach. 1990. Chromosomal location of the Ly-49 (A1, YE1/48) multigene family. Genetic association with the NK 1.1 antigen. *The Journal of Immunology* 145: 2353-2358.
9. Yokoyama, W. M., J. C. Ryan, J. J. Hunter, H. R. Smith, M. Stark, and W. E. Seaman. 1991. cDNA cloning of mouse NKR-P1 and genetic linkage with LY-49. Identification of a natural killer cell gene complex on mouse chromosome 6. *The Journal of Immunology* 147: 3229-3236.
10. Colonna, M., and J. Samaridis. 1995. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science* 268: 405-408.
11. Wagtmann, N., R. Biassoni, C. Cantoni, S. Verdiani, M. S. Malnati, M. Vitale, C. Bottino, L. Moretta, A. Moretta, and E. O. Long. 1995. Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. *Immunity* 2: 439-449.
12. Biron, C., K. Byron, and J. Sullivan. 1989. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med* 320: 1731 - 1735.
13. Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather. 1999. NATURAL KILLER CELLS IN ANTIVIRAL DEFENSE: Function and Regulation by Innate Cytokines. *Annual Review of Immunology* 17: 189-220.
14. Unanue, E. R. 1997. Studies in listeriosis show the strong symbiosis between the innate cellular system and the T-cell response. *Immunological Reviews* 158: 11-25.

15. Scharton, T. M., and P. Scott. 1993. Natural killer cells are a source of interferon gamma that drives differentiation of CD4+ T cell subsets and induces early resistance to Leishmania major in mice. *The Journal of Experimental Medicine* 178: 567-577.
16. Lodoen, M. B., and L. L. Lanier. 2006. Natural killer cells as an initial defense against pathogens. *Current Opinion in Immunology* 18: 391-398.
17. Tyznik, A. J., S. Verma, Q. Wang, M. Kronenberg, and C. A. Benedict. 2014. Distinct Requirements for Activation of NKT and NK Cells during Viral Infection. *The Journal of Immunology*.
18. Della Chiesa, M., E. Marcenaro, S. Sivori, S. Carlomagno, S. Pesce, and A. Moretta. Human NK cell response to pathogens. *Seminars in Immunology*.
19. Diefenbach, A., A. M. Jamieson, S. D. Liu, N. Shastri, and D. H. Raulet. 2000. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat Immunol* 1: 119-126.
20. Seaman, W. E., M. Sleisenger, E. Eriksson, and G. C. Koo. 1987. Depletion of natural killer cells in mice by monoclonal antibody to NK-1.1. Reduction in host defense against malignancy without loss of cellular or humoral immunity. *The Journal of Immunology* 138: 4539-4544.
21. Ruggeri, L., M. Capanni, M. Casucci, I. Volpi, A. Tosti, K. Perruccio, E. Urbani, R. S. Negrin, M. F. Martelli, and A. Velardi. 1999. Role of Natural Killer Cell Alloreactivity in HLA-Mismatched Hematopoietic Stem Cell Transplantation. *Blood* 94: 333-339.
22. Ruggeri, L., M. Capanni, E. Urbani, K. Perruccio, W. D. Shlomchik, A. Tosti, S. Posati, D. Rogaia, F. Frassoni, F. Aversa, M. F. Martelli, and A. Velardi. 2002. Effectiveness of Donor Natural Killer Cell Alloreactivity in Mismatched Hematopoietic Transplants. *Science* 295: 2097-2100.
23. Moffett-King, A. 2002. Natural killer cells and pregnancy. *Nat Rev Immunol* 2: 656-663.
24. Lanier, L. L., J. H. Phillips, J. Hackett, M. Tutt, and V. Kumar. 1986. Natural killer cells: definition of a cell type rather than a function. *The Journal of Immunology* 137: 2735-2739.
25. Moretta, A., C. Bottino, M. C. Mingari, R. Biassoni, and L. Moretta. 2002. What is a natural killer cell? *Nat Immunol* 3: 6-8.
26. Colucci, F., M. A. Caligiuri, and J. P. Di Santo. 2003. What does it take to make a natural killer? *Nat Rev Immunol* 3: 413-425.
27. Trinchieri, G. 1989. Biology of Natural Killer Cells. In *Advances in Immunology*. J. D. Frank, ed. Academic Press. 187-376.
28. Biron, C. A., P. van den Elsen, M. M. Tutt, P. Medveczky, V. Kumar, and C. Terhorst. 1987. Murine natural killer cells stimulated in vivo do not express the T cell receptor alpha, beta, gamma, T3 delta, or T3 epsilon genes. *The Journal of Immunology* 139: 1704-1710.
29. Walzer, T., M. Bléry, J. Chaix, N. Fuseri, L. Chasson, S. H. Robbins, S. Jaeger, P. André, L. Gauthier, L. Daniel, K. Chemin, Y. Morel, M. Dalod, J. Imbert, M. Pierres, A. Moretta, F. Romagné, and E. Vivier. 2007. Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. *Proceedings of the National Academy of Sciences* 104: 3384-3389.
30. Caligiuri, M. 2008. Human natural killer cells. *Blood* 112: 461 - 469.
31. Shi, F.-D., H. Ljunggren, A. La Cava, and L. Van Kaer. 2011. Organ-specific features of natural killer cells. *Nat Rev Immunol* 11: 658 - 671.
32. Grégoire, C., L. Chasson, C. Luci, E. Tomasello, F. Geissmann, E. Vivier, and T. Walzer. 2007. The trafficking of natural killer cells. *Immunological Reviews* 220: 169-182.

33. Lanier, L. 2005. NK cell recognition. *Annu Rev Immunol* 23: 225 - 274.
34. Raulet, D. H., R. E. Vance, and C. W. McMahon. 2001. REGULATION OF THE NATURAL KILLER CELL RECEPTOR REPERTOIRE. *Annual Review of Immunology* 19: 291-330.
35. Hayakawa, Y., J. M. Kelly, J. A. Westwood, P. K. Darcy, A. Diefenbach, D. Raulet, and M. J. Smyth. 2002. Cutting Edge: Tumor Rejection Mediated by NKG2D Receptor-Ligand Interaction Is Dependent upon Perforin. *The Journal of Immunology* 169: 5377-5381.
36. Davis, J. E., M. J. Smyth, and J. A. Trapani. 2001. Granzyme A and B-deficient killer lymphocytes are defective in eliciting DNA fragmentation but retain potent in vivo anti-tumor capacity. *European Journal of Immunology* 31: 39-47.
37. Screpanti, V., R. P. A. Wallin, A. Grandien, and H.-G. Ljunggren. 2005. Impact of FASL-induced apoptosis in the elimination of tumor cells by NK cells. *Molecular Immunology* 42: 495-499.
38. Takeda, K., E. Cretney, Y. Hayakawa, T. Ota, H. Akiba, K. Ogasawara, H. Yagita, K. Kinoshita, K. Okumura, and M. J. Smyth. 2005. *TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver*.
39. Perussia, B., M. M. Tutt, W. Q. Qiu, W. A. Kuziel, P. W. Tucker, G. Trinchieri, M. Bennett, J. V. Ravetch, and V. Kumar. 1989. Murine natural killer cells express functional Fc gamma receptor II encoded by the Fc gamma R alpha gene. *The Journal of Experimental Medicine* 170: 73-86.
40. Orange, J. S., and C. A. Biron. 1996. Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. *The Journal of Immunology* 156: 4746-4756.
41. Vivier, E., D. H. Raulet, A. Moretta, M. A. Caligiuri, L. Zitvogel, L. L. Lanier, W. M. Yokoyama, and S. Ugolini. 2011. Innate or Adaptive Immunity? The Example of Natural Killer Cells. *Science* 331: 44-49.
42. Lucas, M., W. Schachterle, K. Oberle, P. Aichele, and A. Diefenbach. 2007. Dendritic Cells Prime Natural Killer Cells by trans-Presenting Interleukin 15. *Immunity* 26: 503-517.
43. Mortier, E., R. Advincula, L. Kim, S. Chmura, J. Barrera, B. Reizis, B. A. Malynn, and A. Ma. 2009. Macrophage- and Dendritic-Cell-Derived Interleukin-15 Receptor Alpha Supports Homeostasis of Distinct CD8+ T Cell Subsets. *Immunity* 31: 811-822.
44. Guia, S., C. Cognet, L. de Beaucoudrey, M. S. Tessmer, E. Jouanguy, C. Berger, O. Filipe-Santos, J. Feinberg, Y. Camcioglu, J. Levy, S. Al Jumaah, S. Al-Hajjar, J.-L. Stephan, C. Fieschi, L. Abel, L. Brossay, J.-L. Casanova, and E. Vivier. 2008. *A role for interleukin-12/23 in the maturation of human natural killer and CD56+ T cells in vivo*.
45. Igarashi, H., S. C. Gregory, T. Yokota, N. Sakaguchi, and P. W. Kincade. 2002. Transcription from the RAG1 Locus Marks the Earliest Lymphocyte Progenitors in Bone Marrow. *Immunity* 17: 117-130.
46. Kondo, M., I. L. Weissman, and K. Akashi. 1997. Identification of Clonogenic Common Lymphoid Progenitors in Mouse Bone Marrow. *Cell* 91: 661-672.
47. Georgopoulos, K., D. Moore, and B. Derfler. 1992. Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. *Science* 258: 808-812.
48. Molnár, A., and K. Georgopoulos. 1994. The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins. *Molecular and Cellular Biology* 14: 8292-8303.

49. Georgopoulos, K., S. Winandy, and N. Avitahl. 1997. THE ROLE OF THE IKAROS GENE IN LYMPHOCYTE DEVELOPMENT AND HOMEOSTASIS. *Annual Review of Immunology* 15: 155-176.
50. Rothenberg, E. V., and T. Taghon. 2005. MOLECULAR GENETICS OF T CELL DEVELOPMENT. *Annual Review of Immunology* 23: 601-649.
51. Singh, H., K. L. Medina, and J. M. R. Pongubala. 2005. Contingent gene regulatory networks and B cell fate specification. *Proceedings of the National Academy of Sciences of the United States of America* 102: 4949-4953.
52. Georgopoulos, K., M. Bigby, J.-H. Wang, A. Molnar, P. Wu, S. Winandy, and A. Sharpe. 1994. The ikaros gene is required for the development of all lymphoid lineages. *Cell* 79: 143-156.
53. Yokota, Y., A. Mansouri, S. Mori, S. Sugawara, S. Adachi, S.-I. Nishikawa, and P. Gruss. 1999. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature* 397: 702-706.
54. Rosmaraki, E. E., I. Douagi, C. Roth, F. Colucci, A. Cumano, and J. P. Di Santo. 2001. Identification of committed NK cell progenitors in adult murine bone marrow. *European Journal of Immunology* 31: 1900-1909.
55. Samson, S. I., O. Richard, M. Tavian, T. Ranson, C. A. J. Vosshenrich, F. Colucci, J. Buer, F. Grosveld, I. Godin, and J. P. Di Santo. 2003. GATA-3 Promotes Maturation, IFN-γ Production, and Liver-Specific Homing of NK Cells. *Immunity* 19: 701-711.
56. Fathman, J. W., D. Bhattacharya, M. A. Inlay, J. Seita, H. Karsunky, and I. L. Weissman. 2011. Identification of the earliest natural killer cell–committed progenitor in murine bone marrow. *Blood* 118: 5439-5447.
57. Puzanov, I. J., M. Bennett, and V. Kumar. 1996. IL-15 can substitute for the marrow microenvironment in the differentiation of natural killer cells. *The Journal of Immunology* 157: 4282-4285.
58. Zamai, L., M. Ahmad, I. M. Bennett, L. Azzoni, E. S. Alnemri, and B. Perussia. 1998. Natural Killer (NK) Cell–mediated Cytotoxicity: Differential Use of TRAIL and Fas Ligand by Immature and Mature Primary Human NK Cells. *The Journal of Experimental Medicine* 188: 2375-2380.
59. Shibuya, A., K. Nagayoshi, K. Nakamura, and H. Nakauchi. 1995. Lymphokine requirement for the generation of natural killer cells from CD34+ hematopoietic progenitor cells. *Blood* 85: 3538-3546.
60. Suzuki, H., G. S. Duncan, H. Takimoto, and T. W. Mak. 1997. Abnormal Development of Intestinal Intraepithelial Lymphocytes and Peripheral Natural Killer Cells in Mice Lacking the IL-2 Receptor β Chain. *The Journal of Experimental Medicine* 185: 499-506.
61. Vosshenrich, C. A. J., T. Ranson, S. I. Samson, E. Corcuff, F. Colucci, E. E. Rosmaraki, and J. P. Di Santo. 2005. Roles for Common Cytokine Receptor γ-Chain-Dependent Cytokines in the Generation, Differentiation, and Maturation of NK Cell Precursors and Peripheral NK Cells in Vivo. *The Journal of Immunology* 174: 1213-1221.
62. Kim, S., K. Iizuka, H.-S. P. Kang, A. Dokun, A. R. French, S. Greco, and W. M. Yokoyama. 2002. In vivo developmental stages in murine natural killer cell maturation. *Nat Immunol* 3: 523-528.
63. Di Santo, J. P. 2006. NATURAL KILLER CELL DEVELOPMENTAL PATHWAYS: A Question of Balance. *Annual Review of Immunology* 24: 257-286.
64. Chiossone, L., J. Chaix, N. Fuseri, C. Roth, E. Vivier, and T. Walzer. 2009. Maturation of mouse NK cells is a 4-stage developmental program. *Blood* 113: 5488-5496.

