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"Be not simply good, be good for something."

Henry David Thoreau in a letter to Blake, March 27th, 1848.

The cover painting "Unlocking the mystery of the immune system" (2011) is an original painting made especially for this thesis by the talented Anne Peterson Klahr. (http://annepetersonklahr.com)

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ABSTRACT

The immune system needs to respond to danger but remain tolerant to normal cells and tissues. Natural killer cells achieve tolerance to self by the use of inhibitory receptors recognizing MHC class I molecules on healthy cells. Only NK cells expressing such inhibitory receptors for self-MHC class I molecules are allowed to be fully functional through a process of education. Here we have studied this process of education and the nature of the MHC class I mediated influence. We have found that MHC class I molecules exert a quantitative rather than a binary influence on NK cells. This means that NK cells are not “on” or “off” but also everything in-between. We have also found MHC class I molecules to regulate NK cells at multiple levels, such as setting the threshold for activation and determining the quality of NK cell responses to stimulation. Also the formation of the NK cell repertoire is regulated by MHC class I. We propose that this reflects a process in which NK cells continuously sense MHC class I and other relevant inputs and adapt to them. This could serve to maintain NK cell sensitivity to relative changes in stimuli also in a context that is highly dynamic. We have termed this the Rheostat model for NK cell education.

LIST OF PUBLICATIONS INCLUDED IN THESIS

I. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells.
Brodin P, Lakshmikanth T, Johansson S, Kärre K, Höglund P.
Blood. 2009 Mar 12;113(11):2434-41

II. Natural killer cell tolerance persists despite significant reduction of self-MHC class I on normal target cells in mice.
Brodin P, Lakshmikanth T, Mehr R, Johansson MH, Duru AD, Achour A, Salmon-Divon M, Kärre K, Höglund P, Johansson S.
PLoS One. 2010 Oct 4;5(10). pii: e13174.

III. Positive and Negative Selection of NK Cell Subsets by MHC Class I via Regulated Sensitivity to IL-15 and Resistance to Apoptosis.
Brodin P, Lakshmikanth T, Kärre K, Höglund P.
Submitted manuscript.

RELEVANT REVIEW AND OPINION ARTICLES

I. Current perspectives of natural killer cell education by MHC class I molec
Höglund P, **Brodin P.**
Nature Reviews Immunology. 2010 Oct;10(10):724-34.

II. NK cell education: not an on-off switch but a tunable rheostat.
Brodin P, Kärre K, Höglund P.
Trends in Immunology. 2009 Apr;30(4):143-9.

III. Beyond licensing and disarming: a quantitative view on NK cell educatio
Brodin P, Höglund P.
European Journal of Immunology. 2008 Nov;38(11):2934-7.

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LIST OF ABBREVIATIONS

β2m	Beta-2-microglobulin
BCR	B-cell receptor
CD	Cluster of differentiation
CFSE	5,6-Carboxyfluorescein diacetate succinimidyl ester
CMV	Cytomegalovirus
DC	Dendritic cell
Dscam	Down syndrome cell adhesion molecule
ER	Endoplasmic reticulum
Hb	Hemoglobin
HLA	Human leukocyte antigen
HR	Hybrid resistance
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer Immunoglobulin-like receptor
KLRG1	Killer-cell lectin-like receptor G1
LCMV	Lymphocytic choriomeningitis virus
LRC	Lymphocyte receptor complex
MFI	Mean fluorescence intensity
MHC	Major Histocompatibility complex
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKC	Natural killer gene complex
TCR	T-cell receptor
TLR	Toll-like receptor
WT	Wild-type

INTRODUCTION

The immune system is a powerful and utterly complex system consisting of a myriad of cells, interacting via receptors and ligands and an array of soluble factors. Its cells and molecules circulate our bodies through both blood and lymph (*1*). The system is far too complex to be measured as a whole, using any currently available techniques. The immune system is therefore the only functional system lacking adequate laboratory tests of function and as a consequence no definitions of "normal" human immune function exist (*2*). Studies in immunology have been dominated by the use of murine model systems that have provided much important knowledge to date. As novel experimental technologies are becoming available, a shift towards more detailed studies also in human subjects is ongoing (*2-5*).

The evolution of immunity

The human immune system has developed through evolution but the main purpose of this evolution and the relevant selective pressures governing it are not fully understood. It is clear that infectious diseases have placed a particularly heavy burden on human populations in the past, and still does in certain regions (*6*). This burden is exemplified by the fact that life expectancy of humans, before the industrial revolution was only around 25 years (*7*), largely due to infectious diseases. Obviously, such a burden of disease should impose a strong evolutionary pressure on humans to develop counter measures. Interestingly, Charles Darwin did not note the selecting pressure of infectious disease in his works, despite his awareness of the recent advances on the germ theory of disease made by his contemporaries Louis Pasteur and Robert Koch (*8*). Instead, Jack Haldane has been attributed as the first to directly describe the evolutionary pressure by infectious disease on human populations. His most famous work "Genetics and the origin of species" from 1949 contains a speculative hypothesis on the possible resistance to malaria by thalassemia heterozygotes (*8*). This idea gained support when Allison et al described that individuals heterozygous for the Hb-S gene of hemoglobin, i.e. the sickle cell trait, were protected from severe malaria infection (*9, 10*). Since then, many studies have verified these findings and extended them, revealing the strong evolutionary pressure posed by infectious diseases on the evolution of human immune systems (*11*).

Allogenic recognition systems

Contrasting the idea of infectious disease as the main driving force of immune evolution are ideas of anti-microbial defenses as late evolutionary readjustments of a system initially intended for other purposes (*12*). The reactions to infectious pathogens are always examples of xenogeneic recognition while the detection of other members of the same species (conspecifics) represents allogeneic recognition. Some investigators claim that the allogenic recognition systems represent evolutionarily older modes of recognition and that the immune system was actually created originally to maintain individuality against invading cells from other members of the same species. This is important to prevent cells and especially genetic material from contaminating the germ-

line and propagate to the next generation. Important support for this theory, are the many examples of natural chimerism described in humans and its possible links to disease(*13-15*).

Compelling data from colonial marine invertebrate species such as the urochordate Botryllus Schlosseri shows that such primitive creatures, separated from mammalians about 500 million years ago, are crucially dependent on their ability to perform allogenic recognition (*16*). Botryllus Schlosseri colonies grow on natural reefs in the Mediterranean Sea and along the North American coastlines and are constantly facing the threat of predators, spatial limitation and overgrowth. These factors together provide survival benefit to individual zooids growing together in large colonies. Such colonies are formed by fusion of individual zooids (*16*). This fusion is regulated by an intricate allogeneic recognition system that have evolved to allow two proximate colonies either to fuse or reject each other. This response is determined by a single polymorphic locus where only one shared allele will lead to fusion while a mismatch will lead to rejection (*17*).
The Fu/Hc (Fusability/Histocompatibility) locus found in the Botryllus is not a direct homolog of the vertebrate Major Histocompatibility (MHC) genes (*18*) but the similarities between the systems are striking. Similar self/nonself discriminatory systems have since been described also in other invertebrates (*19*) as well as in plants where they prevent self-fertilization (*20, 21*).

Members of the Zipursky lab have described another interesting example of self/nonself discrimination at the level of an individual cell. They have described how growing neurons in the Drosophila brain avoid dendrite connections with its own cell body by using an intricate self-recognition system. In this system, individual neurons express alternatively spliced isoforms of the Dscam receptor and homophilic interactions between identical isoforms ("self-to-self") will lead to a repulsive response and thus "self-avoidance"(*22-24*). This fascinating self-recognition system prevents growing neurons to form connections with its own cell body.

Together these examples show that systems for allogeneic and self/nonself discrimination have evolved several times, and in various shapes and form throughout evolution in order to meet various biological requirements and to provide survival benefit (*25*). Understanding the evolutionary purpose of allogeneic recognition in humans is to me one of the most fascinating issues of immunology.

Hybrid resistance
One particular form of allogeneic recognition response of particular relevance to this thesis is that of "Hybrid Resistance", HR. It was first described in 1958 by George Snell (*26, 27*) and further supported by data from Cudkowicz et al (*28, 29*). Hybrid resistance describes the ability of an F_1 hybrid mouse, which is a progeny of two different inbred mouse strains, to reject transplanted bone marrow cells from either one, or both of its parental strains (*30*).

These finding were originally controversial and in violation with the laws of transplantation formulated by Snell himself just a few years earlier (*31*). These laws

stated that Histocompatibility antigens were codominantly inherited and thus, no foreign allogenic antigens should be expressed on parental cells for the F_1 hybrid immune system to recognize and react to (*31*). Such foreign antigens were believed to be necessary for graft rejection (*31*), and therefore hybrid resistance was unexpected.

The molecular explanation for hybrid resistance would take decades to find and involved the work of many different groups. Snell himself was able to show that MHC related genes were important (*32*) and Cudkowicz showed that an unknown cell type, independent of the thymus but dependent on the bone marrow were responsible for this rejection (*33*).

It was initially proposed that F1 hybrids were able to reject parental hematopoietic, but not tissue grafts (*30*) but more recent data have shown that also reactions to vascular tissue in solid organ grafts follow the rules of hybrid resistance and contribute to graft rejection (*34*).

NK cells and the "missing self" hypothesis

In the mid 1970ies several independent groups had described cytotoxicity against tumor cells by unimmunized lymphocyte populations, implying that some cell other than T cells contributed to target cell rejection (*35*). This inspired Kiessling et al at the Karolinska Institute and Herberman et al at the NIH, to seek and find the first descriptions of a population of large granular lymphocytes with a cytotoxic potential, distinct from cytotoxic T-cells (*36-39*). The cells were termed Natural Killer (NK) cells due to their ability to kill target cells without prior sensitization (*38*).

The specificity of the cells was unknown and initial attempts to identify an activating receptor similar to the clonal receptors on T- and B-lymphocytes failed. Instead, it seemed clear that NK cells and T-cells used completely different modes of target recognition, but both involving MHC class I molecules (*40*).