65. Hayakawa, Y., N. D. Huntington, S. L. Nutt, and M. J. Smyth. 2006. Functional subsets of mouse natural killer cells. *Immunological Reviews* 214: 47-55.
66. Hayakawa, Y., and M. J. Smyth. 2006. CD27 Dissects Mature NK Cells into Two Subsets with Distinct Responsiveness and Migratory Capacity. *The Journal of Immunology* 176: 1517-1524.
67. Watts, T. H. 2005. TNF/TNFR FAMILY MEMBERS IN COSTIMULATION OF T CELL RESPONSES. *Annual Review of Immunology* 23: 23-68.
68. Takeda, K., H. Oshima, Y. Hayakawa, H. Akiba, M. Atsuta, T. Kobata, K. Kobayashi, M. Ito, H. Yagita, and K. Okumura. 2000. CD27-Mediated Activation of Murine NK Cells. *The Journal of Immunology* 164: 1741-1745.
69. Kelly, J., P. Darcy, J. Markby, D. Godfrey, K. Takeda, H. Yagita, and M. Smyth. 2002. Induction of tumor-specific T cell memory by NK cell-mediated tumor rejection. *Nat Immunol* 3: 83 - 90.
70. Solovjov, D. A., E. Pluskota, and E. F. Plow. 2005. Distinct Roles for the α and β Subunits in the Functions of Integrin αMβ2. *Journal of Biological Chemistry* 280: 1336-1345.
71. Cortez, V. S., M. L. Robinette, and M. Colonna. 2015. Innate lymphoid cells: new insights into function and development. *Current Opinion in Immunology* 32: 71-77.
72. Diefenbach, A., M. Colonna, and S. Koyasu. 2014. Development, Differentiation, and Diversity of Innate Lymphoid Cells. *Immunity* 41: 354-365.
73. Artis, D., and H. Spits. 2015. The biology of innate lymphoid cells. *Nature* 517: 293-301.
74. Kim, T.-J., V. Upadhyay, V. Kumar, K.-M. Lee, and Y.-X. Fu. 2014. Innate lymphoid cells facilitate NK cell development through a lymphotoxin-mediated stromal microenvironment. *The Journal of Experimental Medicine* 211: 1421-1431.
75. Nakamura, M. C., S. Hayashi, E. C. Niemi, J. C. Ryan, and W. E. Seaman. 2000. Activating Ly-49d and Inhibitory Ly-49a Natural Killer Cell Receptors Demonstrate Distinct Requirements for Interaction with H2-Dd. *The Journal of Experimental Medicine* 192: 447-454.
75b. Thielens, A., Vivier, E. and Romagne F, 2012. NK cell MHC I specific receptors (KIR): from biology to clinincal intervention.
76. Yokoyama, W. M., and B. F. M. Plougastel. 2003. Immune functions encoded by the natural killer gene complex. *Nat Rev Immunol* 3: 304-316.
77. Drickamer, K., and M. E. Taylor. 1993. Biology of Animal Lectins. *Annual Review of Cell Biology* 9: 237-264.
78. Makrigiannis, A. P., A. T. Pau, P. L. Schwartzberg, D. W. McVicar, T. W. Beck, and S. K. Anderson. 2002. A BAC Contig Map of the Ly49 Gene Cluster in 129 Mice Reveals Extensive Differences in Gene Content Relative to C57BL/6 Mice. *Genomics* 79: 437-444.
79. Proteau, M.-F., E. Rousselle, and A. P. Makrigiannis. 2004. Mapping of the BALB/c Ly49 cluster defines a minimal natural killer cell receptor gene repertoire. *Genomics* 84: 669-677.
80. Yokoyama, W. M., and W. E. Seaman. 1993. The Ly-49 and NKR-P1 Gene Families Encoding Lectin-Like Receptors on Natural Killer Cells: The NK Gene Complex. *Annual Review of Immunology* 11: 613-635.
81. Giorda, R., E. P. Weisberg, T. K. Ip, and M. Trucco. 1992. Genomic structure and strain-specific expression of the natural killer cell receptor NKR-P1. *The Journal of Immunology* 149: 1957-1963.

82. Takei, F., J. Brennan, and D. L. Mager. 1997. The Ly-49 family: genes, proteins and recognition of class I MHC. *Immunological Reviews* 155: 67-77.
83. McQueen, K. L., J. D. Freeman, F. Takei, and D. L. Mager. 1998. Localization of five new Ly49 genes, including three closely related to Ly49c. *Immunogenetics* 48: 174-183.
84. Williams, N. S., A. Kubota, M. Bennett, V. Kumar, and F. Takei. 2000. Clonal analysis of NK cell development from bone marrow progenitors in vitro: orderly acquisition of receptor gene expression. *European Journal of Immunology* 30: 2074-2082.
85. Raulet, D. H., W. Held, I. Correa, J. R. Dorfman, M.-F. Wu, and L. Corral. 1997. Specificity, tolerance and developmental regulation of natural killer cells defined by expression of class I-specific Ly49 receptors. *Immunological Reviews* 155: 41-52.
86. Mason, L. H., J. R. Ortaldo, H. A. Young, V. Kumar, M. Bennett, and S. K. Anderson. 1995. Cloning and functional characteristics of murine large granular lymphocyte-1: a member of the Ly-49 gene family (Ly-49G2). *The Journal of Experimental Medicine* 182: 293-303.
87. Yu, Y. Y. L., T. George, J. R. Dorfman, J. Roland, V. Kumar, and M. Bennett. 1996. The Role of Ly49A and 5E6(Ly49C) Molecules in Hybrid Resistance Mediated by Murine Natural Killer Cells Against Normal T Cell Blasts. *Immunity* 4: 67-76.
88. Hanke, T., H. Takizawa, C. W. McMahon, D. H. Busch, E. G. Pamer, J. D. Miller, J. D. Altman, Y. Liu, D. Cado, F. A. Lemonnier, P. J. Bjorkman, and D. H. Raulet. 1999. Direct Assessment of MHC Class I Binding by Seven Ly49 Inhibitory NK Cell Receptors. *Immunity* 11: 67-77.
89. Michaëlsson, J., A. Achour, M. Salcedo, A. Kåse-Sjöström, J. Sundbäck, R. A. Harris, and K. Kärre. 2000. Visualization of inhibitory Ly49 receptor specificity with soluble major histocompatibility complex class I tetramers. *European Journal of Immunology* 30: 300-307.
90. Scarpellino, L., F. Oeschger, P. Guillaume, J. D. Coudert, F. Lévy, G. Leclercq, and W. Held. 2007. Interactions of Ly49 Family Receptors with MHC Class I Ligands in trans and cis. *The Journal of Immunology* 178: 1277-1284.
91. Barten, R., and J. Trowsdale. 1999. The human <i>Ly-49L gene. *Immunogenetics* 49: 731-734.
92. Parham, P. 2004. Killer cell immunoglobulin-like receptor diversity: balancing signals in the natural killer cell response. *Immunology Letters* 92: 11-13.
93. Litwin, V., J. Gumperz, P. Parham, J. H. Phillips, and L. L. Lanier. 1994. NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. *The Journal of Experimental Medicine* 180: 537-543.
94. Moretta, A., C. Bottino, M. Vitale, D. Pende, R. Biassoni, M. C. Mingari, and L. Moretta. 1996. RECEPTORS FOR HLA CLASS-I MOLECULES IN HUMAN NATURAL KILLER CELLS. *Annual Review of Immunology* 14: 619-648.
95. Karlhofer, F. M., R. K. Ribaudo, and W. M. Yokoyama. 1992. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature* 358: 66-70.
96. Orihuela, M., D. H. Margulies, and W. M. Yokoyama. 1996. The natural killer cell receptor Ly-49A recognizes a peptide-induced conformational determinant on its major histocompatibility complex class I ligand. *Proceedings of the National Academy of Sciences* 93: 11792-11797.
97. Correa, I., and D. H. Raulet. 1995. Binding of diverse peptides to MHC class I molecules inhibits target cell lysis by activated natural killer cells. *Immunity* 2: 61-71.

98. Daniels, B. F., M. C. Nakamura, S. D. Rosen, W. M. Yokoyama, and W. E. Seaman. 1994. Ly-49A, a receptor for H-2Dd, has a functional carbohydrate recognition domain. *Immunity* 1: 785-792.
99. Brennan, J., F. Takei, S. Wong, and D. L. Mager. 1995. Carbohydrate Recognition by a Natural Killer Cell Receptor, Ly-49C. *Journal of Biological Chemistry* 270: 9691-9694.
100. Chang, C., A. Rodríguez, M. Carretero, M. López-Botet, J. H. Phillips, and L. L. Lanier. 1995. Molecular characterization of human CD94: A type II membrane glycoprotein related to the C-type lectin superfamily. *European Journal of Immunology* 25: 2433-2437.
101. Houchins, J. P., T. Yabe, C. McSherry, and F. H. Bach. 1991. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. *The Journal of Experimental Medicine* 173: 1017-1020.
102. Vance, R. E., J. R. Kraft, J. D. Altman, P. E. Jensen, and D. H. Raulet. 1998. Mouse CD94/NKG2A Is a Natural Killer Cell Receptor for the Nonclassical Major Histocompatibility Complex (MHC) Class I Molecule Qa-1b. *The Journal of Experimental Medicine* 188: 1841-1848.
103. Lohwasser, S., P. Hande, D. L. Mager, and F. Takei. 1999. Cloning of murine NKG2A, B and C: second family of C-type lectin receptors on murine NK cells. *European Journal of Immunology* 29: 755-761.
104. Vance, R. E., A. M. Jamieson, and D. H. Raulet. 1999. Recognition of the Class Ib Molecule Qa-1b by Putative Activating Receptors Cd94/Nkg2c and Cd94/Nkg2e on Mouse Natural Killer Cells. *The Journal of Experimental Medicine* 190: 1801-1812.
105. Houchins, J. P., L. L. Lanier, E. C. Niemi, J. H. Phillips, and J. C. Ryan. 1997. Natural killer cell cytolytic activity is inhibited by NKG2-A and activated by NKG2-C. *The Journal of Immunology* 158: 3603-3609.
106. Carretero, M., C. Cantoni, T. Bellón, C. Bottino, R. Biassoni, A. Rodríguez, J. J. Pérez-Villar, L. Moretta, A. Moretta, and M. López-Botet. 1997. The CD94 and NKG2-A C-type lectins covalently assemble to form a natural killer cell inhibitory receptor for HLA class I molecules. *European Journal of Immunology* 27: 563-567.
107. Le Dréan, E., F. Vély, L. Olcese, A. Cambiaggi, S. Guia, G. Krystal, N. Gervois, A. Moretta, F. Jotereau, and E. Vivier. 1998. Inhibition of antigen-induced T cell response and antibody-induced NK cell cytotoxicity by NKG2A: association of NKG2A with SHP-1 and SHP-2 protein-tyrosine phosphatases. *European Journal of Immunology* 28: 264-276.
108. Braud, V. M., D. S. J. Allan, C. A. O'Callaghan, K. Soderstrom, A. D'Andrea, G. S. Ogg, S. Lazetic, N. T. Young, J. I. Bell, J. H. Phillips, L. L. Lanier, and A. J. McMichael. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 391: 795-799.
109. Shum, B. P., L. R. Flodin, D. G. Muir, R. Rajalingam, S. I. Khakoo, S. Cleland, L. A. Guethlein, M. Uhrberg, and P. Parham. 2002. Conservation and Variation in Human and Common Chimpanzee CD94 and NKG2 Genes. *The Journal of Immunology* 168: 240-252.
110. Lazetic, S., C. Chang, J. P. Houchins, L. L. Lanier, and J. H. Phillips. 1996. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. *The Journal of Immunology* 157: 4741-4745.
111. Brooks, A. G., P. E. Posch, C. J. Scorzelli, F. Borrego, and J. E. Coligan. 1997. NKG2A Complexed with CD94 Defines a Novel Inhibitory Natural Killer Cell Receptor. *The Journal of Experimental Medicine* 185: 795-800.