A breakthrough came in the fall of 1981 as Klas Kärre was writing his doctoral thesis. Inspired by the Hybrid resistance theory (*30*), some foreign submarines (*41*) and some data showing that some malignant cells downregulate their MHC expression during tumor progression (*42*), he proposed the "missing-self" hypothesis for NK cell recognition (*43*). His hypothesis stated that NK cells would react to the loss of MHC class I expression on target cells by expressing inhibitory receptors for MHC class I (*43*). A few years later the idea was experimentally supported when tumor cells lacking MHC class I expression was shown to be sensitive to NK cell rejection while cells of the same tumor cell line expressing normal levels of MHC class I was not (*44*). This fundamental idea has since formed the basis for all current theories on NK cell regulation by MHC class I (*45*).

Interestingly, the concept of missing-self recognition is now a general form of innate immune recognition and not exclusive to NK cells. For example it applies to the activation of the complement cascade (*1*). The alternative pathway of complement activation is under negative regulation by several molecules such as Factor H, which is able to distinguish between self (host), and non-self (microbial) sialic acids.

Complement activation will be inhibited in the presence of self but without it complement activation will occur (*46*).

Similarly, phagocytosis by macrophages is under the negative influence of self-recognition by the inhibitory receptor Signal Regulatory Protein alpha (SIRPα) (*47*). This receptor recognizes CD47 on host cells and prevents phagocytosis of "self" allowing phagocytosis of "missing-self"(*48*). Common to all these examples is that self-recognition provides a means of inhibiting an activating signal. Missing-self recognition is thereby dependent on concurrent activating signals, activating signals in NK cells will be discussed in more detail below.

Variable receptors for MHC class I

The molecular explanation to the missing-self hypothesis came when the predicted inhibitory NK cell receptors for MHC class I were identified. The Moretta lab described the Killer Immunoglobulin-like receptor (KIR) genes (*49*) and the Wayne Yokoyama lab the C-type Lectin like Ly49-receptors (*50*). The lectin like receptors (Ly49) are located in a chromosomal region termed the NK complex (NKC) while the Ig-like receptors are confined to the Leukocyte Receptor Complex (LRC). Together with the MHC class I molecules, these are the key orchestrators regulating NK cell biology in mammals. The MHC, NKC and LRC are located on chromosomes 6, 12 and 19 respectively in humans.

From an evolutionary point of view it is interesting to note that the variable receptor genes of the NKC and LRC have been selectively expanded in different ways in different species. In modern humans a large LRC containing 14 KIR-genes is responsible for MHC class I (HLA) recognition and NK cell regulation (*51, 52*). In contrast, the NKC is completely contracted and only encodes a pseudogene in humans (*53*). In mice, the situation is the opposite and the NKC includes many variable genes of the Ly49 family responsible for MHC class I recognition and NK cell regulation (*53*). At the same time in mice the LRC is contracted and only two KIR genes remain but have been moved out of the LRC onto the X chromosome (*53*). There are no known examples of species having expanded both NKC and LRC loci and the reason for why rodents and humans have expanded different loci since their evolutionary separation is not known.

Most likely such selective expansion events reflect past evolutionary crises where expansion of a new MHC class I recognition system favored survival (*54*).

The generally short life span of these evolving receptors throughout evolution has also been viewed as an indication of several competing selecting pressures. For NK cells, protection from microbial pathogens is clearly one such influence and the other could well be reproduction since NK cells play such a major role in reproductive success. Regulating birth weight is crucially important to humans. This is due to bipedalism and the narrow pelvic birth canal that comes with it (*55*). Therefore, strong evolutionary pressures influence factors regulating birth weight in humans. The placenta provides blood and nutrients to the fetus and its establishment through the process of placentation will have a strong impact on birth weight. Placentation is a complicated

process where uterine NK cells play a crucial role by remodeling the maternal spiral arteries providing nutrients to the fetus(*55, 56*). This involves NK cell interactions with MHC class I and certain KIR-HLA compound genotypes have been linked to various outcomes of this process (*57*).

Because NK cells and their variable receptors are important to both reproduction (*55*) and immunity (53) but in different ways, opposing evolutionary pressures from these two could explain why the variable receptor genes have been so transient through evolution (55).

On NK cells, both KIRs in humans and Ly49 receptors in mice are clonally expressed in variable frequency. Receptors are expressed on NK, NKT- and some T-cells. Each receptor is specific for one or a few MHC class I alleles and each NK cell can express one or multiple of these receptors, allowing for a complex repertoire of NK cells with varying MHC class I specificity in a given individual (*45, 58-60*). Thereby the Hybrid resistance mystery was solved when it was shown that NK cells lacking inhibitory receptors specific for parental MHC class I could be responsible for the rejection of parental bone marrow grafts (*61*). Exactly how the complex NK cell receptor repertoire is shaped in an individual is an intense area of research and a key focus of this thesis.

Another important inhibitory receptor, conserved in mice and humans, is the NKG2A/CD94 inhibitory receptor complex (*62*). This receptor binds the non-classical MHC class I molecule HLA-E in humans (*63*) and Qa-1^b in the mouse (*64*). Qa-1^b and HLA-E present leader peptides from classical MHC class I heavy chains, functioning like a survey mechanism of MHC class I transcription. The NKG2A/CD94 receptor complex, expressed early during NK cell development provides enough inhibitory signaling to prevent activation (*65*) but its exact relationship to the variable KIR and Ly49 receptors is not known. It has been proposed that NKG2A could serve as a "buffering" receptor for inhibitory KIR interactions with either too low, or too high MHC class I affinity (*58, 66*). This "buffering" mechanism would serve to balance the inhibition of NK cells, making them optimally responsive but yet self-tolerance (*66*).

Despite the various transmembrane receptors being structurally different, their signaling pathways seem largely conserved (*67-70*). This implies evolutionary re-application of conserved signaling complexes and modules to various types of receptors, possibly with different downstream effects.
The mechanisms of inhibitory signaling from these various receptors are not fully understood but it seems clear that termination of activating signals occurs at multiple levels of the activating cascade (*65, 67, 70-72*).

NK cell self tolerance and the process of education
Since the inhibitory receptors on NK cells and their MHC class I ligands are encoded on different chromosomes, no genetic mechanism could ensure transcription of only inhibitory receptors specific for host MHC class I.

In individuals lacking functional MHC class I expression, such as mice with a targeted mutation of β2-microglobulin (β2m) or MHC class I heavy chains (H-$2K^b$ and D^b) or

the Tap1 gene, as well as humans lacking the Tap1 gene, NK cells still exist in normal numbers and are tolerant to self. This despite NK cells being surrounded by MHC class I deficient target cells. Self-tolerance in these cases is secured by a hyporesponsive NK cell phenotype with an inability to kill target cells via missing-self (73, 74, 75, 117). We now know that also in normal individuals, NK cells exist that lack self-specific inhibitory receptors and display a similarly hyporesponsive and self-tolerant phenotype (*76-78*).

Together, these data have led to the conclusion that NK cells will go through a process of education whereby only NK cells with an ability to be inhibited by self MHC class I will be allowed full functionality (*45*). Three different models have been put forward to explain this phenomenon. 1) The Licensing model propose a process of "licensing" in which NK cells with an ability to sense MHC class I gain function (*79*), 2) The Disarming model propose that NK cells are by default responsive and auto reactive cells unable to sense MHC class I mediated inhibition will instead be "disarmed" and made hyporesponsive and thereby tolerant (*80*). 3) The Rheostat model can be considered a unifying model of quantitative tuning of NK cell responsiveness where NK cells are more or less responsive depending on the strength of signal received from MHC class I in a continuous and reversible way as described further below (*81*).

NK cell activation

NK cells were named based on their ability to be triggered without prior sensitization (*38*). As such, NK cells were clearly different from T-cells. More recent studies have shown that in a context of an immune response NK cells do actually need a priming signal delivered in the lymph node in the form of trans-presented IL-15 by $CD11c^{hi}$ Dendritic cells (*82*). Also a signal from the cytokine IL-18 seems to be able to provide a priming signal for NK cells to respond with IFN-γ upon subsequent IL-12 stimulation (*83*). Whether these reflect absolute requirements or just a lowering of thresholds for activation making stimulation easier is not known.

In contrast to T- and B-cells, NK cells lack clonally distributed receptors for activation. Instead many different receptors contribute to triggering NK cell activation through a complex interplay of multiple different activating signaling pathways (*68*). Interesting studies have described the interplay between activating receptors showing that activation of one single receptor is not enough for triggering of resting NK cells but instead simultaneous co-activating receptor ligation is usually needed (*84, 85*). One exception to this rule is the triggering of the Fc-receptor CD16 (FcγRIIIb) by antibodies stimulating Antibody Dependent Cellular Cytotoxicity (ADCC). This form of activation does not require co-activation receptors (*84*). In general the co-activation theory implies that a certain threshold level of activating signal is needed to overcome the threshold for activation. The influences regulating the level of this threshold are still unknown, but its molecular wiring is being revealed (*71*).

The ligands for activating receptors on NK cells are diverse and only partially known. The NKG2D receptor (*86*) expressed by all NK cells, γδT-cells and some CD8+ T-cells bind various ligands (*87*) upregulated upon cellular stress such as tumor transformation (*88, 89*) and DNA-damage (*90*). It seems increasingly clear that the induction of these

ligands is more complex than initially proposed. It is probable that different ligands are expressed in different tissues and induced by different stressors and also in a variety of cell types. For example skin-specific NKG2D ligands have been cloned and proposed to regulate wound repair (*91, 92*). Interesting ideas from the Hayday group have stated that NKG2D ligands induced in keratinocytes of the skin, recognized by local lymphocytes including NK cells, which could then activate dendritic cells to induce adaptive immunity (*93*). This would thus represent a novel form of immunosurveillance where these NKG2D ligands play a central role (*93-95*).

Also, the importance of these ligands in viral infection is illustrated by the fact that Cytomegaloviruses (CMV) has developed mechanisms of targeting certain NKG2D ligands as way of interfering with host immune responses (*96, 97*).

Another group of triggering receptors are the Natural Cytotoxicity receptors (NCRs)(*98*), NKp46 (*99*), NKp44 (*100*), NKp30 (*101*). Proposed ligand for the NKp46 receptor is haemagglutinins of Influenza virus (*102*), and for NKp30 the B7-family member B7-H6, a ligand that seems to be expressed on tumor cells but not normal cells (*103*). Also another tumor associated molecule, BAT3 has been proposed to be a ligand for NKp30 suggesting that triggering of this receptor occurs upon tumor cell interactions (*104*).

An interesting NK cell receptor with both activating and inhibitory signaling capacity is the SLAM family receptor CD244 (2B4)(*105*). This receptor is expressed by all NK cell and recognizes the CD48 receptor expressed broadly on hematopoietic cells and regulated in part by viral products (*106*). The fact that the receptor is expressed on all NK cells and that its ligand is abundant allow for interesting regulatory influence of this receptor which will be discussed in more detail below.

NK cells express a vast number of additional receptors with the ability to trigger responses to various targets but many are still poorly understood and will not be described further here.

NK cell heterogeneity

Today, NK cells are known to be more than just “Natural Killer” cells. The population bearing the typical surface markers CD56 in humans and NK1.1 in the mouse, lacking the expression of the CD3ε marker of T-cells, have proven to be a more heterogeneous population of cells than initially thought and with a variety of functions.

NK cells are circulating both blood and lymph and are residents of most tissues in the body (*107*). They perform various functions beyond cytolysis. For example during pregnancy an NK cell subset, distinct from peripheral blood NK cells (*108*) is abundant in the uterus, responsible for remodeling of the spiral arteries necessary for correct placentation and essential for successful reproduction. The details of this process are still unknown but interactions with MHC class I are important. Interestingly, the only MHC class I allele expressed in this tissue is HLA-C and large epidemiological studies have shown beneficial effects of certain compound genotypes of MHC class I and their corresponding NK cell receptor genes with relation to reproductive success (*55, 57*).

Other interesting examples of tissue specific NK cell subsets are the mucosa-associated cells with similarities to NK cells. These cells are found in humans and mice and contribute to mucosal homeostasis and immune defenses by producing cytokines such as IL-22 in response to infection, regulating the epithelial homeostasis and possibly also providing tissue protection from inflammatory responses (*109-111*). Also, liver NK cells are interesting with a distinct phenotype and possibly able to exhibit immunological memory(*112, 113*). Similarly, our group studied pancreatic NK cells and found phenotypical differences to splenic NK cells, both in diabetic (NOD) and wt mice(*114*). Together it seems clear that NK cells are a heterogeneous population of cells widely distributed and well adjusted to its local tissue niche(*107*).

GENERAL AIMS OF THIS THESIS

MHC class I molecules regulate NK cell activation upon target cell encounter. These molecules are also essential regulators of various aspects of NK cell biology. Our general aim has been to understand the nature of this MHC class I mediated influence. In this thesis I have studied the regulation of NK cell function, differentiation and repertoire formation by MHC class I. I have studied the quantitative influence of MHC class I and together with my supervisors, proposed a model for NK cell education termed the Rheostat model.

The three articles included in this thesis represent the main focus of my work as a PhD student. However, a lot more work has been done with some interesting findings but not yet formulated into manuscripts. In the following sections of my thesis I will describe and discuss the results presented in the enclosed papers as well some of the unpublished data.

In the first section I will focus on the concept of NK cell education and the various models proposed to explain this process. I will discuss the implications of my work related to the MHC class I mediated regulation of NK cell function and various aspects of NK cell functional regulation by MHC class I.

In the second section, I will describe the findings of **paper II-III** on the influence of MHC class I expression level in target cell recognition and education and then **Paper III** extends this analysis and includes studies of the formation of the NK cell repertoire. This process is shown to be regulated by MHC class I can be considered yet another layer of regulation provided by MHC class I.

METHODOLOGICAL DEVELOPMENT

The work presented in this thesis is based on the use of a novel reductionist experimental approach. It builds around various murine models with controlled expression of MHC class I molecules and a high-dimension flow cytometry setup allowing for detailed studies of very well defined NK cell populations.

Mice with altered MHC class I expression

The first mouse model without functional MHC class I expression was the β2-microglobulin knock-out mouse created independently in 1989 by Rudolf Jaenisch and colleagues at MIT (*115*), and by the Smithies group at the University of North Carolina (*116*). The resulting MHC class I deficiency of all nucleated cells in these mice paved the way for research in the field of NK cell education by allowing studies describing the self-tolerance and hyporesponsiveness of $\beta 2m^{-/-}$ NK cells (*73, 117*).

Interestingly, Bieberich et al created a transgenic mouse expressing a H-$2D^d$ transgene on the H-2^b background (*118*). Using this mouse model Höglund et al were able to show that MHC class I directly regulates the specificity of NK cells by inducing the ability to reject H-2^b lymphoma cells (*119*) as well as normal bone-marrow cells (*74*).

For the studies described in this thesis MHC class I heavy chain knockout mice (H-$2K^{b-/-}D^{b-/-}$) were used as the negative control for any MHC class I influence. By crossing these mice to various MHC class I expressing mice, mice hemizygous for a given MHC class I allele were generated and back-crossed so that single MHC class I allele expressing mice (Homozygous) were generated (*120*). These mice include H-$2K^b$, H-$2D^b$, H-$2L^d$ and H-$2D^d$ single MHC class I allele mice. They will hereafter be denoted by their allelic names throughout the thesis. By using such single MHC class I allele expressing mice, the influence of a given MHC class I allele on NK cell function could be assessed (*45, 120*). As described in **Paper II and III**, we also compared the mice expressing a given single MHC allele in a hemi- and homozygous fashion providing a system to study the influence of MHC class I expression level on NK cell biology.

Polychromatic flow cytometry

The second aspect of our reductionist system involved a detailed characterization of the expression of MHC class I specific receptors. This was required since NK cells often express multiple receptors with varying, and often overlapping, specificity for MHC class I alleles. Our desire to understand the outcome of individual Receptor-Ligand interactions therefore urged us to develop an advanced flow cytometry protocol involving the simultaneous detection of 6-7 inhibitory receptors for MHC class I on individual NK cells. This tedious exercise involved testing of multiple antibody-flourochrome combinations as well as staining protocols before finally being established. Together with the surface receptor detection parameters, we also include markers for NK cells, viable cells, forward and side-scatter parameters as well as 2-3 functional parameters resulting in a flow cytometry protocol of 13-15 parameters in

total. For efficient and standardized analysis of such large data sets we developed gating algorithms based on Boolean-statistics using the software suite FlowJo (Threestar, California U.S).

Single cell activation assays

Another important technological advancement important to our studies is the recent development of assays based on flow cytometry for the functional assessment of individual NK cells. These assays are performed *in vitro* where an activating stimulus such as a target cell or a plate bound antibody to an activating NK cell receptor, is present. NK cells will respond with upregulation of activation markers such as the CD69 antigen (*121*). They will also, depending on the stimulus respond by secreting cytokines such as IFN-γ and chemokines into the supernatant. The amount of cytokine or chemokine secreted by individual NK cells can now be directly quantified by the addition of a chemical inhibitor of exocytosis such as Brefeldin A (*122*). Another important advancement allowing for single-cell measurement of function was the identification of the marker CD107a/Lamp1 found in the membrane of vesicles secreted from cells, such as the cytotoxic granules on cytotoxic T- and NK cells (*123*). By using this antibody during stimulation of NK cells, the amount of cytotoxic responses by individual cells can be estimated from the amount of CD107a staining. Such staining is dependent on the release of cytotoxic granules, exposing the antibody labeling epitope leading to antibody binding. This important technical advancement has been instrumental to the work of this thesis allowing the assessment of function of individual NK cells rather than population oriented assays of function.

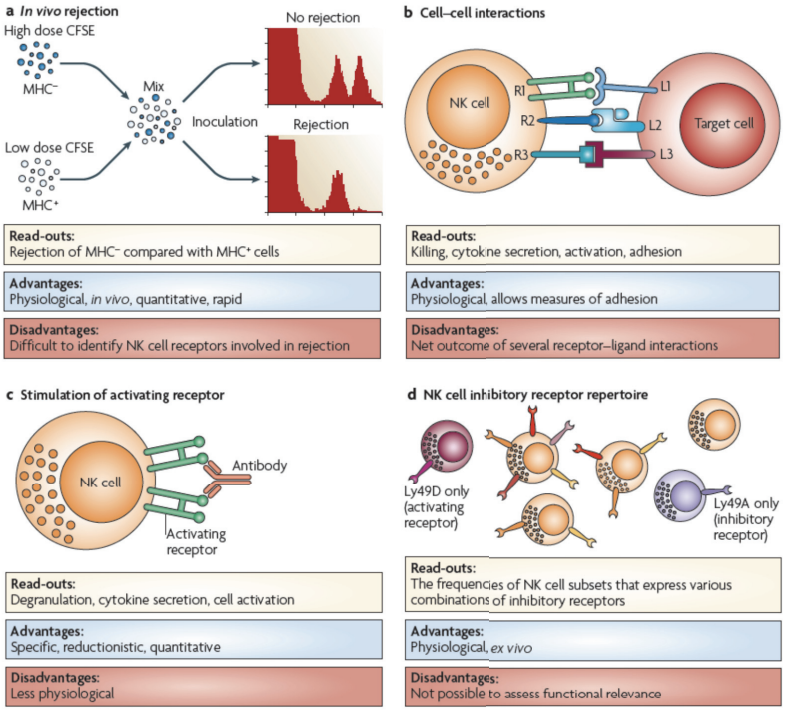

FIGURE 1. *Four ways of measuring NK cell function and the influence of MHC class I.*
A) In vivo rejection of CFSE-labelled target cells inoculated in a 1:1 ratio with syngeneic controls is physiological but does not provide single-cell resolution. B) NK cell interaction with target cells involves multiple receptor-ligand interactions, which is physiological but can be hard to interpret. C) Antibody mediated cross-linking of activating receptors triggers the targeted activating pathway only, which is a reductionist but non-physiological approach. D) Measurements of the NK cell repertoire composition can be an indirect measurement of NK cell education. It is physiological but still descriptive and hard to translate into functionally relevant terms.
Figure (45) is reused with permission from the Nature Publishing Group.

RESULTS AND DISCUSSION

Discussion on the models of NK cell education

When I started working in the laboratory of Klas Kärre and Petter Höglund, the field of NK cell education was focused on the most recent results by Wayne Yokoyama and David Raulet showing the existence of NK cells in normal mice, lacking the expression of self-specific inhibitory receptors for MHC class I. These cells were shown to be self-tolerant due to a hyporesponsive phenotype similar to the one of MHC class I deficient NK cells described above. These results supported the notion that hyporesponsiveness is induced or maintained through NK cell education in order to secure self-tolerance and that this occurs even in normal mice and not just in the artificial setting of MHC class I deficiency (*76, 77*). Similar observations were also made in normal human subjects (*78*).