112. Borrego, F., M. Ulbrecht, E. H. Weiss, J. E. Coligan, and A. G. Brooks. 1998. Recognition of Human Histocompatibility Leukocyte Antigen (HLA)-E Complexed with HLA Class I Signal Sequence–derived Peptides by CD94/NKG2 Confers Protection from Natural Killer Cell–mediated Lysis. *The Journal of Experimental Medicine* 187: 813-818.
113. Lee, N., M. Llano, M. Carretero, A. Ishitani, F. Navarro, M. Lopez-Botet, and D. Geraghty. 1998. HLA-E is a major ligand for the NK inhibitory receptor CD94/NKG2A. *Proc Natl Acad Sci USA* 95: 5199 - 5204.
114. Braud, V. M., D. S. J. Allan, and A. J. McMichael. 1999. Functions of nonclassical MHC and non-MHC-encoded class I molecules. *Current Opinion in Immunology* 11: 100-108.
115. Aldrich, C. J., A. DeCloux, A. S. Woods, R. J. Cotter, M. J. Soloski, and J. Forman. 1994. Identification of a tap-dependent leader peptide recognized by alloreactive T cells specific for a class Ib antigen. *Cell* 79: 649-658.
116. Ryan, J. C., J. Turck, E. C. Niemi, W. M. Yokoyama, and W. E. Seaman. 1992. Molecular cloning of the NK1.1 antigen, a member of the NKR-P1 family of natural killer cell activation molecules. *The Journal of Immunology* 149: 1631-1635.
117. Plougastel, B. F. M., and W. M. Yokoyama. 2006. Extending Missing-Self? Functional Interactions Between Lectin-like Nkrp1 Receptors on NK Cells with Lectin-like Ligands. In *Immunobiology of Natural Killer Cell Receptors*. R. W. Compans, M. D. Cooper, T. Honjo, H. Koprowski, F. Melchers, M. B. A. Oldstone, S. Olsnes, M. Potter, P. K. Vogt, H. Wagner, E. Vivier, and M. Colonna, eds. Springer Berlin Heidelberg. 77-89.
118. Lanier, L. L., C. Chang, and J. H. Phillips. 1994. Human NKR-P1A. A disulfide-linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. *The Journal of Immunology* 153: 2417-2428.
119. Giorda, R., and M. Trucco. 1991. Mouse NKR-P1. A family of genes selectively coexpressed in adherent lymphokine-activated killer cells. *The Journal of Immunology* 147: 1701-1708.
120. Carlyle, J. R., A. Martin, A. Mehra, L. Attisano, F. W. Tsui, and J. C. Zúñiga-Pflücker. 1999. Mouse NKR-P1B, a Novel NK1.1 Antigen with Inhibitory Function. *The Journal of Immunology* 162: 5917-5923.
121. Iizuka, K., O. V. Naidenko, B. F. M. Plougastel, D. H. Fremont, and W. M. Yokoyama. 2003. Genetically linked C-type lectin-related ligands for the NKRP1 family of natural killer cell receptors. *Nat Immunol* 4: 801-807.
122. Plougastel, B., C. Dubbelde, and W. Yokoyama. 2001. Cloning of Clr, a new family of lectin-like genes localized between mouse Nkrp1a and Cd69. *Immunogenetics* 53: 209-214.
123. Plougastel, B., K. Matsumoto, C. Dubbelde, and W. Yokoyama. 2001. Analysis of a 1-Mb BAC contig overlapping the mouse Nkrp1 cluster of genes: cloning of three new Nkrp1 members, Nkrp1d, Nkrp1e, and Nkrp1f. *Immunogenetics* 53: 592-598.
124. Carlyle, J. R., A. Mesci, B. Ljutic, S. Belanger, L.-H. Tai, E. Rousselle, A. D. Troke, M.-F. Proteau, and A. P. Makrigiannis. 2006. Molecular and Genetic Basis for Strain-Dependent NK1.1 Alloreactivity of Mouse NK Cells. *The Journal of Immunology* 176: 7511-7524.
125. Carlyle, J. R., A. M. Jamieson, S. Gasser, C. S. Clingan, H. Arase, and D. H. Raulet. 2004. Missing self-recognition of Ocil/Clr-b by inhibitory NKR-P1 natural killer cell receptors. *Proceedings of the National Academy of Sciences of the United States of America* 101: 3527-3532.

126. Hanke, T., L. Corral, R. E. Vance, and D. H. Raulet. 1998. 2F1 antigen, the mouse homolog of the rat “mast cell function-associated antigen”, is a lectin-like type II transmembrane receptor expressed by natural killer cells. *European Journal of Immunology* 28: 4409-4417.
127. Blaser, C., M. Kaufmann, and H. Pircher. 1998. Cutting Edge: Virus-Activated CD8 T Cells and Lymphokine-Activated NK Cells Express the Mast Cell Function-Associated Antigen, An Inhibitory C-Type Lectin. *The Journal of Immunology* 161: 6451-6454.
128. Corral, L., T. Hanke, R. E. Vance, D. Cado, and D. H. Raulet. 2000. NK cell expression of the killer cell lectin-like receptor G1 (KLRG1), the mouse homolog of MAFA, is modulated by MHC class I molecules. *European Journal of Immunology* 30: 920-930.
129. Hofmann, M., O. Schweier, and H. Pircher. 2012. Different inhibitory capacities of human and mouse KLRG1 are linked to distinct disulfide-mediated oligomerizations. *European Journal of Immunology* 42: 2484-2490.
130. Gründemann, C., S. Schwartzkopff, M. Koschella, O. Schweier, C. Peters, D. Voehringer, and H. Pircher. 2010. The NK receptor KLRG1 is dispensable for virus-induced NK and CD8+ T-cell differentiation and function in vivo. *European Journal of Immunology* 40: 1303-1314.
131. Huntington, N. D., H. Tabarias, K. Fairfax, J. Brady, Y. Hayakawa, M. A. Degli-Esposti, M. J. Smyth, D. M. Tarlinton, and S. L. Nutt. 2007. NK Cell Maturation and Peripheral Homeostasis Is Associated with KLRG1 Up-Regulation. *The Journal of Immunology* 178: 4764-4770.
132. Fogel, L. A., M. M. Sun, T. L. Geurs, L. N. Carayannopoulos, and A. R. French. 2013. Markers of Nonselective and Specific NK Cell Activation. *The Journal of Immunology* 190: 6269-6276.
133. Gründemann, C., M. Bauer, O. Schweier, N. von Oppen, U. Lässing, P. Saudan, K.-F. Becker, K. Karp, T. Hanke, M. F. Bachmann, and H. Pircher. 2006. Cutting Edge: Identification of E-Cadherin as a Ligand for the Murine Killer Cell Lectin-Like Receptor G1. *The Journal of Immunology* 176: 1311-1315.
134. Robbins, S. H., K. B. Nguyen, N. Takahashi, T. Mikayama, C. A. Biron, and L. Brossay. 2002. Cutting Edge: Inhibitory Functions of the Killer Cell Lectin-Like Receptor G1 Molecule During the Activation of Mouse NK Cells. *The Journal of Immunology* 168: 2585-2589.
135. Ito, M., T. Maruyama, N. Saito, S. Koganei, K. Yamamoto, and N. Matsumoto. 2006. Killer cell lectin-like receptor G1 binds three members of the classical cadherin family to inhibit NK cell cytotoxicity. *The Journal of Experimental Medicine* 203: 289-295.
136. Tessmer, M. S., C. Fugere, F. Stevenaert, O. V. Naidenko, H. J. Chong, G. Leclercq, and L. Brossay. 2007. KLRG1 binds cadherins and preferentially associates with SHIP-1. *International Immunology* 19: 391-400.
137. Garni-Wagner, B. A., A. Purohit, P. A. Mathew, M. Bennett, and V. Kumar. 1993. A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells. *The Journal of Immunology* 151: 60-70.
138. Valiante, N. M., and G. Trinchieri. 1993. Identification of a novel signal transduction surface molecule on human cytotoxic lymphocytes. *The Journal of Experimental Medicine* 178: 1397-1406.
139. Engel, P., M. J. Eck, and C. Terhorst. 2003. The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease. *Nat Rev Immunol* 3: 813-821.

140. Romero, X., D. Benítez, S. March, R. Vilella, M. Miralpeix, and P. Engel. 2004. Differential expression of SAP and EAT-2-binding leukocyte cell-surface molecules CD84, CD150 (SLAM), CD229 (Ly9) and CD244 (2B4). *Tissue Antigens* 64: 132-144.
141. Latchman, Y., P. F. McKay, and H. Reiser. 1998. Cutting Edge: Identification of the 2B4 Molecule as a Counter-Receptor for CD48. *The Journal of Immunology* 161: 5809-5812.
142. Dong, Z., M.-E. Cruz-Munoz, M.-C. Zhong, R. Chen, S. Latour, and A. Veillette. 2009. Essential function for SAP family adaptors in the surveillance of hematopoietic cells by natural killer cells. *Nat Immunol* 10: 973-980.
143. Lee, K.-M., M. E. McNerney, S. E. Stepp, P. A. Mathew, J. D. Schatzle, M. Bennett, and V. Kumar. 2004. 2B4 Acts As a Non–Major Histocompatibility Complex Binding Inhibitory Receptor on Mouse Natural Killer Cells. *The Journal of Experimental Medicine* 199: 1245-1254.
144. Lanier, L. L., A. M. Le, J. H. Phillips, N. L. Warner, and G. F. Babcock. 1983. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. *The Journal of Immunology* 131: 1789-1796.
145. Nakamura, M. C., P. A. Linnemeyer, E. C. Niemi, L. H. Mason, J. R. Ortaldo, J. C. Ryan, and W. E. Seaman. 1999. Mouse Ly-49D Recognizes H-2Dd and Activates Natural Killer Cell Cytotoxicity. *The Journal of Experimental Medicine* 189: 493-500.
146. Silver, E. T., D.-E. Gong, C. S. Chang, A. Amrani, P. Santamaria, and K. P. Kane. 2000. Ly-49P Activates NK-Mediated Lysis by Recognizing H-2Dd 1. *The Journal of Immunology* 165: 1771-1781.
147. Silver, E. T., D.-E. Gong, B. Hazes, and K. P. Kane. 2001. Ly-49W, an Activating Receptor of Nonobese Diabetic Mice With Close Homology to the Inhibitory Receptor Ly-49G, Recognizes H-2Dk and H-2Dd. *The Journal of Immunology* 166: 2333-2341.
148. Daniels, K. A., G. Devora, W. C. Lai, C. L. O'Donnell, M. Bennett, and R. M. Welsh. 2001. Murine Cytomegalovirus Is Regulated by a Discrete Subset of Natural Killer Cells Reactive with Monoclonal Antibody to Ly49h. *The Journal of Experimental Medicine* 194: 29-44.
149. Tripathy, S. K., H. R. C. Smith, E. A. Holroyd, J. T. Pingel, and W. M. Yokoyama. 2006. Expression of m157, a Murine Cytomegalovirus-Encoded Putative Major Histocompatibility Class I (MHC-I)-Like Protein, Is Independent of Viral Regulation of Host MHC-I. *Journal of Virology* 80: 545-550.
150. Arase, H., E. S. Mocarski, A. E. Campbell, A. B. Hill, and L. L. Lanier. 2002. Direct Recognition of Cytomegalovirus by Activating and Inhibitory NK Cell Receptors. *Science* 296: 1323-1326.
151. Pegram, H. J., D. M. Andrews, M. J. Smyth, P. K. Darcy, and M. H. Kershaw. 2011. Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol* 89: 216-224.
152. Brusilovsky, M., B. Rosental, A. Shemesh, M. Y. Appel, and A. Porgador. Human NK cell recognition of target cells in the prism of natural cytotoxicity receptors and their ligands. *Journal of Immunotoxicology* 0: 1-8.
153. Pogge von Strandmann, E., V. Simhadri, B. von Tresckow, S. Sasse, K. Reiners, H. Hansen, A. Rothe, B. Boll, V. Simhadri, and P. Borchmann. 2007. Human leukocyte antigen-B-associated transcript 3 is released from tumor cells and engages the NKp30 receptor on natural killer cells. *Immunity* 27: 965 - 974.
154. Mandelboim, O., N. Lieberman, M. Lev, L. Paul, T. I. Arnon, Y. Bushkin, D. M. Davis, J. L. Strominger, J. W. Yewdell, and A. Porgador. 2001. Recognition of haemagglutinins

on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* 409: 1055-1060.
155. Kruse, P. H., J. Matta, S. Ugolini, and E. Vivier. 2014. Natural cytotoxicity receptors and their ligands. *Immunol Cell Biol* 92: 221-229.
156. Biassoni, R., A. Pessino, C. Bottino, D. Pende, L. Moretta, and A. Moretta. 1999. The murine homologue of the human NKp46, a triggering receptor involved in the induction of natural cytotoxicity. *European Journal of Immunology* 29: 1014-1020.
157. Halfteck, G. G., M. Elboim, C. Gur, H. Achdout, H. Ghadially, and O. Mandelboim. 2009. Enhanced In Vivo Growth of Lymphoma Tumors in the Absence of the NK-Activating Receptor NKp46/NCR1. *The Journal of Immunology* 182: 2221-2230.
158. Saether, P. C., S. E. Hoelsbrekken, S. Fossum, and E. Dissen. 2011. Rat and Mouse CD94 Associate Directly with the Activating Transmembrane Adaptor Proteins DAP12 and DAP10 and Activate NK Cell Cytotoxicity. *The Journal of Immunology* 187: 6365-6373.
159. Lee, J., T. Zhang, I. Hwang, A. Kim, L. Nitschke, M. Kim, Jeannine M. Scott, Y. Kamimura, Lewis L. Lanier, and S. Kim. 2015. Epigenetic Modification and Antibody-Dependent Expansion of Memory-like NK Cells in Human Cytomegalovirus-Infected Individuals. *Immunity* 42: 431-442.
160. Lanier, L. L., B. Corliss, J. Wu, and J. H. Phillips. 1998. Association of DAP12 with Activating CD94/NKG2C NK Cell Receptors. *Immunity* 8: 693-701.
161. Guerra, N., Y. X. Tan, N. T. Joncker, A. Choy, F. Gallardo, N. Xiong, S. Knoblaugh, D. Cado, N. R. Greenberg, and D. H. Raulet. 2008. NKG2D-Deficient Mice Are Defective in Tumor Surveillance in Models of Spontaneous Malignancy. *Immunity* 28: 571-580.
162. Hayakawa, Y., and M. J. Smyth. 2006. NKG2D and cytotoxic effector function in tumor immune surveillance. *Seminars in Immunology* 18: 176-185.
163. Ho, E. L., L. N. Carayannopoulos, J. Poursine-Laurent, J. Kinder, B. Plougastel, H. R. C. Smith, and W. M. Yokoyama. 2002. Costimulation of Multiple NK Cell Activation Receptors by NKG2D. *The Journal of Immunology* 169: 3667-3675.
164. Diefenbach, A., E. Tomasello, M. Lucas, A. M. Jamieson, J. K. Hsia, E. Vivier, and D. H. Raulet. 2002. Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. *Nat Immunol* 3: 1142-1149.
165. Gilfillan, S., E. L. Ho, M. Cella, W. M. Yokoyama, and M. Colonna. 2002. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. *Nat Immunol* 3: 1150-1155.
166. Lanier, L. L. 2008. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* 9: 495-502.
167. Billadeau, D. D., J. L. Upshaw, R. A. Schoon, C. J. Dick, and P. J. Leibson. 2003. NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nat Immunol* 4: 557-564.
168. Wu, J., Y. Song, A. B. H. Bakker, S. Bauer, T. Spies, L. L. Lanier, and J. H. Phillips. 1999. An Activating Immunoreceptor Complex Formed by NKG2D and DAP10. *Science* 285: 730-732.
169. Diefenbach, A., E. R. Jensen, A. M. Jamieson, and D. H. Raulet. 2001. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* 413: 165-171.
170. Cerwenka, A., A. B. H. Bakker, T. McClanahan, J. Wagner, J. Wu, J. H. Phillips, and L. L. Lanier. 2000. Retinoic Acid Early Inducible Genes Define a Ligand Family for the Activating NKG2D Receptor in Mice. *Immunity* 12: 721-727.

171. Raulet, D. H., S. Gasser, B. G. Gowen, W. Deng, and H. Jung. 2013. Regulation of Ligands for the NKG2D Activating Receptor. *Annual Review of Immunology* 31: 413-441.
172. Gasser, S., S. Orsulic, E. Brown, and D. Raulet. 2005. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* 436: 1186 - 1190.
173. Raulet, D. H. 2003. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol* 3: 781-790.
174. Smyth, M. J., K. Y. T. Thia, S. E. A. Street, E. Cretney, J. A. Trapani, M. Taniguchi, T. Kawano, S. B. Pelikan, N. Y. Crowe, and D. I. Godfrey. 2000. Differential Tumor Surveillance by Natural Killer (Nk) and Nkt Cells. *The Journal of Experimental Medicine* 191: 661-668.
175. Cosman, D., J. Müllberg, C. L. Sutherland, W. Chin, R. Armitage, W. Fanslow, M. Kubin, and N. J. Chalupny. 2001. ULBPs, Novel MHC Class I–Related Molecules, Bind to CMV Glycoprotein UL16 and Stimulate NK Cytotoxicity through the NKG2D Receptor. *Immunity* 14: 123-133.
176. Steinle, A., P. Li, D. Morris, V. Groh, L. Lanier, R. Strong, and T. Spies. 2001. Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse RAE-1 protein family. *Immunogenetics* 53: 279-287.
177. Zhang, J., F. Basher, and J. D. Wu. 2015. NKG2D Ligands in Tumor Immunity: Two Sides of a Coin. *Frontiers in Immunology* 6: 97.
178. Deng, W., B. G. Gowen, L. Zhang, L. Wang, S. Lau, A. Iannello, J. Xu, T. L. Rovis, N. Xiong, and D. H. Raulet. 2015. A shed NKG2D ligand that promotes natural killer cell activation and tumor rejection. *Science* 348: 136-139.
179. Iguchi-Manaka, A., H. Kai, Y. Yamashita, K. Shibata, S. Tahara-Hanaoka, S.-i. Honda, T. Yasui, H. Kikutani, K. Shibuya, and A. Shibuya. 2008. Accelerated tumor growth in mice deficient in DNAM-1 receptor. *The Journal of Experimental Medicine* 205: 2959-2964.
180. Welch, M. J., J. R. Teijaro, H. A. Lewicki, M. Colonna, and M. B. A. Oldstone. 2012. CD8 T cell defect of TNF-α and IL-2 in DNAM-1 deficient mice delays clearance in vivo of a persistent virus infection. *Virology* 429: 163-170.
181. Enqvist, M., E. H. Ask, E. Forslund, M. Carlsten, G. Abrahamsen, V. Béziat, S. Andersson, M. Schaffer, A. Spurkland, Y. Bryceson, B. Önfelt, and K.-J. Malmberg. 2015. Coordinated Expression of DNAM-1 and LFA-1 in Educated NK Cells. *The Journal of Immunology* 194: 4518-4527.
182. Bryceson, Y. T., M. E. March, H.-G. Ljunggren, and E. O. Long. 2006. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* 107: 159-166.
183. Bida, A. T., J. L. Upshaw Neff, C. J. Dick, R. A. Schoon, A. Brickshawana, C. C. Chini, and D. D. Billadeau. 2011. 2B4 utilizes ITAM-containing receptor complexes to initiate intracellular signaling and cytolysis. *Molecular Immunology* 48: 1149-1159.
184. Bryceson, Y. T., and E. O. Long. 2008. Line of attack: NK cell specificity and integration of signals. *Current Opinion in Immunology* 20: 344-352.
185. Alter, G., J. M. Malenfant, and M. Altfeld. 2004. CD107a as a functional marker for the identification of natural killer cell activity. *Journal of Immunological Methods* 294: 15-22.
186. Long, E. O., H. Sik Kim, D. Liu, M. E. Peterson, and S. Rajagopalan. 2013. Controlling Natural Killer Cell Responses: Integration of Signals for Activation and Inhibition. *Annual Review of Immunology* 31: 227-258.

187. Watzl, C., and E. O. Long. 2010. Signal Transduction During Activation and Inhibition of Natural Killer Cells. *Current protocols in immunology / edited by John E. Coligan ... [et al.]* 0 11: 10.1002/0471142735.im0471141109bs0471142790.
188. Vivier, E., J. A. Nunès, and F. Vély. 2004. Natural Killer Cell Signaling Pathways. *Science* 306: 1517-1519.
189. Vely, F., and E. Vivier. 2005. Natural Killer Cell Receptor Signaling Pathway. *Sci. STKE* 2005: cm6-.
190. Ralston, K. J., S. L. Hird, X. Zhang, J. L. Scott, B. Jin, R. F. Thorne, M. C. Berndt, A. W. Boyd, and G. F. Burns. 2004. The LFA-1-associated Molecule PTA-1 (CD226) on T Cells Forms a Dynamic Molecular Complex with Protein 4.1G and Human Discs Large. *Journal of Biological Chemistry* 279: 33816-33828.
191. Shibuya, K., L. L. Lanier, J. H. Phillips, H. D. Ochs, K. Shimizu, E. Nakayama, H. Nakauchi, and A. Shibuya. 1999. Physical and Functional Association of LFA-1 with DNAM-1 Adhesion Molecule. *Immunity* 11: 615-623.
192. Shibuya, A., L. L. Lanier, and J. H. Phillips. 1998. Protein Kinase C Is Involved in the Regulation of Both Signaling and Adhesion Mediated by DNAX Accessory Molecule-1 Receptor. *The Journal of Immunology* 161: 1671-1676.
193. Campbell, K. S., and J. Hasegawa. 2013. Natural killer cell biology: An update and future directions. *Journal of Allergy and Clinical Immunology* 132: 536-544.
194. Burshtyn, D. N., A. M. Scharenberg, N. Wagtmann, S. Rajagopalan, K. Berrada, T. Yi, J.-P. Kinet, and E. O. Long. 1996. Recruitment of Tyrosine Phosphatase HCP by the Killer Cell Inhibitory Receptor. *Immunity* 4: 77-85.
195. Peterson, M. E., and E. O. Long. 2008. Inhibitory Receptor Signaling via Tyrosine Phosphorylation of the Adaptor Crk. *Immunity* 29: 578-588.
196. Pérez-Quintero, L.-A., R. Roncagalli, H. Guo, S. Latour, D. Davidson, and A. Veillette. 2014. EAT-2, a SAP-like adaptor, controls NK cell activation through phospholipase Cγ, Ca++, and Erk, leading to granule polarization. *The Journal of Experimental Medicine* 211: 727-742.
197. Veillette, A. 2006. NK cell regulation by SLAM family receptors and SAP-related adapters. *Immunological Reviews* 214: 22-34.
198. Dong, Z., D. Davidson, Luis A. Pérez-Quintero, T. Kurosaki, W. Swat, and A. Veillette. 2012. The Adaptor SAP Controls NK Cell Activation by Regulating the Enzymes Vav-1 and SHIP-1 and by Enhancing Conjugates with Target Cells. *Immunity* 36: 974-985.
199. Chlewicki, L. K., C. A. Velikovsky, V. Balakrishnan, R. A. Mariuzza, and V. Kumar. 2008. Molecular Basis of the Dual Functions of 2B4 (CD244). *The Journal of Immunology* 180: 8159-8167.
200. Taniguchi, R. T., D. Guzior, and V. Kumar. 2007. 2B4 inhibits NK-cell fratricide. *Blood* 110: 2020-2023.
201. Georgoudaki, A.-M., S. Khodabandeh, S. Puiac, C. M. Persson, M. K. Larsson, M. Lind, O. Hammarfjord, T. H. Nabatti, R. P. A. Wallin, U. Yrlid, M. Rhen, V. Kumar, and B. J. Chambers. 2015. CD244 is expressed on dendritic cells and regulates their functions. *Immunol Cell Biol* 93: 581-590.
202. Kim, S., J. Poursine-Laurent, S. M. Truscott, L. Lybarger, Y.-J. Song, L. Yang, A. R. French, J. B. Sunwoo, S. Lemieux, T. H. Hansen, and W. M. Yokoyama. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 436: 709-713.
203. Fernandez, N. C., E. Treiner, R. E. Vance, A. M. Jamieson, S. Lemieux, and D. H. Raulet. 2005. A subset of natural killer cells achieves self-tolerance without

expressing inhibitory receptors specific for self-MHC molecules. *Blood* 105: 4416-4423.
204. Brodin, P., T. Lakshmikanth, K. Kärre, and P. Höglund. 2012. Skewing of the NK Cell Repertoire by MHC Class I via Quantitatively Controlled Enrichment and Contraction of Specific Ly49 Subsets. *The Journal of Immunology* 188: 2218-2226.
205. Dorfman, J. R., and D. H. Raulet. 1998. Acquisition of Ly49 Receptor Expression by Developing Natural Killer Cells. *The Journal of Experimental Medicine* 187: 609-618.
206. Salcedo, M., F. Colucci, P. J. Dyson, L. A. Cotterill, F. A. Lemonnier, P. Kourilsky, J. P. Di Santo, H.-G. Ljunggren, and J.-P. Abastado. 2000. Role of Qa-1b-binding receptors in the specificity of developing NK cells. *European Journal of Immunology* 30: 1094-1101.
207. Toomey, J. A., M. Salcedo, L. A. Cotterill, M. M. Millrain, Z. Chrzanowska-Lightowlers, J. Lawry, K. Fraser, F. Gays, J. H. Robinson, S. Shrestha, P. J. Dyson, and C. G. Brooks. 1999. Stochastic Acquisition of Qa1 Receptors During the Development of Fetal NK Cells In Vitro Accounts in Part But Not in Whole for the Ability of These Cells to Distinguish Between Class I-Sufficient and Class I-Deficient Targets. *The Journal of Immunology* 163: 3176-3184.
208. Held, W., J. D. Coudert, and J. Zimmer. 2003. The NK cell receptor repertoire: formation, adaptation and exploitation. *Current Opinion in Immunology* 15: 233-237.
209. Roth, C., J. R. Carlyle, H. Takizawa, and D. H. Raulet. 2000. Clonal Acquisition of Inhibitory Ly49 Receptors on Developing NK Cells Is Successively Restricted and Regulated by Stromal Class I MHC. *Immunity* 13: 143-153.
210. Kennedy, M. K., M. Glaccum, S. N. Brown, E. A. Butz, J. L. Viney, M. Embers, N. Matsuki, K. Charrier, L. Sedger, C. R. Willis, K. Brasel, P. J. Morrissey, K. Stocking, J. C. L. Schuh, S. Joyce, and J. J. Peschon. 2000. Reversible Defects in Natural Killer and Memory Cd8 T Cell Lineages in Interleukin 15–Deficient Mice. *The Journal of Experimental Medicine* 191: 771-780.
211. Held, W., J. Roland, and D. H. Raulet. 1995. Allelic exclusion of Ly49-family genes encoding class I MHC-specific receptors on NK cells. *Nature* 376: 355-358.
212. Held, W., and B. Kunz. 1998. An allele-specific, stochastic gene expression process controls the expression of multiple Ly49family genes and generates a diverse, MHC-specific NK cell receptor repertoire. *European Journal of Immunology* 28: 2407-2416.
213. Held, W., B. Kunz, V. Ioannidis, and B. Lowin-Kropf. 1999. Mono-allelic Ly49 NK cell receptor expression. *Seminars in Immunology* 11: 349-355.
214. Held, W., B. Kunz, B. Lowin-Kropf, M. van de Wetering, and H. Clevers. 1999. Clonal Acquisition of the Ly49A NK Cell Receptor Is Dependent on the trans-Acting Factor TCF-1. *Immunity* 11: 433-442.
215. Kunz, B., and W. Held. 2001. Positive and Negative Roles of the Trans-Acting T Cell Factor-1 for the Acquisition of Distinct Ly-49 MHC Class I Receptors by NK Cells. *The Journal of Immunology* 166: 6181-6187.
216. Rouhi, A., L. Gagnier, F. Takei, and D. L. Mager. 2006. Evidence for Epigenetic Maintenance of Ly49a Monoallelic Gene Expression. *The Journal of Immunology* 176: 2991-2999.
217. Tanamachi, D. M., T. Hanke, H. Takizawa, A. M. Jamieson, and D. H. Raulet. 2001. Expression of Natural Killer Receptor Alleles at Different Ly49 Loci Occurs Independently and Is Regulated by Major Histocompatibility Complex Class I Molecules. *The Journal of Experimental Medicine* 193: 307-316.