Two seemingly conflicting models were proposed to describe the functional development of NK cells under the influence of MHC class I mediated signals. Firstly, the "Licensing model" proposed that NK cells are non-functional or hyporesponsive by default and only acquire functional competence or "a license to kill" upon MHC class I mediated signals during development (*76, 79*). In contrast to this, Raulet et al proposed a "Disarming" model in which NK cells are considered functionally responsive by default but would be "disarmed" in the absence of MHC class I mediated inhibition during development (*77, 80*).

Initially it seemed contra-intuitive that signals from inhibitory receptors would transmit a "positive" signal inducing "licensing" as proposed by the Yokoyama model (*76*). Also, Raulet and colleagues claimed that the "Disarming model" was more compatible with older experimental data (*80*). The licensing model seemed to predict that NK cell interactions with surrounding cells lacking MHC class I, i.e. missing-self targets, would not be relevant to NK cell licensing.

This fact seemed incompatible with data from Wu et al who created Bone-Marrow chimeras between MHC class I negative and MHC class I-positive bone marrow cells. In these chimeric mice tolerance to MHC deficient cells was dominantly induced by the presence of MHC class I deficient cells in the chimera. Also data from our own group showed similar results when a D^d-transgene introduced in a H-2^b mouse model accidently was expressed in a mosaic fashion on 20-80% of cells. Also in these DL6 mice, tolerance to D^d-negative target cells was dominantly induced by the presence of D^d-negative cells (*124*). Again, this result suggested that NK cell education and the development of self-tolerance to co-existing missing-self targets depends on interactions with such targets and would possibly be more compatible with the disarming model of NK cell education (*80*).

Quantitative NK cell education

In a study performed by our laboratory just before I joined the group, Johansson et al showed that mice expressing different single MHC class I alleles individually were differently efficient at rejecting MHC class I deficient cells *in vivo* (*120*). This result implied that different MHC class I alleles were differently efficient at educating NK cells, an effect termed "Educating impact" of individual MHC class I alleles (*120*).

This result implied that NK cell education was quantitative rather than binary in nature and inspired the work I was to perform during my thesis work. First, as shown in **Paper I**, we tested the degree of missing-self rejection of MHC class I deficient cells in mice expressing none ($MHC^{-/-}$), one (D^b), two (K^bD^b) or three ($K^bD^bD^d$) MHC class I alleles. The idea behind this was that the presence of multiple MHC class I alleles could provide more inhibitory signal and educate the NK cell system more efficiently. The result showed that the ability to reject MHC deficient cells *in vivo* was clearly regulated in a quantitative way. When comparing mice expressing two (K^bD^b) to those expressing only one (D^b) MHC class I allele, there was a marked increase in the ability to reject MHC class I deficient cells (**Paper I, Fig. 1**).

At the time, when preparing paper I, we didn't compare all single MHC class I expressing mice to the mice expressing two and three alleles with regards to *in vivo* rejection of MHC class I deficient targets. We were thus unable to say whether the number of MHC class I alleles per se was the determining factor or whether the type of MHC class I alleles involved would also be important. The latter would be expected from our previous work since the different single MHC class I alleles were shown to differ in their educating impact (*120*). As shown here in **FIGURE 2**, the two single MHC class I alleles K^b and D^d shown to have the highest educating impact of the single alleles, educated the NK cell system with an educating impact well comparable to that of mice expressing two alleles K^bD^b but lower than mice expressing three alleles ($K^bD^bD^d$). This result suggests that the total educating impact of MHC class I in an individual is not only dependent on the number of MHC class I alleles involved but also qualitative aspects of individual MHC class I alleles. This could involve receptor affinities varying between MHC class I ligands and also MHC class I stability and thus the duration of NK cell interactions differing between MHC alleles

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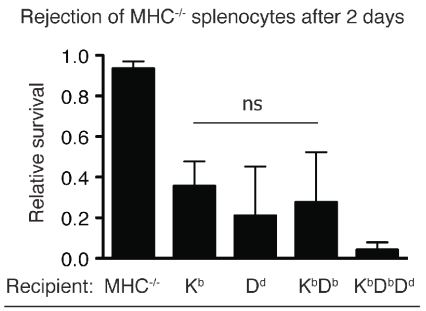

***FIGURE 2**. Comparable Educating impact of one strong single MHC class I allele and two co-expressed alleles.*
In vivo rejection of MHC class I deficient target cells in indicated mice after 2 days as measured by relative frequency to syngeneic control cells in peripheral blood. (Brodin et al, Unpublished data)

As another test of our prediction that NK cell education is quantitatively determined by the strength of MHC class I mediated input, we tested the responsiveness of NK cells expressing single and multiple Ly49-receptors specific for self-MHC class I. The reasoning was that NK cells expressing multiple receptors would receive more MHC class I mediated signal and thus be more responsive than cells expressing only one receptor. As shown in **Paper I, Fig. 6**, combinations of Ly49/NKG2A receptors did provide synergistic effects on NK cell responsiveness as measured by the amount of degranulation supporting the idea that NK cell responsiveness is dependent on the strength of inhibitory input during NK cell education. These data were later supported by data from Joncker et al also showing synergistic effects of co-expressed inhibitory receptors on NK cell responsiveness (*125*). A study from the Parham group on human NK cells provided support for quantitative NK cell education in humans by showing that NK cells expressing multiple self-specific KIR receptors responded more strongly to missing-self target cells than did single KIR expressing cells (*66*). These data again suggest that NK cell education in both mice and humans is quantitatively regulated by the signals from MHC class I.

All tests described above compared NK cells educated via different MHC class I interactions involving multiple receptors or MHC alleles making it impossible to exclude that differences between different Ly49-MHC interactions would not account for the differences seen. We therefor sought to develop a system where the MHC class I ligand-receptor interactions would be qualitatively similar but quantitatively different. Based on this we crossed our single MHC class I mice to $MHC^{-/-}$ mice to generate MHC hemizygous mice. In these mice the cell surface expression levels of MHC class I molecules K^b, D^b and D^d were heavily reduced (**Paper II, Fig. 1 and Paper III, Fig. 1A**). For the D^d allele the reduction was about 50% percent (**Paper III, Fig. 1A**), providing an optimal system to dissect quantitative NK cell education by allowing us to study the consequences of interactions between a given Ly49 receptor and its MHC class I ligand expressed at either a high, or a lower level.

As shown in **Paper III, Fig. 1B**, when inoculating MHC deficient cells into $D^{d+/-}$ and $D^{d+/+}$ mice respectively, we saw a striking difference in global NK cell mediated rejection of MHC class I deficient target cells. NK cells educated by a D^d-ligand expressed at a high surface level ($D^{d+/+}$) rejected the target cells twice as efficiently as $D^{d+/-}$ NK cells over time. These data imply that NK cells are sensitive to the expression level of its MHC class I ligands during education and that during this quantitative process, the ability of the NK cell system to reject missing-self targets is set proportional to its MHC class I input signals.

Differences between differently responsive NK cells

After seeing the quantitative differences at the population level, we sought to understand whether these differences between NK cell populations educated in the context of a high educating impact, as compared to a lower educating impact would, be reflected by changes at the level of the individual NK cell. The higher level of missing-self reactivity *in vivo* in mice with a higher educating impact could in theory be explained by 1) a higher frequency of educated NK cells responding to target cells, 2) a stronger response by the cells responding or, 3) a combination of both. To delineate

these effects, we stimulated NK cells from these various mice *in vitro* and measured single cell responsiveness by flow cytometry.

We found that a high educating impact, such as the presence of multiple MHC class I alleles or one strong vs. one weak MHC class I allele, led to a higher frequency of NK cells responsive to a given activating stimuli (**Paper I, Figs. 2, 3 and 4**). Also $D^{d+/+}$, as compared to $D^{d+/-}$ NK cells, made more NK cells responsive to stimulation with antibody mediated cross-linking of activating receptor NKp46 (**Paper III, Figs. 1C**). Combined these results suggested that NK cell education quantitatively regulates the frequency of NK cells responsive to a given activating stimulus. It also suggests that the more efficient missing-self rejection in mice with a high educating impact could be explained, at least in part by a higher frequency of NK cells responding to the target cells in these mice (**Paper I and III**).

We also measured the quality of the responses elicited by these different NK cell populations. We measured the Mean Fluorescence Intensity (MFI) of cytokines produced and the simultaneous release of both cytotoxic granules and cytokines and in some cases, chemokines.

The rationale for this approach came from recent data on T-cells. Studies had shown that the per cell amount of cytokine produced by a given T-cell, as measured by the MFI of that cytokine, positively correlated with the ability of the T-cell to simultaneously produce complex responses, called polyfunctionality (*126*). This involved a simultaneous production of different cytokines or chemokines and the release of cytotoxic granules (*127*). It was also proposed that such polyfunctionality represented a higher quality of T-cell response as compared to a more simple response (*128, 129*). One study had also shown that the presence of polyfunctional T-cell clones correlated with a better clinical outcome of Leishmania Major infection (*127, 130*). Also, a study on human NK cells suggested a qualitative difference in responses to Influenza A virus infected target cells. This differences was suggested to be linked to differences in MHC class I mediated NK cell education (*131*).

We therefor tested the quality of responses produced by NK cells subsets educated in the context of a high and a low educating impact. We found that upon stimulation of activating NK cell receptors using cross-linking antibodies, NK cells from mice expressing multiple as compared to a single MHC class I allele, one strong vs. a weak allele or one given allele in a homo- as compared to a hemizygous fashion, all responded with more cytokine or chemokine on a per cell basis as measured as MFI for the given parameter upon intracellular staining (**Paper I, Fig. 5, Paper III, Fig. S1C**). Also, the frequency of NK cells displaying polyfunctionality as simultaneous cytokine and cytotoxicity (**Paper I, Fig. 5**) and chemokine secretion (**Paper III, Fig. 1C**). These data suggests that NK cell education, not only determines the frequency of cells responding to a given stimuli, but also the quality of responses produced by these NK cells. Our data together with the data on qualitative responses in T-cells, suggests that polyfunctionality is a common feature of highly efficient lymphocyte populations.

The molecular regulation of polyfunctionality is currently unknown. One interesting clue comes from an experimental setting of T-cell receptor (TCR) gene transfer and the

creation of transgenic T-cells for use in immunotherapy. In a recent study, Perro et al showed that treatment of TCR-transduced cells with a combination of IL-15 and IL-21 induced polyfunctionality in a large fraction of such T-cells, as measured upon subsequent antigenic challenge (*132*). It is possible that such combined cytokine pre-stimulation in general induces polyfunctionality but also that the essential survival factor IL-15 alone could induce polyfunctionality in NK cells. This should be tested experimentally in the future.

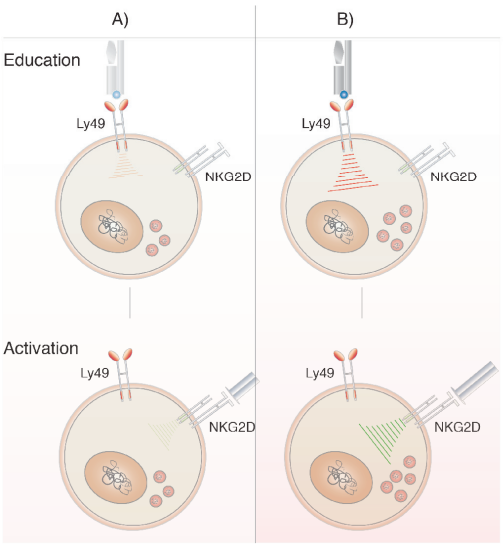

***FIGURE 3**. NK cells balance their activation threshold to inhibition. When NK cells receive a weak signal during education, the responsiveness to stimuli later is low (A) but for an NK cell that receives a stronger signal during education a stronger response will be elicited upon subsequent stimulation (B).*

The threshold for activation of NK cells

The results described above show that education regulates the threshold of activation of NK cells so that cells educated in the context of a high educating impact will be more likely to respond to stimulation, than NK cells educated in the context of a lower educating impact. This implies that the threshold for activation of NK cells are calibrated during education and set according to the input from inhibitory receptors ligating MHC class I. Our view is that NK cells will interpret a net signal from relevant inhibitory and activating receptors and set their threshold for activation in order to be able to respond to stimuli but at the same time be tolerant to normal self. This is illustrated in **FIGURE 3** above.

NK cell activation is believed to occur through an integration of activating and inhibitory signals (*68*). Inhibition is overruled and activation occurs, either due to the loss of inhibitory ligands as in missing-self recognition, (*133*) or due to an induced expression of activating ligands, i.e. induced-self recognition. The latter have been described for certain tumor cell interactions (*134-137*).

The exact amounts of activating ligands needed to trigger NK cells are unknown. It is also poorly understood exactly what levels of MHC class I are needed to protect cells from NK cell mediated rejection. We have tested this in our mouse models where MHC class I is expressed in a hemi- or homozygous fashion (**Paper II, Fig.1**).

When we injected MHC class I hemizygous splenocytes into mice expressing the same MHC class I alleles but in a homozygous fashion, no rejection was seen over 4 days of follow-up. This clearly show that a 50% reduction of MHC class I expression on normal healthy cells does not trigger missing-self rejection *in vivo* (**Paper II, Fig. 2A-C**). To test if we could break this tolerance by inducing a higher state of general immune activation *in vivo*, we pretreated the recipient mice with the Type I Interferon inducer Tilorone (Sigma-Aldrich, Stockholm). This drug acts as an Interferon inducer when administered orally (*138*) and stimulates NK cells to be more responsive to target cells and to cytokine stimulation. Despite pre-activation NK cells were still completely tolerant to inoculated cells expressing half their level of MHC class I, **Paper II, Fig. 2D**. From these results we conclude that NK cell tolerance is robust and a loss of more than 50% of MHC class I expression is required to trigger missing-self rejection, even if NK cells are pre-activated by interferon signals *in vivo*.

Next we tried to investigate NK cell rejection of splenocytes expressing even less MHC class I on their cell surface. We developed a systems using cells lacking functional the peptide transporter Tap1, necessary for transport of cytosolic peptides into the Endoplasmic reticulum (ER) where MHC class I processing and assembly occurs (*139, 140*). In such cells, the empty MHC class I complexes assemble and reach the cell surface but are instable and rapidly fall apart (*141*). Addition of peptides in the extracellular fluid can stabilize MHC class I expression if the peptides bind and stabilize these empty MHC complexes (*142*). We took advantage of this and incubated $Tap1^{-/-}$ splenocytes with a stabilizing peptide in a titrated range of concentrations to induce various levels of surface expression of MHC class I. We were able to stabilize a surface expression level of the MHC class I allele K^b between 10-80% of the surface level in K^b homozygous mice. When these target cells were inoculated into $K^{b+/+}$ mice, all cells expressing a level of K^b at 20% or more of $K^{b+/+}$ recipients were tolerated while cells expressing less than that were rejected, **Paper II, Fig. 3**.

We conclude from this that there seems to be a threshold for activation in NK cells and that this threshold is robustly calibrated to maintain tolerance to normal cells expressing a wide range of MHC class I. It is highly possible that cells expressing more ligands for activating receptors, such as tumor cells or virally infected cells, upregulating stress ligands etc. would trigger NK cell activation more easily than the normal splenocytes used in this study. A different pattern has previously been described in a study measuring tumor cell rejection by human NK cells. It was found that B-lymphoblastoid tumor cells transfected with MHC class I molecules were protected from NK cell killing and that protection was quantitative and positively correlated with the amount of MHC class I expression on the transfected cells (*143*). There was therefor a discrepancy to our data on normal target cells showing more of a threshold effect on NK cell resistance. One possible explanation for this discrepancy is the abundance of ligands for activating NK cell receptors presumably present on these B-lymphoblastoid cells. Such ligands would probably provide a much stronger activating signal than the healthy splenocytes used in our experiments. Thus, in the presence of abundant activating signals NK cells are more likely to respond and the response becomes quantitatively correlated with the amount of MHC class I expressed. It was also shown in this study that MHC class I transfected tumor cells were only protected from naïve but not

interferon or cytokine (IL-2) pre-stimulated NK cells (*143*). This also suggests that pre-stimulated NK cells are easier to trigger than naïve cells. Something we did not see in our studies, presumably due to the very robust tolerance to normal splenocytes.

Together these results suggest that there is a threshold for activation in individual NK cells. Triggering of NK cells requires the net integrated signal from activating and inhibitory receptors to overcome this threshold. The threshold for activation seems calibrated through education but pre-activation of NK cells with cytokines can lower the threshold for activation.

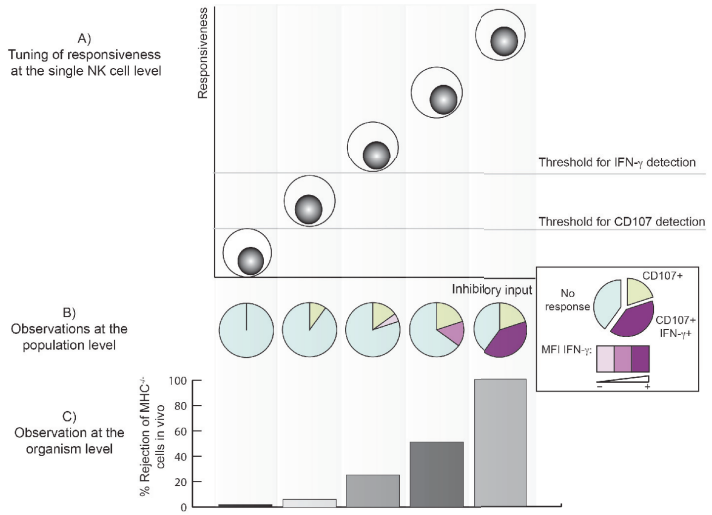

FIGURE 4. *The Rheostat model of NK cell tuning of activation thresholds*
NK cell education and the input from MHC class I quantitatively regulates NK cell function at the cellular level (A) where the strength of interactions determine the responsiveness of the individual NK cell, (B) at the NK cell subset level where education determines the quantity and quality of responses that the NK cell population will elicit and, (B) at the organism level where this will translate into a global NK cell mediated function. Figure (81) is reused with permission from Elsevier Press.

The Rheostat Model- Tuning of thresholds for activation
Evident from our studies described above is that NK cells seem to miss certain MHC class I alleles more than others when absent. This means that some MHC class I alleles educate NK cells better than others and induce stronger missing-self responses when their expression is subsequently lost (*120, 144*). We have interpreted this as evidence for NK cell education as a quantitative rather than a binary "on/off" process. Also, our data showing that individual NK cells are more likely to respond, and respond more potently to stimuli if educated by MHC class I with a high educating impact also support the quantitative education hypothesis (*66, 81, 125, 144-146*).

FIGURE 4 illustrates our interpretation of our data on a quantitative MHC class I influence at the cellular, population and organism level respectively (*81*). We propose that individual NK cells adapt their level of responsiveness to the strength of MHC class I mediated inhibitory signals during education. We believe, the purpose of this would be to secure robust tolerance to self by only allowing cells able to sense inhibition from MHC class I to be fully responsive. However, this does not explain the quantitative nature of the process of education. Tolerance does not in theory at least, require a quantitative process of education but should be achieved also by a binary process where cells able to sense adequate inhibition only would be allowed any responsiveness, i.e. "a license to kill". Instead, our data clearly show that the NK cell education system is quantitative.

Could there be a possible selective benefit of having a NK cell population made up of more or less potent cells?
One possible reason might be that it is an important feature for the system to be able to call in more potent killers in the event of a more serious challenge. This means that having highly efficient cells around is a valuable feature for the systems. However, such highly potent cells would need to be kept tightly regulated to prevent autoreactivity, and this could in theory be achieved by allowing NK cells to balance their responsiveness to their inhibitory signal. In this way the most potent killers will also be most tightly regulated, i.e. having the highest threshold for activation due to the expression of multiple inhibitory receptors for MHC class I. In this way the NK cell population includes cells with different responsiveness and a level of regulation that is proportional to this in order to maintain self-tolerance.

Although this idea is highly speculative it could be tested experimentally. So far we have mainly tested responsiveness of individual NK cells using antibody-mediated cross-linking of activating receptors. This form of stimulation bypasses the inhibitory receptors completely and will only test the potency of the activating signaling pathway itself. If NK cell subsets were instead triggered by a target cell expressing some MHC class I but also enough ligands for activating receptors to override this inhibition and trigger the NK cells, one could test whether NK cells expressing only one inhibitory receptor would be more likely to respond as compared to NK cells expressing multiple receptors specific for the MHC class I molecules on the target cells. One could also test the prediction that the cells expressing multiple receptors and still do respond, would respond more potently than NK cells expressing only single receptors. This would be predicted from our data on NK cells triggered using antibody mediated cross-linking of activating receptors, **Paper I, Fig. 6**.