218. Smith, H. R. C., H. H. Chuang, L. L. Wang, M. Salcedo, J. W. Heusel, and W. M. Yokoyama. 2000. Nonstochastic Coexpression of Activation Receptors on Murine Natural Killer Cells. *The Journal of Experimental Medicine* 191: 1341-1354.
219. Held, W., and D. H. Raulet. 1997. Expression of the Ly49A gene in murine natural killer cell clones is predominantly but not exclusively mono-allelic. *European Journal of Immunology* 27: 2876-2884.
220. Gays, F., S. Taha, and C. G. Brooks. 2015. The Distal Upstream Promoter in Ly49 Genes, Pro1, Is Active in Mature NK Cells and T Cells, Does Not Require TATA Boxes, and Displays Enhancer Activity. *The Journal of Immunology* 194: 6068-6081.
221. Held, W., J. R. Dorfman, M.-F. Wu, and D. H. Raulet. 1996. Major histocompatibility complex class I-dependent skewing of the natural killer cell Ly49 receptor reportoire. *European Journal of Immunology* 26: 2286-2292.
222. Salcedo, M., A. D. Diehl, M. Y. Olsson-Alheim, J. Sundbäck, L. Van Kaer, K. Kärre, and H. G. Ljunggren. 1997. Altered expression of Ly49 inhibitory receptors on natural killer cells from MHC class I-deficient mice. *The Journal of Immunology* 158: 3174-3180.
223. Held, W., and D. H. Raulet. 1997. Ly49A Transgenic Mice Provide Evidence for a Major Histocompatibility Complex–dependent Education Process in Natural Killer Cell Development. *The Journal of Experimental Medicine* 185: 2079-2088.
224. Fahlén, L., U. Lendahl, and C. L. Sentman. 2001. MHC Class I-Ly49 Interactions Shape the Ly49 Repertoire on Murine NK Cells. *The Journal of Immunology* 166: 6585-6592.
225. Hanke, T., H. Takizawa, and D. H. Raulet. 2001. MHC-dependent shaping of the inhibitory Ly49 receptor repertoire on NK cells: evidence for a regulated sequential model. *European Journal of Immunology* 31: 3370-3379.
226. Johansson, S., M. Salmon-Divon, M. H. Johansson, Y. Pickman, P. Brodin, K. Kärre, R. Mehr, and P. Höglund. 2009. Probing Natural Killer Cell Education by Ly49 Receptor Expression Analysis and Computational Modelling in Single MHC Class I Mice. *PLoS ONE* 4: e6046.
227. Höglund, P., C. Ohlén, E. Carbone, L. Franksson, H. G. Ljunggren, A. Latour, B. Koller, and K. Kärre. 1991. Recognition of beta 2-microglobulin-negative (beta 2m-) T-cell blasts by natural killer cells from normal but not from beta 2m- mice: nonresponsiveness controlled by beta 2m- bone marrow in chimeric mice. *Proceedings of the National Academy of Sciences* 88: 10332-10336.
228. Bix, M., N.-S. Liao, M. Zijlstra, J. Loring, R. Jaenisch, and D. Raulet. 1991. Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. *Nature* 349: 329-331.
229. Liao, N., M. Bix, M. Zijlstra, R. Jaenisch, and D. Raulet. 1991. MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. *Science* 253: 199-202.
230. Grigoriadou, K., C. Ménard, B. Pérarnau, and F. A. Lemonnier. 1999. MHC class Ia molecules alone control NK-mediated bone marrow graft rejection. *European Journal of Immunology* 29: 3683-3690.
231. Johansson, M. H., C. Bieberich, G. Jay, K. Kärre, and P. Höglund. 1997. Natural Killer Cell Tolerance in Mice with Mosaic Expression of Major Histocompatibility Complex Class I Transgene. *The Journal of Experimental Medicine* 186: 353-364.
232. Brodin, P., K. Kärre, and P. Höglund. 2009. NK cell education: not an on-off switch but a tunable rheostat. *Trends in Immunology* 30: 143-149.

233. Brodin, P., T. Lakshmikanth, S. Johansson, K. Kärre, and P. Höglund. 2009. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. *Blood* 113: 2434-2441.
234. Brodin, P., and P. Höglund. 2008. Beyond licensing and disarming: A quantitative view on NK-cell education. *European Journal of Immunology* 38: 2934-2937.
235. Johansson, S., M. Johansson, E. Rosmaraki, G. Vahlne, R. Mehr, M. Salmon-Divon, F. Lemonnier, K. Kärre, and P. Höglund. 2005. Natural killer cell education in mice with single or multiple major histocompatibility complex class I molecules. *The Journal of Experimental Medicine* 201: 1145-1155.
236. Nakamura, S., K. Kuroki, I. Ohki, K. Sasaki, M. Kajikawa, T. Maruyama, M. Ito, Y. Kameda, M. Ikura, K. Yamamoto, N. Matsumoto, and K. Maenaka. 2009. Molecular Basis for E-cadherin Recognition by Killer Cell Lectin-like Receptor G1 (KLRG1). *Journal of Biological Chemistry* 284: 27327-27335.
237. Li, Y., M. Hofmann, Q. Wang, L. Teng, L. K. Chlewicki, H. Pircher, and R. A. Mariuzza. 2009. Structure of Natural Killer Cell Receptor KLRG1 Bound to E-Cadherin Reveals Basis for MHC-Independent Missing Self Recognition. *Immunity* 31: 35-46.
238. Lee, J. E., S. A. Lim, T.-J. Kim, K. Kim, J. Ng, Y. H. Kim, I. J. Jang, S. B. Oh, J.-C. Lee, C. Yee, V. Kumar, and K.-M. Lee. 2014. NKG2D ligation relieves 2B4-mediated NK-cell self-tolerance in mice. *European Journal of Immunology* 44: 1802-1813.
239. Andrews, D. M., L. C. Sullivan, N. Baschuk, C. J. Chan, R. Berry, C. L. Cotterell, J. Lin, H. Halse, S. V. Watt, J. Poursine-Laurent, C.-R. Wang, A. A. Scalzo, W. M. Yokoyama, J. Rossjohn, A. G. Brooks, and M. J. Smyth. 2012. Recognition of the nonclassical MHC class I molecule H2-M3 by the receptor Ly49A regulates the licensing and activation of NK cells. *Nat Immunol* 13: 1171-1177.
240. Chen, P., O. A. Aguilar, M. M. A. Rahim, D. S. J. Allan, J. H. Fine, C. L. Kirkham, J. Ma, M. Tanaka, M. M. Tu, A. Wight, V. Kartsogiannis, M. T. Gillespie, A. P. Makrigiannis, and J. R. Carlyle. 2015. Genetic Investigation of MHC-Independent Missing-Self Recognition by Mouse NK Cells Using an In Vivo Bone Marrow Transplantation Model. *The Journal of Immunology* 194: 2909-2918.
241. Rahim, M. M. A., P. Chen, A. N. Mottashed, A. B. Mahmoud, M. J. Thomas, Q. Zhu, C. G. Brooks, V. Kartsogiannis, M. T. Gillespie, J. R. Carlyle, and A. P. Makrigiannis. 2015. *The mouse NKR-P1B:Clr-b recognition system is a negative regulator of innate immune responses*.
242. Olsson, M. Y., K. Kärre, and C. L. Sentman. 1995. Altered phenotype and function of natural killer cells expressing the major histocompatibility complex receptor Ly-49 in mice transgenic for its ligand. *Proceedings of the National Academy of Sciences* 92: 1649-1653.
243. Karlhofer, F. M., R. Hunziker, A. Reichlin, D. H. Margulies, and W. M. Yokoyama. 1994. Host MHC class I molecules modulate in vivo expression of a NK cell receptor. *The Journal of Immunology* 153: 2407-2416.
244. Kåse, A., M. H. Johansson, M. Y. Olsson-Alheim, K. Kärre, and P. Höglund. 1998. External and Internal Calibration of the MHC Class I-Specific Receptor Ly49A on Murine Natural Killer Cells. *The Journal of Immunology* 161: 6133-6138.
245. Sjöström, A., M. Eriksson, C. Cerboni, M. H. Johansson, C. L. Sentman, K. Kärre, and P. Höglund. 2001. Acquisition of External Major Histocompatibility Complex Class I Molecules by Natural Killer Cells Expressing Inhibitory Ly49 Receptors. *The Journal of Experimental Medicine* 194: 1519-1530.
246. Zimmer, J., V. Ioannidis, and W. Held. 2001. H-2D Ligand Expression by Ly49A+ Natural Killer (NK) Cells Precludes Ligand Uptake from Environmental Cells:

Implications for NK Cell Function. *The Journal of Experimental Medicine* 194: 1531-1539.
247. Doucey, M.-A., L. Scarpellino, J. Zimmer, P. Guillaume, I. F. Luescher, C. Bron, and W. Held. 2004. Cis association of Ly49A with MHC class I restricts natural killer cell inhibition. *Nat Immunol* 5: 328-336.
248. Andersson, K. E., G. S. Williams, D. M. Davis, and P. Höglund. 2007. Quantifying the reduction in accessibility of the inhibitory NK cell receptor Ly49A caused by binding MHC class I proteins in cis. *European Journal of Immunology* 37: 516-527.
249. Chalifour, A., L. Scarpellino, J. Back, P. Brodin, E. Devèvre, F. Gros, F. Lévy, G. Leclercq, P. Höglund, F. Beermann, and W. Held. 2009. A Role for cis Interaction between the Inhibitory Ly49A Receptor and MHC Class I for Natural Killer Cell Education. *Immunity* 30: 337-347.
250. Ebihara, T., A. H. Jonsson, and W. M. Yokoyama. 2013. Natural killer cell licensing in mice with inducible expression of MHC class I. *Proceedings of the National Academy of Sciences* 110: E4232-E4237.
251. Bessoles, S., G. S. Angelov, J. Back, G. Leclercq, E. Vivier, and W. Held. 2013. Education of Murine NK Cells Requires Both cis and trans Recognition of MHC Class I Molecules. *The Journal of Immunology* 191: 5044-5051.
252. O'Leary, J. G., M. Goodarzi, D. L. Drayton, and U. H. von Andrian. 2006. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat Immunol* 7: 507-516.
253. Smith, H. R. C., J. W. Heusel, I. K. Mehta, S. Kim, B. G. Dorner, O. V. Naidenko, K. Iizuka, H. Furukawa, D. L. Beckman, J. T. Pingel, A. A. Scalzo, D. H. Fremont, and W. M. Yokoyama. 2002. Recognition of a virus-encoded ligand by a natural killer cell activation receptor. *Proceedings of the National Academy of Sciences* 99: 8826-8831.
254. Lee, S.-H., S. Girard, D. Macina, M. Busa, A. Zafer, A. Belouchi, P. Gros, and S. M. Vidal. 2001. Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. *Nat Genet* 28: 42-45.
255. Sjölin, H., E. Tomasello, M. Mousavi-Jazi, A. Bartolazzi, K. Kärre, E. Vivier, and C. Cerboni. 2002. Pivotal Role of KARAP/DAP12 Adaptor Molecule in the Natural Killer Cell–mediated Resistance to Murine Cytomegalovirus Infection. *The Journal of Experimental Medicine* 195: 825-834.
256. Dokun, A. O., S. Kim, H. R. C. Smith, H.-S. P. Kang, D. T. Chu, and W. M. Yokoyama. 2001. Specific and nonspecific NK cell activation during virus infection. *Nat Immunol* 2: 951-956.
257. Sun, J. C., J. N. Beilke, and L. L. Lanier. 2009. Adaptive immune features of natural killer cells. *Nature* 457: 557-561.
258. Orr, M. T., W. J. Murphy, and L. L. Lanier. 2010. 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nat Immunol* 11: 321-327.
259. Jamieson, A. M., P. Isnard, J. R. Dorfman, M. C. Coles, and D. H. Raulet. 2004. Turnover and Proliferation of NK Cells in Steady State and Lymphopenic Conditions. *The Journal of Immunology* 172: 864-870.
260. Cooper, M. A., J. M. Elliott, P. A. Keyel, L. Yang, J. A. Carrero, and W. M. Yokoyama. 2009. Cytokine-induced memory-like natural killer cells. *Proceedings of the National Academy of Sciences* 106: 1915-1919.
261. Cooper, M. A., and W. M. Yokoyama. 2010. Memory-like responses of natural killer cells. *Immunological Reviews* 235: 297-305.

262. Gumá, M., A. Angulo, C. Vilches, N. Gómez-Lozano, N. Malats, and M. López-Botet. 2004. *Imprint of human cytomegalovirus infection on the NK cell receptor repertoire*.
263. Béziat, V., L. L. Liu, J.-A. Malmberg, M. A. Ivarsson, E. Sohlberg, A. T. Björklund, C. Retière, E. Sverremark-Ekström, J. Traherne, P. Ljungman, M. Schaffer, D. A. Price, J. Trowsdale, J. Michaëlsson, H.-G. Ljunggren, and K.-J. Malmberg. 2013. NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs. *Blood* 121: 2678-2688.
264. Béziat, V., O. Dalgard, T. Asselah, P. Halfon, P. Bedossa, A. Boudifa, B. Hervier, I. Theodorou, M. Martinot, P. Debré, N. K. Björkström, K.-J. Malmberg, P. Marcellin, and V. Vieillard. 2012. CMV drives clonal expansion of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. *European Journal of Immunology* 42: 447-457.
265. Foley, B., S. Cooley, M. R. Verneris, M. Pitt, J. Curtsinger, X. Luo, S. Lopez-Vergès, L. L. Lanier, D. Weisdorf, and J. S. Miller. 2012. *Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function*.
266. Schlums, H., F. Cichocki, B. Tesi, J. Theorell, V. Beziat, Tim D. Holmes, H. Han, Samuel C. C. Chiang, B. Foley, K. Mattsson, S. Larsson, M. Schaffer, K.-J. Malmberg, H.-G. Ljunggren, Jeffrey S. Miller, and Yenan T. Bryceson. 2015. Cytomegalovirus Infection Drives Adaptive Epigenetic Diversification of NK Cells with Altered Signaling and Effector Function. *Immunity* 42: 443-456.
267. Thomas, L. 1959. Cellular and Humoral Aspects of Hypersensitivity.
268. Burnet, F. 1957. Cancer - A biological approach. 1. The process of control. *Br Med J* 1: 779 - 782.
269. Kärre, K., G. O. Klein, R. Kiessling, G. Klein, and J. C. Roder. 1980. In vitro NK-activity and in vivo resistance to leukemia: Studies of beige, beige/nude and wild-type hosts on C57BL background. *International Journal of Cancer* 26: 789-797.
270. Talmadge, J. 1980. Role of NK cells in tumour growth and metastasis in beige mice. *Nature* 284: 622 - 624.
271. Haliotis, T. 1985. Spontaneous and induced primary oncogenesis in natural killer (NK)-cell-deficient beige mutant mice. *Int J Cancer* 35: 505 - 513.
272. van den Broek, M. F., D. Kägi, R. M. Zinkernagel, and H. Hengartner. 1995. Perforin dependence of natural killer cell-mediated tumor control in vivo. *European Journal of Immunology* 25: 3514-3516.
273. Smyth, M. J., K. Y. T. Thia, E. Cretney, J. M. Kelly, M. B. Snook, C. A. Forbes, and A. A. Scalzo. 1999. Perforin Is a Major Contributor to NK Cell Control of Tumor Metastasis. *The Journal of Immunology* 162: 6658-6662.
274. Kim, S., K. Iizuka, H. L. Aguila, I. L. Weissman, and W. M. Yokoyama. 2000. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. *Proceedings of the National Academy of Sciences* 97: 2731-2736.
275. Okumura, K., S. Habu, and M. Kasai. 1982. The Role of NK Cells in Resistance of In Vivo Tumors. In *Macrophages and Natural Killer Cells*. S. Normann, and E. Sorkin, eds. Springer US. 773-784.
276. Tu, M. M., A. B. Mahmoud, A. Wight, A. Mottashed, S. Bélanger, M. M. A. Rahim, E. Abou-Samra, and A. P. Makrigiannis. 2014. Ly49 Family Receptors Are Required for Cancer Immunosurveillance Mediated by Natural Killer Cells. *Cancer Research* 74: 3684-3694.
277. Rosenberg, S. A., M. T. Lotze, J. C. Yang, S. L. Topalian, A. E. Chang, D. J. Schwartzentruber, P. Aebersold, S. Leitman, W. M. Linehan, C. A. Seipp, D. E. White,

and S. M. Steinberg. 1993. Prospective Randomized Trial of High-Dose Interleukin-2 Alone or in Conjunction With Lymphokine-Activated Killer Cells for the Treatment of Patients With Advanced Cancer. *Journal of the National Cancer Institute* 85: 622-632.

278. Levy, E. M., M. P. Roberti, and J. Mordoh. 2011. Natural Killer Cells in Human Cancer: From Biological Functions to Clinical Applications. *Journal of Biomedicine and Biotechnology* 2011: 676198.
279. Luevano, M., A. Madrigal, and A. Saudemont. 2012. Generation of natural killer cells from hematopoietic stem cells in vitro for immunotherapy. *Cellular and Molecular Immunology* 9: 310-320.
280. Tonn, T., S. Becker, R. Esser, D. Schwabe, and E. Seifried. 2001. Cellular Immunotherapy of Malignancies Using the Clonal Natural Killer Cell Line NK-92. *Journal of Hematotherapy & Stem Cell Research* 10: 535-544.
281. Tonn, T., D. Schwabe, H. G. Klingemann, S. Becker, R. Esser, U. Koehl, M. Suttorp, E. Seifried, O. G. Ottmann, and G. Bug. 2013. Treatment of patients with advanced cancer with the natural killer cell line NK-92. *Cytotherapy* 15: 1563-1570.
282. Arai, S., R. Meagher, M. Swearingen, H. Myint, E. Rich, J. Martinson, and H. Klingemann. 2008. Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial. *Cytotherapy* 10: 625-632.
283. Ruggeri, L., A. Mancusi, M. Capanni, E. Urbani, A. Carotti, T. Aloisi, M. Stern, D. Pende, K. Perruccio, E. Burchielli, F. Topini, E. Bianchi, F. Aversa, M. F. Martelli, and A. Velardi. 2007. *Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value*.
284. Stern, M., L. Ruggeri, A. Mancusi, M. E. Bernardo, C. de Angelis, C. Bucher, F. Locatelli, F. Aversa, and A. Velardi. 2008. *Survival after T cell–depleted haploidentical stem cell transplantation is improved using the mother as donor*.
285. Pende, D., S. Marcenaro, M. Falco, S. Martini, M. E. Bernardo, D. Montagna, E. Romeo, C. Cognet, M. Martinetti, R. Maccario, M. C. Mingari, E. Vivier, L. Moretta, F. Locatelli, and A. Moretta. 2009. *Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and redefinition of inhibitory KIR specificity*.
286. Mancusi, A., L. Ruggeri, E. Urbani, A. Pierini, M. S. Massei, A. Carotti, A. Terenzi, F. Falzetti, A. Tosti, F. Topini, S. Bozza, L. Romani, R. Tognellini, M. Stern, F. Aversa, M. F. Martelli, and A. Velardi. 2015. *Haploidentical hematopoietic transplantation from KIR ligand–mismatched donors with activating KIRs reduces nonrelapse mortality*.
287. Foley, B., D. De Santis, L. Lathbury, F. Christiansen, and C. Witt. 2008. KIR2DS1-mediated activation overrides NKG2A-mediated inhibition in HLA-C C2-negative individuals. *International Immunology* 20: 555-563.
288. Giebel, S., F. Locatelli, T. Lamparelli, A. Velardi, S. Davies, G. Frumento, R. Maccario, F. Bonetti, J. Wojnar, M. Martinetti, F. Frassoni, G. Giorgiani, A. Bacigalupo, and J. Holowiecki. 2003. *Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors*.
289. Davies, S. M., L. Ruggieri, T. DeFor, J. E. Wagner, D. J. Weisdorf, J. S. Miller, A. Velardi, and B. R. Blazar. 2002. *Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants*.
290. Farag, S. S., A. Bacigalupo, M. Eapen, C. Hurley, B. Dupont, M. A. Caligiuri, C. Boudreau, G. Nelson, M. Oudshoorn, J. van Rood, A. Velardi, M. Maiers, M. Setterholm, D. Confer, P. E. Posch, C. Anasetti, N. Kamani, J. S. Miller, D. Weisdorf, and S. M. Davies. 2006. The Effect of KIR Ligand Incompatibility on the Outcome of

Unrelated Donor Transplantation: A Report from the Center for International Blood and Marrow Transplant Research, the European Blood and Marrow Transplant Registry, and the Dutch Registry. *Biology of Blood and Marrow Transplantation* 12: 876-884.

291. Willemze, R., C. A. Rodrigues, M. Labopin, G. Sanz, G. Michel, G. Socie, B. Rio, A. Sirvent, M. Renaud, L. Madero, M. Mohty, C. Ferra, F. Garnier, P. Loiseau, J. Garcia, L. Lecchi, G. Kogler, Y. Beguin, C. Navarrete, T. Devos, I. Ionescu, K. Boudjedir, A. L. Herr, E. Gluckman, and V. Rocha. 2009. KIR-ligand incompatibility in the graft-versus-host direction improves outcomes after umbilical cord blood transplantation for acute leukemia. *Leukemia* 23: 492-500.
292. Brunstein, C. G., J. E. Wagner, D. J. Weisdorf, S. Cooley, H. Noreen, J. N. Barker, T. DeFor, M. R. Verneris, B. R. Blazar, and J. S. Miller. 2009. *Negative effect of KIR alloreactivity in recipients of umbilical cord blood transplant depends on transplantation conditioning intensity*.
293. Yu, J., J. M. Venstrom, X.-R. Liu, J. Pring, R. S. Hasan, R. J. O'Reilly, and K. C. Hsu. 2009. Breaking tolerance to self, circulating natural killer cells expressing inhibitory KIR for non-self HLA exhibit effector function after T cell–depleted allogeneic hematopoietic cell transplantation. *Blood* 113: 3875-3884.
294. Björklund, A. T., M. Schaffer, C. Fauriat, O. Ringdén, M. Remberger, C. Hammarstedt, A. J. Barrett, P. Ljungman, H.-G. Ljunggren, and K.-J. Malmberg. 2010. *NK cells expressing inhibitory KIR for non–self-ligands remain tolerant in HLA-matched sibling stem cell transplantation*.
295. Haas, P., P. Loiseau, R. Tamouza, J.-M. Cayuela, H. Moins-Teisserenc, M. Busson, G. Henry, C. S. Falk, D. Charron, G. Socié, A. Toubert, and N. Dulphy. 2011. NK-cell education is shaped by donor HLA genotype after unrelated allogeneic hematopoietic stem cell transplantation. *Blood* 117: 1021-1029.
296. Pende, D., G. M. Spaggiari, S. Marcenaro, S. Martini, P. Rivera, A. Capobianco, M. Falco, E. Lanino, I. Pierri, R. Zambello, A. Bacigalupo, M. C. Mingari, A. Moretta, and L. Moretta. 2005. *Analysis of the receptor-ligand interactions in the natural killer–mediated lysis of freshly isolated myeloid or lymphoblastic leukemias: evidence for the involvement of the Poliovirus receptor (CD155) and Nectin-2 (CD112)*.
297. Burns, L. J., D. J. Weisdorf, T. E. DeFor, D. H. Vesole, T. L. Repka, B. R. Blazar, S. R. Burger, A. Panoskaltsis-Mortari, C. A. Keever-Taylor, M. J. Zhang, and J. S. Miller. 0000. IL-2-based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: a phase I//II trial. *Bone Marrow Transplant* 32: 177-186.
298. Raulet, D. H., and W. Held. 1995. Natural killer cell receptors: The offs and ons of NK cell recognition. *Cell* 82: 697-700.
299. Karre, K. 1995. Express yourself or die: peptides, MHC molecules, and NK cells. *Science* 267: 978-979.
300. Miller, J. S., Y. Soignier, A. Panoskaltsis-Mortari, S. A. McNearney, G. H. Yun, S. K. Fautsch, D. McKenna, C. Le, T. E. Defor, L. J. Burns, P. J. Orchard, B. R. Blazar, J. E. Wagner, A. Slungaard, D. J. Weisdorf, I. J. Okazaki, and P. B. McGlave. 2005. *Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer*.
301. Curti, A., L. Ruggeri, A. D'Addio, A. Bontadini, E. Dan, M. R. Motta, S. Trabanelli, V. Giudice, E. Urbani, G. Martinelli, S. Paolini, F. Fruet, A. Isidori, S. Parisi, G. Bandini, M. Baccarani, A. Velardi, and R. M. Lemoli. 2011. *Successful transfer of alloreactive*

*haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute myeloid leukemia patients*.