Reversible and continuous tuning of responsiveness
Our Rheostat model of tuning of activation thresholds could reconcile the two previous and contradictory models of NK cell education, the "Licensing"(*76, 79*) and "Disarming"(*77, 80*) models. Bidirectional tuning of NK cell thresholds for activation could serve to increase ("Licensing") and reduce responsiveness ("Disarming") as a continuously active process of adaptation, **FIGURE 5**(*81*). We propose that NK cell education is in fact such a continuous process of adaptation rather than just a process limited to NK cell development and maturation. In this way, NK cell tuning of

responsiveness could serve to maintain an optimal level of responsiveness in the NK cell system over a wide range of MHC class I expression levels.

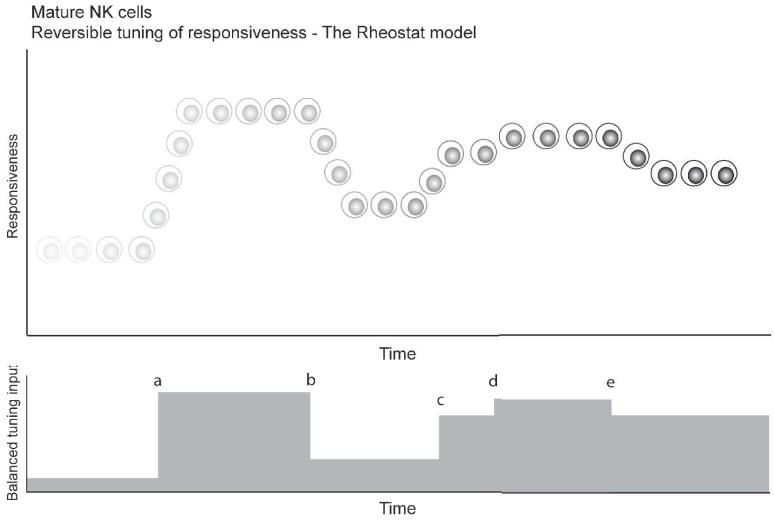

FIGURE 5. *Bidirectional and continuous tuning of NK cell thresholds for activation*
An individual NK cell adjusts its threshold for activation continuously as a consequence of quantitative changes in its tuning input.
The figure (81) is reused with permission from Elsevier Press.

Variation in MHC class I expression is significant between tissues and also between different cell types within a given tissue. This fact is illustrated in **FIGURE 6** showing MHC class I expression in different tissues as HLA-B staining in 65 human cell types from 12 different tissue types (*147*). As NK cells circulate both blood and lymph throughout the body (*107*), they will face cells expressing various levels of MHC class I. We believe that this is one reason for why NK cells do not react to normal splenocytes unless about 80% of their MHC class I expression is lost, **Paper II, Fig. 2-3**.

Moreover, in the event of an immune response both type I and II Interferons released will have the potential to induce MHC class I expression in various cell types (*148*). In the context of an immune response this might serve to induce NK cell function by tuning of activation thresholds in parallel with its effects on improved antigen presentation to T-cells etc. Interestingly, NK cells themselves are efficient producer of IFN-γ known to induce MHC class I expression (*148*). As NK cells produce large amounts of this cytokine early during an immune response it might self-propagate its own activation in this way.

The first evidence of MHC class I directed tuning of NK cell responses came from studies in our group in the late 1990ies. The mouse model DL6 described above had a H-2D^{d} transgene introduced on a H-2^{b} background that accidently became expressed in

a mosaic fashion on 20-80% of cells in the mouse. As mentioned above, the D^{d+} NK cells in these mice were tolerant to D^{d-} cells in contrast to NK cells from mice expressing the same D^{d} transgene on all of its cells (*124*).

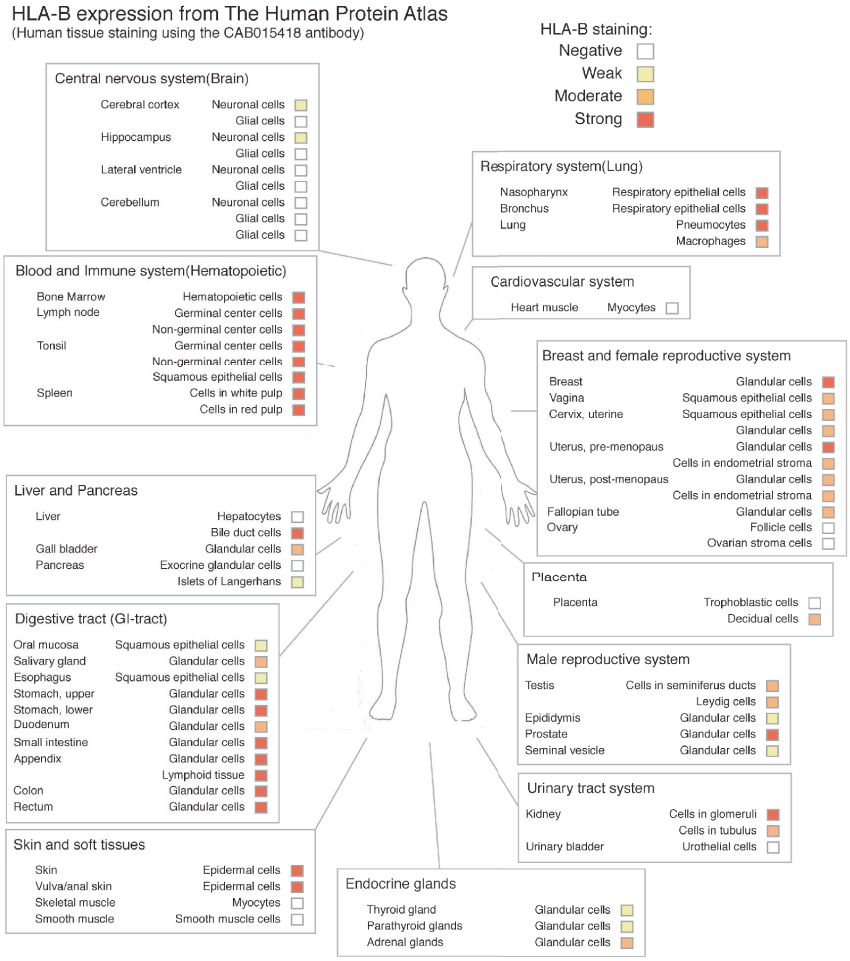

FIGURE 6. *HLA-B expression levels in various human tissues*
Antibody staining of HLA-B illustrating the variable expression level of HLA-B molecules at the resting state. Data from the Human Protein Atlas (147).

One interesting observation made in this study was that if D^{d+} and D^{d-} cells were sorted and cultured separately in the presence of IL-2, then the D^{d+} cells regained their ability to reject H-2^{b} (D^{d-}) cells in a standard Chromium (Cr^{51}) release assay. It was not formally defined exactly how long the separated cells needed to be apart for tolerance to be broken, but it didn't matter if they were separated before or after the 4-day IL-2 culture (*124*). At least a few hours separation time was required for tolerance to be

broken prior to the challenge with H-2^b target cells. This intriguing finding clearly suggests that NK cells can tune their responsiveness and that tolerance is reversible.

In the DL6 mosaic mouse model NK cells expressing inhibitory receptors specific for the D^d-transgene but not for the other MHC class I molecules H-$2K^b$ and D^b would fail to sense inhibition from D^{d-} cells. These NK cells should thus be recognizing D^{d-} cells as missing-self targets and respond. At the time of the original study, no available technique could identify NK cell subsets at this level of resolution. As mentioned in the section on methodological development I have developed such a technique for the work in this thesis and we therefore applied this multiparameter flow cytometry protocol to study NK cells in the DL6 mouse. We were able to identify such Ly49A and G2 single positive (sp) cells and as shown in **FIGURE 7,** these NK cells from the DL6 mosaic mice displayed a reduced level of responsiveness as compared to the same cells from the D8 (non mosaic) control mouse. The cells were not fully hyporesponsive since their responsiveness was clearly higher than that of $MHC^{-/-}$ cells and this probably reflects interactions with other MHC class I molecules present in the DL6 mice, K^b or D^b. Ly49A (*149, 150*) has been proposed to bind D^b *in vitro* and results from on of our recent studies suggested that Ly49 specificities *in vivo* might be much more promiscuous than previously appreciated where most Ly49 receptors might bind most MHC class I alleles albeit with varying affinity(*59*).

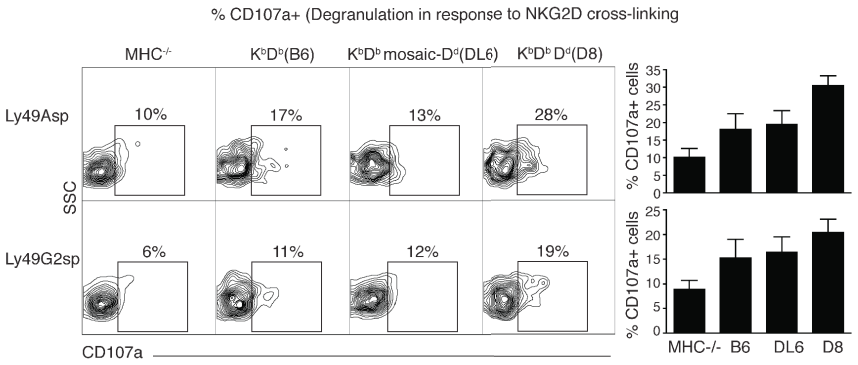

FIGURE 7. *Hyporesponsive Ly49Asp and -G2sp NK cells from DL6 (D^d mosaic) mice.*
Cells expressing the D^d binding Ly49A and -G2 receptors only are less responsive in the presence of D^d- negative cells in the H-2^bD^d mosaic DL6 mouse as compared to the fully D^d transgenic H-2^b mouse D8 mice.(Brodin et al, Unpublished data)

Two recent studies independently of each other supported our initial finding in the DL6 model by transferring NK cells from MHC-deficient mice into MHC-expressing mice showing that function of NK cells were restored in the new context (*151, 152*). This positive tuning of NK cell responsiveness occurred both in proliferating and non-proliferating cells of mature phenotype (*151*). One of the studies also showed that responsive NK cells could be tuned down to a hyporesponsive phenotype upon transfer into a MHC deficient context (*151*).

Activation induced tuning of NK cell responsiveness

Several studies have described reduced NK cell responsiveness upon continuous stimulation. Non-obese diabetic (NOD) mice (*153*) display an NK cell defect, which has partly been explained by a reduced expression level and dethatched signaling pathway of the activating receptor NKG2D, possibly due to continuous ligand stimulation *in vivo (154)*. Also when NK cells are co-cultured with ligand expressing tumor cells in vitro there is a gradual loss of function of the NKG2D receptor over time (*155*). Whether these findings reflected a tuning of global NK cell responsiveness or just an isolated defect in one overstimulated pathway was not clear at this point.

However, when Oppenheim et al expressed the NKG2D ligand Rae1ε as a transgene, either in squamous epithelium or broadly in all tissues, broader NK cell defects were shown. The rejection of $b2m^{-/-}$ cells *in vivo* was impaired in mice expressing Rae1ε broadly but interestingly enough, even more impaired in mice expressing this NKG2D ligand in squamous epithelia only. This suggests that NK cell responsiveness was tuned down as NK cells interacted with Rae1e expressing cells, a tuning that could be reversed by global NK cell activation using the TLR3-ligand Poly I:C (*156*). This defect is probably not due to a direct role for NKG2D since $NKG2D^{-/-}$ mice exhibit normal missing-self reactivity (*157*). Instead this suggests that prolonged stimulation via NKG2D leads to a reduced responsiveness to other stimuli. Supporting this, Coudert et al incubated NK cells with tumor cells transfected with another murine NKG2D ligand H60 and found that this led to a markedly reduced responsiveness to various different stimuli including RMA/S cells, i.e. missing-self targets (*158*).

In line with these data on NKG2D engagement, Sun et al and Tripathy et al published independent reports of mice expressing the viral glycoprotein m157, a ligand for the activating Ly49H receptor in $H\text{-}2^{b}$ mice. Also this led to the impairment of Ly49H signaling and in some cases to broader NK cell defects involving other activating receptor stimuli (*159*). It also suggested an inability of NK cells to proliferate and expand in the context of a viral infection (*160*), suggesting that tuning mechanisms also influences such features.

Tuning and cellular adaptation in biology

Many different cell types use an adaptation or tuning ability to be able to respond to relative changes in their surrounding, rather than absolute levels of signals or ligands. This adaptation must be determined by continuous input and therefor not only influenced by the same input that is strong enough to trigger full activation of cells. Previous triggering events would not provide current information on the current contextual environment of the cell. Instead more continuous interactions are needed.

It has been widely discussed by Grossman and Paul (*161-164*) that T-cells might adapt to their surrounding by sub-threshold stimulation of their antigen receptor by self-antigens. This input from self-antigens would be too weak to cause full activation but still allow for continuous tuning of the activation threshold. In this way, T-cell anergy has been proposed to be a result of a tuning event where the threshold for activation has been tuned very high due to the sudden strong stimulus in the absence of co-stimulation (*164*). Also, thymic selection has been proposed to represent a process of tuning where

T-cells need to tune up their responsiveness slowly as they pass through the stages of selection to be allowed to exit into the periphery (*165*).

Similarly, nerve cells stimulated by light will adapt to a prolonged stimulation in order to maintain sensitivity to subsequent alterations in light stimuli (*166*). This is what happens when we enter a dark room. At first we are blind but after a few seconds of adaptation, our visual system is adapted to the lower level of background stimulation and we are able to discriminate contours if there is only a little bit of light present.

We believe that similar adaptation processes occur in NK cells. Our hypothesis does not only imply input from MHC class I as the tuning input even though that has been the focus of our studies. Instead, all possible stimuli that NK cells can sense and respond to, would be expected to cause tuning. This includes cytokines such as IL-15, necessary for proliferation and survival of NK cells as well as inputs from various activating receptors. We believe that the tuning could occur for all these inputs either as an integrated net input signal that would induce one master tuning process of adaptation, or possibly as individual inputs separately cause tuning of their respective signaling pathway with individual adaptive circuits connected to them.

The idea of adaptation in signal transduction pathways is well established. It can be accomplished by combinations of various feedback and feed-forward loops where protein molecules and transduction pathways can act as computational elements in living cells (*167*).

The murine NK cell repertoire

From the discussions above it is clear that the actions of NK cells are highly determined by the MHC class I specific receptors they express. These receptors determine specificity and responsiveness of NK cells through interactions of its MHC class I ligands. The composition of the NK cell repertoire in an individual is thus a key determinant of global NK cell mediated function. As illustrated by Hybrid resistance described above, a variable NK cell repertoire where NK cells express different combinations of receptors is a prerequisite for allogeneic recognition of partially MHC mismatched cells and not only MHC class I deficient cells (*168*).

Whether there exist mechanisms regulating the formation of this NK cell repertoire or not has been a matter of debate for years. Held et al found in 1996 that NK cells expressing two inhibitory receptors for an MHC class I allele were less frequent in mice expressing that allele than in other mice (*169*). This have since been confirmed by others (*59, 170-172*). Also in Ly49A-transgenic mice expressing the D^d ligand for this receptor, the frequency of cells expressing another D^d specific receptor Ly49G2 was reduced (*173*).

Ly49 receptor genes are assumed to be stably expressed once turned on. This is based on data where sorted Ly49+ cells were transferred and followed over 10 days without any observed changes in Ly49 expression (*174*). Based on this assumption and the data above two developmental schemes were proposed by Raulet et al. First, the sequential expression model proposes that every developing NK cell expresses individual

receptors in a cumulative way but in random order. At the same time interactions with MHC class I occur and as soon as the NK cell senses adequate inhibitory signal it will continue to mature with a stable Ly49 expression (*168*). The second model is a two step selection model in which NK cells with a stochastic Ly49 expression will be tested first for its ability to bind MHC class I and secondly that this binding is not too strong (*168*). In a recent analysis using mathematical modeling of these two processes fitted to experimental data from multiple MHC class I transgenic mice, we found that the two-step selection fit the experimental data better than the sequential acquisition model (*59*). It was also shown that an open parameter in the simulations improved fit in many instances suggesting that additional mechanisms would contribute to the shaping of the repertoire. Such mechanisms could involve influence on subset proliferation and/or survival.

Our high dimension flow cytometry protocol allows for the first time for full characterization of the near complete Ly49/NKG2A repertoires from MHC class I deficient and sufficient mice. In **Paper III** we present our data and model for the shaping of the NK cell repertoire in mice. To focus on quantitative MHC class I effects we use the $D^{d-/-}$, $D^{d+/-}$ and $D^{d+/+}$ model system whereby we can measure the influence of a given, well-characterized MHC class I allele on NK cell repertoire formation. When we stained individually for the 4 Ly49 receptors and NKG2A, measuring the frequencies of all 32 possible NK cell subsets a pattern was clear. Our results show that NK cell subsets expressing multiple receptors (3-5) are less frequent as compared to MHC class I deficient mice while the opposite is true for NK cells expressing 1 or 2 receptors, **Paper III, Fig. 2**.

To investigate a possible mechanism for this pattern we studied population dynamics of NK cell subsets and found that the frequency of pre-apoptotic cells at a given time in the spleen of the different mice correlated with the selection patterns, **Paper III, Fig. 3**. As this suggested that there might be a link between population survival, turnover and possibly proliferation and selection we decided to study this further. We found that NK cells positively selected were more responsive to IL-15 stimulation, proliferated more at limiting concentration of IL-15 *in vitro* and had a regulated expression level of the proapototic factor bim upon cytokine deprival, **Paper III, Fig. 4**.

We conclude from these data that there seems to be a link between MHC class I interactions, the formation of the NK cell repertoire and the sensitivity of NK cells to IL-15.

Future work should focus on establishing the molecular link between these different signaling pathways central to NK cell biology. Cross-regulation between the IL-15 signaling pathway and the NKG2D activating receptor pathway have recently been proposed. Horng et al disrupted the NKG2D pathway by directly targeting the adaptor DAP10 for ubiquitination. This made NK cells unresponsive to NKG2D stimuli but also to IL-15 (*175*). It is theoretically possible that the necessary tuning signal supporting NK cell function and survival to regulate function and repertoire formation is a complex signal of several simultaneous interactions. NK cell priming in the lymph node during an immune response requires the trans-presentation of IL-15 by dendritic cells (*82*). Trans-presentation of cytokines in contrast to signals from soluble cytokines

have been proposed to serve the purpose of giving context dependent signals, that is allowing for cytokine stimulation in conjunction with other simultaneous signals.

My hypothesis would thus be that NK cell interactions with dendritic cells trans-presenting IL-15 could provide the unique signaling context needed for NK cell tuning. If MHC class I interactions and possibly a third signal from NKG2D or other receptors occur simultaneously with IL-15 in this unique context of interaction, a different downstream signal could result as compared to when these stimuli are given separately. This in turn could provide the tuning signal and the adjustment of thresholds for activation, cytokine sensitivity etc.

NK cell repertoires in mice and men

The role of MHC class I in shaping the murine NK cell repertoire is well established even if the details of this process still remain unclear.

The repertoire of Killer Immunoglobulin-like Receptors (KIRs) in humans have been challenging to study due to the variation between individuals as a consequence of genetic variations excluded from the inbred laboratory mouse for which population genetics is largely ignored. Despite the difficulties, several groups have provided high-resolution data on NK cell repertoires in humans with interesting patterns (*58, 60, 66, 176-178*). It is not yet clear whether a MHC class I dependent mechanism shaping the KIR repertoire exists or not, some data suggest this.

To understand this better ongoing analysis by us in collaboration with the Malmberg, Uhrberg, Parham and Mehr groups will hopefully bring some clarity to this issue. We are working to reanalyze all generated data on human KIR repertoires and murine Ly49 repertoires in relation to MHC class I using comparable methods of calculation. In doing so we hope to find common and disparate patterns for future study. Using the increased power of calculations we hope to be able to settle the issue of MHC class I mediated control of the KIR repertoire in humans. Preliminary results confirm the expected pattern that interindividual variability is significantly greater in human data sets as compared to the murine data (Simon et al manuscript in preparation).