302. Romee, R., J. W. Leong, and T. A. Fehniger. 2014. Utilizing Cytokines to Function-Enable Human NK Cells for the Immunotherapy of Cancer. *Scientifica* 2014: 18.
303. Tam, Y. K., G. Maki, B. Miyagawa, B. Hennemann, T. Tonn, and H. G. Klingemann. 1999. Characterization of Genetically Altered, Interleukin 2-Independent Natural Killer Cell Lines Suitable for Adoptive Cellular Immunotherapy. *Human Gene Therapy* 10: 1359-1373.
304. Müller, T., C. Uherek, G. Maki, K. Chow, A. Schimpf, H.-G. Klingemann, T. Tonn, and W. Wels. 2008. Expression of a CD20-specific chimeric antigen receptor enhances cytotoxic activity of NK cells and overcomes NK-resistance of lymphoma and leukemia cells. *Cancer Immunol Immunother* 57: 411-423.
305. Imai, C., S. Iwamoto, and D. Campana. 2005. *Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells*.
306. Wennerberg, E., D. Sarhan, M. Carlsten, V. O. Kaminskyy, P. D'Arcy, B. Zhivotovsky, R. Childs, and A. Lundqvist. 2013. Doxorubicin sensitizes human tumor cells to NK cell- and T-cell-mediated killing by augmented TRAIL receptor signaling. *International Journal of Cancer*: n/a-n/a.
307. Champsaur, M., and L. L. Lanier. 2010. Effect of NKG2D ligand expression on host immune responses. *Immunological Reviews* 235: 267-285.
308. Hervieu, A., C. Rebe, F. Vegran, F. Chalmin, M. Bruchard, P. Vabres, L. Apetoh, F. Ghiringhelli, and G. Mignot. 2013. Dacarbazine-Mediated Upregulation of NKG2D Ligands on Tumor Cells Activates NK and CD8 T Cells and Restrains Melanoma Growth. *J Invest Dermatol* 133: 499-508.
309. Khallouf, H., A. Marten, S. Serba, V. Teichgraber, M. W. Buchler, D. Jager, and J. Schmidt. 2012. 5-Fluorouracil and Interferon-[alpha] Immunochemotherapy Enhances Immunogenicity of Murine Pancreatic Cancer Through Upregulation of NKG2D Ligands and MHC Class I. *Journal of Immunotherapy* 35: 245-253.
310. Martner, A., F. B. Thorén, J. Aurelius, J. Söderholm, M. Brune, and K. Hellstrand. 2010. Immunotherapy with histamine dihydrochloride for the prevention of relapse in acute myeloid leukemia. *Expert Review of Hematology* 3: 381-391.
311. Brune, M., S. Castaigne, J. Catalano, K. Gehlsen, A. D. Ho, W.-K. Hofmann, D. E. Hogge, B. Nilsson, R. Or, A. I. Romero, J. M. Rowe, B. Simonsson, R. Spearing, E. A. Stadtmauer, J. Szer, E. Wallhult, and K. Hellstrand. 2006. *Improved leukemia-free survival after postconsolidation immunotherapy with histamine dihydrochloride and interleukin-2 in acute myeloid leukemia: results of a randomized phase 3 trial*.
312. Martner, A., F. B. Thorén, J. Aurelius, and K. Hellstrand. 2013. Immunotherapeutic strategies for relapse control in acute myeloid leukemia. *Blood Reviews* 27: 209-216.
313. Aurelius, J., A. Martner, M. Brune, L. Palmqvist, M. Hansson, K. Hellstrand, and F. B. Thoren. 2012. Remission maintenance in acute myeloid leukemia: impact of functional histamine H(2) receptors expressed by leukemic cells. *Haematologica* 97: 1904-1908.
314. Brune, M., M. Hansson, U. H. Mellqvist, S. Hermodsson, and K. Hellstrand. 1996. NK cell-mediated killing of AML blasts: role of histamine, monocytes and reactive oxygen metabolites. *European Journal of Haematology* 57: 312-319.
315. Mellqvist, U.-H., M. Hansson, M. Brune, C. Dahlgren, S. Hermodsson, and K. Hellstrand. 2000. *Natural killer cell dysfunction and apoptosis induced by chronic myelogenous leukemia cells: role of reactive oxygen species and regulation by histamine*.

316. Hamaguchi, Y., Y. Xiu, K. Komura, F. Nimmerjahn, and T. F. Tedder. 2006. Antibody isotype-specific engagement of Fcγ receptors regulates B lymphocyte depletion during CD20 immunotherapy. *The Journal of Experimental Medicine* 203: 743-753.
317. Minard-Colin, V., Y. Xiu, J. C. Poe, M. Horikawa, C. M. Magro, Y. Hamaguchi, K. M. Haas, and T. F. Tedder. 2008. *Lymphoma depletion during CD20 immunotherapy in mice is mediated by macrophage FcγRI, FcγRIII, and FcγRIV*.
318. Takai, T., M. Li, D. Sylvestre, R. Clynes, and J. V. Ravetch. 1994. FcR γ chain deletion results in pleiotrophic effector cell defects. *Cell* 76: 519-529.
319. Clynes, R., Y. Takechi, Y. Moroi, A. Houghton, and J. V. Ravetch. 1998. Fc receptors are required in passive and active immunity to melanoma. *Proceedings of the National Academy of Sciences* 95: 652-656.
320. Clynes, R. A., T. L. Towers, L. G. Presta, and J. V. Ravetch. 2000. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. *Nat Med* 6: 443-446.
321. Busfield, S. J., M. Biondo, M. Wong, H. S. Ramshaw, E. M. Lee, S. Ghosh, H. Braley, C. Panousis, A. W. Roberts, S. Z. He, D. Thomas, L. Fabri, G. Vairo, R. B. Lock, A. F. Lopez, and A. D. Nash. 2014. Targeting of acute myeloid leukemia in vitro and in vivo with an anti-CD123 mAb engineered for optimal ADCC. *Leukemia* 28: 2213-2221.
322. Koh, C. Y., B. R. Blazar, T. George, L. A. Welniak, C. M. Capitini, A. Raziuddin, W. J. Murphy, and M. Bennett. 2001. Augmentation of antitumor effects by NK cell inhibitory receptor blockade in vitro and in vivo. *Blood* 97: 3132-3137.
323. Koh, C. Y., A. Raziuddin, L. A. Welniak, B. R. Blazar, M. Bennett, and W. J. Murphy. 2002. NK inhibitory-receptor blockade for purging of leukemia: Effects on hematopoietic reconstitution. *Biology of Blood and Marrow Transplantation* 8: 17-25.
324. Koh, C. Y., J. R. Ortaldo, B. R. Blazar, M. Bennett, and W. J. Murphy. 2003. NK-cell purging of leukemia: superior antitumor effects of NK cells H2 allogeneic to the tumor and augmentation with inhibitory receptor blockade. *Blood* 102: 4067-4075.
325. Pardoll, D. M. 2012. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 12: 252-264.
326. Hamid, O., C. Robert, A. Daud, F. S. Hodi, W.-J. Hwu, R. Kefford, J. D. Wolchok, P. Hersey, R. W. Joseph, J. S. Weber, R. Dronca, T. C. Gangadhar, A. Patnaik, H. Zarour, A. M. Joshua, K. Gergich, J. Elassaiss-Schaap, A. Algazi, C. Mateus, P. Boasberg, P. C. Tumeh, B. Chmielowski, S. W. Ebbinghaus, X. N. Li, S. P. Kang, and A. Ribas. 2013. Safety and Tumor Responses with Lambrolizumab (Anti–PD-1) in Melanoma. *The New England journal of medicine* 369: 134-144.
327. Buckley, R. H., R. I. Schiff, S. E. Schiff, M. L. Markert, L. W. Williams, T. O. Harville, J. L. Roberts, and J. M. Puck. 1997. Human severe combined immunodeficiency: Genetic, phenotypic, and functional diversity in one hundred eight infants. *The Journal of Pediatrics* 130: 378-387.
328. Janka, G. E., and K. Lehmberg. 2014. Hemophagocytic syndromes — An update. *Blood Reviews* 28: 135-142.
329. Russell, J. H., and T. J. Ley. 2002. LYMPHOCYTE-MEDIATED CYTOTOXICITY. *Annual Review of Immunology* 20: 323-370.
330. Cho, B. K., C. Wang, S. Sugawa, H. N. Eisen, and J. Chen. 1999. Functional differences between memory and naive CD8 T cells. *Proceedings of the National Academy of Sciences of the United States of America* 96: 2976-2981.
331. Göransdotter Ericson, K., B. Fadeel, S. Nilsson-Ardnor, C. Söderhäll, A. Samuelsson, G. Janka, M. Schneider, A. Gürgey, N. Yalman, T. Révész, R. M. Egeler, K. Jahnukainen, I. Storm-Mathiesen, Á. Haraldsson, J. Poole, G. de Saint Basile, M.

Nordenskjöld, and J.-I. Henter. 2001. Spectrum of Perforin Gene Mutations in Familial Hemophagocytic Lymphohistiocytosis. *American Journal of Human Genetics* 68: 590-597.
332. Stepp, S. E., R. Dufourcq-Lagelouse, F. L. Deist, S. Bhawan, S. Certain, P. A. Mathew, J.-I. Henter, M. Bennett, A. Fischer, G. d. S. Basile, and V. Kumar. 1999. Perforin Gene Defects in Familial Hemophagocytic Lymphohistiocytosis. *Science* 286: 1957-1959.
333. Katano, H., M. A. Ali, A. C. Patera, M. Catalfamo, E. S. Jaffe, H. Kimura, J. K. Dale, S. E. Straus, and J. I. Cohen. 2004. *Chronic active Epstein-Barr virus infection associated with mutations in perforin that impair its maturation*.
334. Clementi, R., F. Locatelli, L. Dupré, A. Garaventa, L. Emmi, M. Bregni, G. Cefalo, A. Moretta, C. Danesino, M. Comis, A. Pession, U. Ramenghi, R. Maccario, M. Aricò, and M. G. Roncarolo. 2005. *A proportion of patients with lymphoma may harbor mutations of the perforin gene*.
335. Risma, K. A., R. W. Frayer, A. H. Filipovich, and J. Sumegi. 2006. Aberrant maturation of mutant perforin underlies the clinical diversity of hemophagocytic lymphohistiocytosis. *The Journal of Clinical Investigation* 116: 182-192.
336. Orange, J. S. 2013. Natural killer cell deficiency. *Journal of Allergy and Clinical Immunology* 132: 515-525.
337. Orange, J. 2002. Human natural killer cell deficiencies and susceptibility to infection. *Microbes Infect* 4: 1545 - 1558.
338. Wood, S., H.-G. Ljunggren, and Y. Bryceson. 2011. Insights into NK cell biology from human genetics and disease associations. *Cell. Mol. Life Sci.* 68: 3479-3493.
339. Gilmour, K. C., H. Fujii, T. Cranston, E. G. Davies, C. Kinnon, and H. B. Gaspar. 2001. *Defective expression of the interleukin-2/interleukin-15 receptor β subunit leads to a natural killer cell–deficient form of severe combined immunodeficiency*.
340. Bélanger, S., M. M. Tu, M. M. A. Rahim, A. B. Mahmoud, R. Patel, L.-H. Tai, A. D. Troke, B. T. Wilhelm, J.-R. Landry, Q. Zhu, K. S. Tung, D. H. Raulet, and A. P. Makrigiannis. 2012. Impaired natural killer cell self-education and "missing-self" responses in Ly49-deficient mice. *Blood* 120: 592-602.
341. Kim, S., J. Poursine-Laurent, S. Truscott, L. Lybarger, Y. Song, L. Yang, A. French, J. Sunwoo, S. Lemieux, T. Hansen, and W. Yokoyama. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 436: 709 - 713.
342. Lowin-Kropf, B., B. Kunz, F. Beermann, and W. Held. 2000. Impaired Natural Killing of MHC Class I-Deficient Targets by NK Cells Expressing a Catalytically Inactive Form of SHP-1. *The Journal of Immunology* 165: 1314-1321.
343. Viant, C., A. Fenis, G. Chicanne, B. Payrastre, S. Ugolini, and E. Vivier. 2014. SHP-1-mediated inhibitory signals promote responsiveness and anti-tumour functions of natural killer cells. *Nat Commun* 5.
344. Salcedo, M., M. Andersson, S. Lemieux, L. Van Kaer, B. J. Chambers, and H.-G. Ljunggren. 1998. Fine tuning of natural killer cell specificity and maintenance of self tolerance in MHC class I-deficient mice. *European Journal of Immunology* 28: 1315-1321.
345. Gismondi, A., L. Cifaldi, C. Mazza, S. Giliani, S. Parolini, S. Morrone, J. Jacobelli, E. Bandiera, L. Notarangelo, and A. Santoni. 2004. *Impaired natural and CD16-mediated NK cell cytotoxicity in patients with WAS and XLT: ability of IL-2 to correct NK cell functional defect*.
346. Cerwenka, A., J. L. Baron, and L. L. Lanier. 2001. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a

MHC class I-bearing tumor in vivo. *Proceedings of the National Academy of Sciences* 98: 11521-11526.
347. Tomasello, E., P.-O. Desmoulins, K. Chemin, S. Guia, H. Cremer, J. Ortaldo, P. Love, D. Kaiserlian, and E. Vivier. 2000. Combined Natural Killer Cell and Dendritic Cell Functional Deficiency in KARAP/DAP12 Loss-of-Function Mutant Mice. *Immunity* 13: 355-364.
348. Öberg, L., S. Johansson, J. Michaëlsson, E. Tomasello, E. Vivier, K. Kärre, and P. Höglund. 2004. Loss or mismatch of MHC class I is sufficient to trigger NK cell-mediated rejection of resting lymphocytes in vivo – role of KARAP/DAP12-dependent and -independent pathways. *European Journal of Immunology* 34: 1646-1653.
349. Coudert, J. D., L. Scarpellino, F. Gros, E. Vivier, and W. Held. 2008. Sustained NKG2D engagement induces cross-tolerance of multiple distinct NK cell activation pathways. *Blood* 111: 3571-3578.
350. Oppenheim, D. E., S. J. Roberts, S. L. Clarke, R. Filler, J. M. Lewis, R. E. Tigelaar, M. Girardi, and A. C. Hayday. 2005. Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. *Nat Immunol* 6: 928-937.
351. Zafirova, B., S. Mandarić, R. Antulov, A. Krmpotić, H. Jonsson, W. M. Yokoyama, S. Jonjić, and B. Polić. 2009. Altered NK Cell Development and Enhanced NK Cell-Mediated Resistance to Mouse Cytomegalovirus in NKG2D-Deficient Mice. *Immunity* 31: 270-282.
352. Bolanos, F. D., and S. K. Tripathy. 2011. Activation Receptor-Induced Tolerance of Mature NK Cells In Vivo Requires Signaling through the Receptor and Is Reversible. *The Journal of Immunology* 186: 2765-2771.
353. Narni-Mancinelli, E., B. N. Jaeger, C. Bernat, A. Fenis, S. Kung, A. De Gassart, S. Mahmood, M. Gut, S. C. Heath, J. Estellé, E. Bertosio, F. Vely, L. N. Gastinel, B. Beutler, B. Malissen, M. Malissen, I. G. Gut, E. Vivier, and S. Ugolini. 2012. Tuning of Natural Killer Cell Reactivity by NKp46 and Helios Calibrates T Cell Responses. *Science* 335: 344-348.
354. Sheppard, S., C. Triulzi, M. Ardolino, D. Serna, L. Zhang, D. H. Raulet, and N. Guerra. 2013. Characterization of a novel NKG2D and NKp46 double-mutant mouse reveals subtle variations in the NK cell repertoire. *Blood* 121: 5025-5033.
355. Tassi, I., R. Presti, S. Kim, W. M. Yokoyama, S. Gilfillan, and M. Colonna. 2005. Phospholipase C-γ2 Is a Critical Signaling Mediator for Murine NK Cell Activating Receptors. *The Journal of Immunology* 175: 749-754.
356. Regunathan, J., Y. Chen, S. Kutlesa, X. Dai, L. Bai, R. Wen, D. Wang, and S. Malarkannan. 2006. Differential and Nonredundant Roles of Phospholipase Cγ2 and Phospholipase Cγ1 in the Terminal Maturation of NK Cells. *The Journal of Immunology* 177: 5365-5376.
357. Caraux, A., N. Kim, S. E. Bell, S. Zompi, T. Ranson, S. Lesjean-Pottier, M. E. Garcia-Ojeda, M. Turner, and F. Colucci. 2006. Phospholipase C-γ2 is essential for NK cell cytotoxicity and innate immunity to malignant and virally infected cells. *Blood* 107: 994-1002.
358. Bell, S. E., E. Vigorito, S. McAdam, H. M. Reynolds, A. Caraux, F. Colucci, and M. Turner. 2004. PLCγ2 regulates Bcl-2 levels and is required for survival rather than differentiation of marginal zone and follicular B cells. *European Journal of Immunology* 34: 2237-2247.

359. Hashimoto, A., K. Takeda, M. Inaba, M. Sekimata, T. Kaisho, S. Ikehara, Y. Homma, S. Akira, and T. Kurosaki. 2000. Cutting Edge: Essential Role of Phospholipase C-γ2 in B Cell Development and Function. *The Journal of Immunology* 165: 1738-1742.
360. Enoksson, S. L., E. K. Grasset, T. Hägglöf, N. Mattsson, Y. Kaiser, S. Gabrielsson, T. L. McGaha, A. Scheynius, and M. C. I. Karlsson. 2011. The inflammatory cytokine IL-18 induces self-reactive innate antibody responses regulated by natural killer T cells. *Proceedings of the National Academy of Sciences* 108: E1399–E1407.
361. Bessoles, S., C. Grandclément, E. Alari-Pahissa, J. Gehrig, B. Jeevan-Raj, and W. Held. 2014. ADAPTATIONS OF NATURAL KILLER CELLS TO SELF-MHC CLASS I. *Frontiers in Immunology* 5.
362. Elliott, J. M., J. A. Wahle, and W. M. Yokoyama. 2010. MHC class I–deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I–sufficient environment. *The Journal of Experimental Medicine* 207: 2073-2079.
363. Joncker, N. T., N. Shifrin, F. Delebecque, and D. H. Raulet. 2010. Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment. *The Journal of Experimental Medicine* 207: 2065-2072.
364. Coudert, J. D., J. Zimmer, E. Tomasello, M. Cebecauer, M. Colonna, E. Vivier, and W. Held. 2005. Altered NKG2D function in NK cells induced by chronic exposure to NKG2D ligand–expressing tumor cells. *Blood* 106: 1711-1717.
365. Hayakawa, Y., S. V. Watt, K. Takeda, and M. J. Smyth. 2008. Distinct receptor repertoire formation in mouse NK cell subsets regulated by MHC class I expression. *Journal of Leukocyte Biology* 83: 106-111.
366. Höglund, P., J. Sundbäck, M. Y. Olsson-Alheim, M. Johansson, M. Salcedo, C. Öhién, H.-G. Ljunggren, C. L. Sentman, and K. Kärre. 1997. Host MHC class I gene control of NK-cell specificity in the mouse. *Immunological Reviews* 155: 11-28.
367. Romagné, F., P. André, P. Spee, S. Zahn, N. Anfossi, L. Gauthier, M. Capanni, L. Ruggeri, D. M. Benson, B. W. Blaser, M. Della Chiesa, A. Moretta, E. Vivier, M. A. Caligiuri, A. Velardi, and N. Wagtmann. 2009. *Preclinical characterization of 1-7F9, a novel human anti–KIR receptor therapeutic antibody that augments natural killer–mediated killing of tumor cells*.
368. Sola, C., P. André, C. Lemmers, N. Fuseri, C. Bonnafous, M. Bléry, N. R. Wagtmann, F. Romagné, E. Vivier, and S. Ugolini. 2009. Genetic and antibody-mediated reprogramming of natural killer cell missing-self recognition in vivo. *Proceedings of the National Academy of Sciences* 106: 12879-12884.
369. Vey, N., J.-H. Bourhis, N. Boissel, D. Bordessoule, T. Prebet, A. Charbonnier, A. Etienne, P. Andre, F. Romagne, D. Benson, H. Dombret, and D. Olive. 2012. A phase 1 trial of the anti-inhibitory KIR mAb IPH2101 for AML in complete remission. *Blood* 120: 4317-4323.
370. Benson, D. M., C. E. Bakan, S. Zhang, S. M. Collins, J. Liang, S. Srivastava, C. C. Hofmeister, Y. Efebera, P. Andre, F. Romagne, M. Bléry, C. Bonnafous, J. Zhang, D. Clever, M. A. Caligiuri, and S. S. Farag. 2011. IPH2101, a novel anti-inhibitory KIR antibody, and lenalidomide combine to enhance the natural killer cell versus multiple myeloma effect. *Blood* 118: 6387-6391.
371. Benson, D. M., C. C. Hofmeister, S. Padmanabhan, A. Suvannasankha, S. Jagannath, R. Abonour, C. Bakan, P. Andre, Y. Efebera, J. Tiollier, M. A. Caligiuri, and S. S. Farag. 2012. A phase 1 trial of the anti-KIR antibody IPH2101 in patients with relapsed/refractory multiple myeloma. *Blood* 120: 4324-4333.
372. Kohrt, H. E., A. Thielens, A. Marabelle, I. Sagiv-Barfi, C. Sola, F. Chanuc, N. Fuseri, C. Bonnafous, D. Czerwinski, A. Rajapaksa, E. Waller, S. Ugolini, E. Vivier, F. Romagné, R.

Levy, M. Bléry, and P. André. 2014. Anti-KIR antibody enhancement of anti-lymphoma activity of natural killer cells as monotherapy and in combination with anti-CD20 antibodies. *Blood* 123: 678-686.

373. Tarek, N., J.-B. Le Luduec, M. M. Gallagher, J. Zheng, J. M. Venstrom, E. Chamberlain, S. Modak, G. Heller, B. Dupont, N.-K. V. Cheung, and K. C. Hsu. 2012. Unlicensed NK cells target neuroblastoma following anti-GD2 antibody treatment. *The Journal of Clinical Investigation* 122: 3260-3270.
374. Benson, D. M., C. E. Bakan, A. Mishra, C. C. Hofmeister, Y. Efebera, B. Becknell, R. A. Baiocchi, J. Zhang, J. Yu, M. K. Smith, C. N. Greenfield, P. Porcu, S. M. Devine, R. Rotem-Yehudar, G. Lozanski, J. C. Byrd, and M. A. Caligiuri. 2010. *The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti–PD-1 antibody*.
375. Davies, F. E., N. Raje, T. Hideshima, S. Lentzsch, G. Young, Y.-T. Tai, B. Lin, K. Podar, D. Gupta, D. Chauhan, S. P. Treon, P. G. Richardson, R. L. Schlossman, G. J. Morgan, G. W. Muller, D. I. Stirling, and K. C. Anderson. 2001. *Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma*.
376. Kijima, M., N. Gardiol, and W. Held. 2011. Natural Killer Cell Mediated Missing-Self Recognition Can Protect Mice from Primary Chronic Myeloid Leukemia. *PLoS ONE* 6: e27639.
377. Sun, J. C., and L. L. Lanier. 2008. Cutting Edge: Viral Infection Breaks NK Cell Tolerance to “Missing Self”. *The Journal of Immunology* 181: 7453-7457.
378. Ardolino, M., C. S. Azimi, A. Iannello, T. N. Trevino, L. Horan, L. Zhang, W. Deng, A. M. Ring, S. Fischer, K. C. Garcia, and D. H. Raulet. 2014. Cytokine therapy reverses NK cell anergy in MHC-deficient tumors. *The Journal of Clinical Investigation* 124: 4781-4794.
379. Mace EM, Hsu AP, Monaco-Shawver L, Makedonas G, Rosen JB, Dropulic L, et al. 2013. Mutations in GATA2 cause human NK cell deficiency with specific loss of the CD56(bright) subset. *Blood*;121:2669-77.
380. Gineau L, Cognet C, Kara N, Lach FP, Dunne J, Veturi U, et al. 2012. Partial MCM4 deficiency in patients with growth retardation, adrenal insufficiency, and natural killer cell deficiency. *The Journal of Clinical Investigation*;122:821-32
381. Freud AG, Becknell B, Roychowdhury S, Mao HC, Ferketich AK, Nuovo GJ, et al. 2005. A human CD34(1) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. *Immunity*;22:295-304
382. Jawahar S, Moody C, Chan M, Finberg R, Geha R, Chatila T. 1996. Natural Killer (NK) cell deficiency associated with an epitope-deficient Fc receptor type IIIA (CD16-II). *Clin Exp Immunol*;103:408-13
383. Komiyama A, Kawai H, Yabuhara A, Mitsuhiko Y, Miyagawa Y, Ota M, et al. 1990. Natural killer cell immunodeficiency in siblings: defective killing in the absence of natural killer cytotoxic factor activity in natural killer and lymphokineactivated killer cytotoxicities. *Pediatrics*;85:323-30.
384. Tamm A, Schmidt RE. 1996. The binding epitopes of human CD16 (Fc gamma RIII) monoclonal antibodies. Implications for ligand binding. *Journal of Immunology*; 157:1576-81
385. de Vries E, Koene HR, Vossen JM, Gratama J-W, von dem Borne AEGK, Waaijer JLM, et al. 1996 Identification of an unusual Fcg receptor IIIa (CD16) on natural killer cells in a patient with recurrent infections. *Blood* ;88:3022-7
386. Stanietsky N, Rovis TL, Glasner A, Seidel E, Tsukerman P, Yamin R, Enk J,

Jonjic S and Mandelboim O. 2013. Mouse TIGIT inhibits NK-cell cytotoxicity upon interaction with PVR. *European Journal of Immunology*; 43:2138–2150.

389. Anfossi N, Andre P, Guia S, Falk CS, Roetynck S, Stewart A, Breso V, Frassati C, Reviron D, Middelton D, Romagne F, Ugolini S and Vivier E. 2006. Human NK Cell Education by Inhibitory Receptors for MHC Class I. *Immunity*; 25;331-342.