Non-MHC class I signals in the formation of the NK cell repertoire

NK cells are surrounded by cells expressing MHC class I and these molecules will continuously be ligated by NK cells, possibly providing continuous tuning input regulating both function and repertoire formation. Also, other abundant molecules could also in theory influence NK cells similarly.

CD244 (2B4) is a receptor expressed on all NK cells recognizing the CD2 family receptor CD48 expressed abundantly on most hematopoietic cells. Thus, NK cells could in theory be surrounded by cells expressing ligands for 2B4 and continuous interactions with such cells could contribute and possibly influence the continuous signals from MHC class I. As shown in **FIGURE 8**, we found that the Ly49 receptor repertoire in $2B4^{-/-}$ mice follow the same pattern as a $2B4^{+/+}$ (B6) mouse, as expected from the fact that they share the same MHC class I expression ($H\text{-}2^{b}$). The pattern of

selection of the NK cell repertoire is reminiscent of that described above and in Paper III for D^d-single mice with a enrichment of cells expressing individual Ly49 receptors, especially the strong self-receptor Ly49C and simultaneously a decrease of cells expressing multiple receptors. The interesting finding from these experiments were instead that the pattern of selection imposed by the presence of K^bD^b seems enhanced in the $2B4^{-/-}$ mice suggesting a balancing role for 2B4 signals on this selection. This is the first example of non-MHC class I mediated signals influencing the shaping of the Ly49/NKG2A receptor repertoire (Brodin, Chambers, Kumar, Manuscript in preparation).

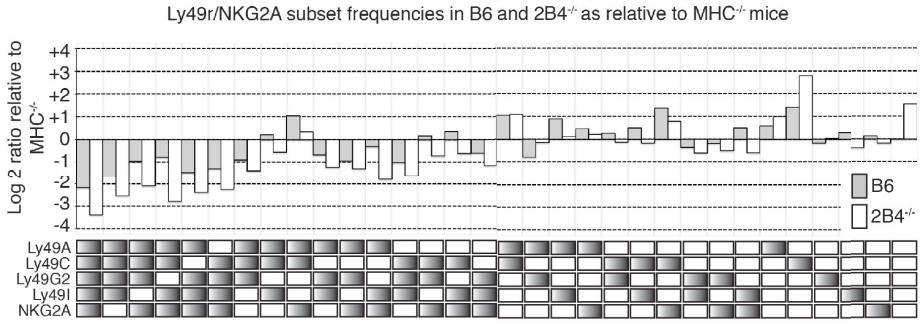

FIGURE 8. *Enhanced skewing of Ly49/NKG2A repertoire in 2B4 k.o mice.*
Relative frequencies of all NK cell subsets in 2B4 k.o and B6(wt) mice as relative log2ratios of the respective subset frequency in $MHC^{-/-}$ mice. (Brodin, Chambers, Kumar Manuscript in preparation)

Molecular mechanisms of NK cell tuning

The molecular mechanisms for how MHC class I mediated signals are translated into functional regulation in NK cells are unknown. The patterns of induced or suppressed NK cell function described above have not been explained at the molecular level.

The hyporesponsive phenotype of MHC deficient NK cells have been extensively studied, but no mechanistic explanation for its unresponsiveness to stimulation have been found. The activating receptor expression remains unaltered (*77, 144*) and the proximal signaling adaptors DAP10 and DAP12 are expressed at normal levels (*179*). Several gene expression profiles have been generated but none have provided a clear answer to NK cell hyporesponsiveness.

Also the signals downstream of Ly49-receptors providing the necessary signals for NK cell education remains elusive. It has been shown that the Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) motif of Ly49 is necessary for NK cell education (*76*) but its associated phosphatase SHP-1 was not. However, this has been controversial since other groups have described a hyporesponsive phenotype of NK cells from $SHP\text{-}1^{-/-}$ mice (*179*) and mice with a catalytically inactive form of SHP-1 (*180*). Clearly, more detailed studies of inhibitory and activating signaling pathways in hyporesponsive NK cells are needed.

Hyporesponsive NK cells can only be defined by functional tests. A surface marker would be highly useful to better characterize molecular differences between differently responsive NK cells. Hyporesponsive NK cells share some similarities with anergic T-cells and specific surface markers have been described for anergic T-cells (*181*). We therefor tested the expression of surface markers CD98, 4-IBBL and FasL (*181*) without finding any significant differences between MHC-deficient and wt NK cells (Data not shown).

Instead the best marker for NK cell responsiveness seems to be the inhibitory surface receptor Killer-cell lectin-like receptor G1 (KLRG1)(*182*) expressed at a higher level on educated than MHC-deficient NK cells (*151, 183*) and also quantitatively regulated in correlation with NK cell responsiveness on $D^{d+/-}$ and $D^{d+/+}$ NK cells, **Paper III, Fig. S2.** KLRG1 has been shown to be associated with a senescent T-cell phenotype (*182, 184*) but its function is still unclear. Its presumed ligands are cadherin family members. Interestingly, KLRG1 k.o mice exhibit normal responses to Lymphocytic choriomeningitis virus (LCMV) and MCMV, but also normal RMA/S rejection and IFN-γ responses to antibody mediated cross-linking of the activating NK1.1 receptor on NK cells (*185*). One study showed that NK cells on free-living, wild mice express higher levels of KLRG1, possibly suggesting a role for external influences on KLRG1 expression, such as infections not occurring in laboratory mice (*186*).

Clearly more work is needed before any mechanism of NK cell education can be described. The potential for using the KLRG1 surface marker to sort out cells with different MHC class I experience is very encouraging and should inspire more advances towards this end.

Cis-interaction between Ly49 receptors and MHC class I in tuning of NK cell responsiveness

It is well established that NK cells can interact with MHC class I both in trans on other cells as well as in cis where MHC class I ligands are expressed on the NK cell itself (*187-189*). The importance of the cis-interaction has been elusive but in a recent study Held et al have proposed a possible role for cis-interaction in regulating quantitatively NK cell responsiveness (*190*). The model was inspired by data showing how the ITIM-containing receptor CD22, a negative regulator of B-cell receptor (BCR) signaling (*191*), is in turn regulated by cis-interacting sialic acids in the B-cell membrane (*192, 193*). In normal situations most CD22 molecules are located in clusters distant from the BCR complex, unable to inhibit B-cell activation. These clusters are maintained by cis-interaction with sialic acids regulating the number of CD22 molecules in proximity of the BCR with a potential to inhibit activating signals is regulated (*192*).

Translating this to NK cells, Chalifour et al showed that cis-interactions between MHC class I and Ly49 receptors in the NK cell membrane serve to sequester Ly49 receptors away from the activating synapse where triggering occurs (*190*). Unengaged Ly49 receptors were able, by unknown mechanism to inhibit NK cell activation when accessing the synapse, even without ligating MHC class I on target cells (*190*). The model suggests that NK cells can quantitatively regulate their responsiveness by regulating the amount free or unengaged (in cis) Ly49 receptors (*190*).

One prediction form this model is that the expression level of MHC class I on NK cells could regulate responsiveness by the degree of Cis-interaction with Ly49 receptors. We tested this for NK cells expressing D^d in a hemi or homozygous fashion. To measure the amount of free, accessible Ly49 receptors available to interact in trans with MHC class I on other cells, we took advantage of the trogocytosis phenomenon where NK cells acquire MHC class I molecules ligated in trans (*194*). By co-incubation of NK cells with target cells expressing a D^d-GFP construct, NK cells will acquire D^d-GFP molecules depending on the amount of accessible Ly49 receptors on the NK cell. By measuring the amount of D^d-GFP on the NK cell accessible Ly49 receptors can be quantifie9

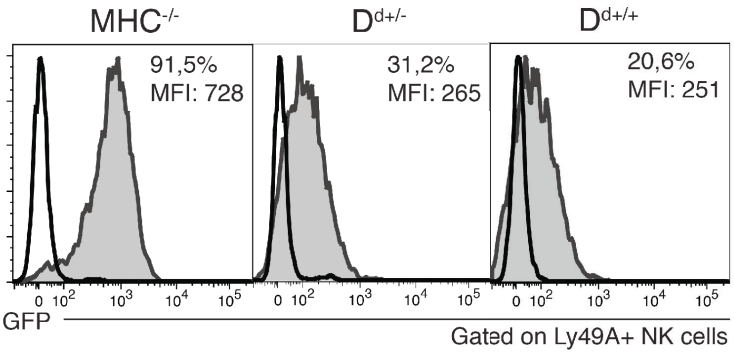

FIGURE 9. *Ly49A accessibility and thereby D^d acquisition depends on MHC class I expression on NK cells D^d-GFP fluorescence on Ly49A+ NK cells from the indicated mice after co-culture with EL4 D^d-GFP cells (Gray Histogram) or negative control (Open Histogram), Brodin, Kärre and Höglund, Manuscript in preparation.*

We found that $MHC^{-/-}$ NK cells all acquired D^d-GFP as expected from their lack of cis interaction and thus all Ly49 receptors would be accessible for trans-binding and trogocytosis. Interestingly, $D^{d+/-}$ NK cells expressing the Ly49A receptor picked up more D^d-GFP than did their $D^{d+/+}$ counterpart, **FIGURE 9.** This result shows that the expression level of MHC class I on the NK cell do in fact correlate with the amount of accessible Ly49 receptors for trans binding and possibly inhibition in the immune synapse. By inference this might also indicate that the Cis-binding hypothesis could explain the differences in responsiveness seen between identical Ly49 subsets from $D^{d+/+}$ and $D^{d+/-}$ mice, **Paper III**.

CONCLUDING REMARKS

In this thesis and over the years of work we have realized several important features of the MHC class I mediated regulation of NK cells. Firstly, the influence is clearly quantitative rather than binary meaning that NK cells are never either or, on or off but always everywhere in-between.

Also the influence of MHC class I affect multiple aspects of NK cell biology, from thresholds to activation, quality of responses to the shaping of the NK cell repertoire. We have proposed a theoretical model in which all these processes reflect a process of adaptation in the NK cell system to its current context. We have suggested this to be a continuously ongoing process of adaptation similar to other adaptive processes described in biology and that such adaptation is important for NK cells to remain sensitive to challenges even in a context that is always changing. Maybe similar processes of adaptation are continuously ongoing in all cells of the immune system and the body as a whole and maybe our ideas of NK cells will add a little piece to that never-ending puzzle of wonder that is biology.

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