NATURAL KILLER CELL INHIBITORY AND ACTIVATING RECEPTORS

– REGULATORY ROLE IN EFFECTOR FUNCTIONS AGAINST NORMAL AND TUMOR CELLS

Gustaf Vahlne

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LIST OF PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by their roman numerals:


III. Catrine M. Persson, Erika Assarsson, Gustaf Vahlne, Petter Brodin and Benedict J. Chambers. The non classical MHC molecule Qa1\(^b\) plays a critical role in the protection of mature dendritic cells from NK cell mediated killing. *Scandinavian Journal of Immunology (in press)*.

IV. Gustaf Vahlne, Sofie Becker, Petter Brodin and Maria H. Johansson. IFN-γ production and degranulation is differently regulated in response to stimulation in murine NK cells. *Scandinavian Journal of Immunology (in press)*.
In memory of my beloved mother
Cecilia Vahlne
ABSTRACT

Natural killer (NK) cells can mediate effector functions as cytokine production and degranulation dependent cytotoxicity against cells that have become aberrant by mutagenesis or by infection. As part of the innate immunity, the NK cells are most important in the direct early defense against infections, and also in indirect shaping of the later adaptive response. NK cells monitor cells by the use of inhibitory and activating receptors which interact with MHC class I and other ligands, some of which can be induced during cellular stress or by infections. The net outcome of the signals transduced via the inhibitory and activating receptors will either lead to activation of the NK cells and delivery of effector functions, or if the inhibitory signals predominate the NK cell will leave the target cell unaffected and continue to screen other cells. The most important ligands for NK cell inhibitory receptors are the major histocompatibility complex (MHC) class I molecules. One important feature of the system is that the NK cell in its quest of seeking out and destroying aberrant cells does not kill or harm cells which are healthy, a mechanism that is referred to as NK cell self-tolerance. Tolerance is in part maintained by the expression of self MHC class I molecules on normal cells which NK cells interact with continuously. However, upon an infection, cellular mutagenesis or dysfunction, the MHC class I expression can be impaired, rendering cells susceptible to NK cell attack. This is referred to as missing self recognition. NK cells can also attack cells from other individuals, e.g. after haematopoetic transplantation. Even though allogeneic cells express MHC class I molecules, they often fail to present the “self” MHC class I molecules that the NK system has been educated to scan for. Many, but not all, tumors downregulate MHC class I expression or upregulate activating ligands for NK cells. Tumor cells are therefore often not recognized as aberrant cells by the NK cells.

The first studies in this thesis have utilized mice expressing different single MHC class I genes in order to characterize the quantitative and qualitative impact that different MHC class I alleles have on the education of missing self recognition by NK cells. In particular, these studies focused on the influence of host MHC on expression of inhibitory NK cell receptors in the Ly49 family. A second set of studies addressed whether, once tolerance has been established by MHC guided education, it is possible to break it by antibody blockade of inhibitory Ly49 receptors in such a way that tumor rejection is induced or enhanced, while tolerance is maintained towards healthy cells? The focus was then set on NK cell inhibitory receptors in interactions with dendritic cells (DCs). These studies addressed whether NK cells can discriminate between mature and naïve DCs, and in particular the role played by the non-classical MHC molecule Qa-1 on dendritic cells in interactions with NKG2A⁺ NK cells? A final series of studies addressed whether two main effector functions, IFN-γ secretion and degranulation (associated with cytotoxicity), are coordinately regulated during NK cell maturation and under different conditions of stimulation.

Different MHC class I alleles exerted different educating impact for missing self recognition (the strength by which NK cells reject cells missing the relevant MHC gene). Furthermore, the observations suggested some rules for how this impact is affected when two or more MHC class I alleles are expressed together. The data also
suggested that the educating impact of each MHC gene depends on the number of educated NK cells as well as the efficacy state that each NK cell has been educated to by the gene. Regarding attempts to interfere with NK cell tolerance, the studies demonstrated that blockade of the inhibitory NK cell Ly49C/I receptors in B6 mice induced NK cell mediated elimination of MHC class I expressing tumor cells without breaking of tolerance towards autologous healthy haematopoietic cells. This effect persisted during continuous receptor blockade for two week. Tolerance of NK cells towards mature autologous DCs depended on the interaction of the inhibitory receptor NKG2A and the ligand Qa-1 on the DCs. Last but not least, studies at the single cell level demonstrated that secretion of IFN-γ and degranulation in response to different stimuli are not coordinately regulated within the total NK cell population. The effector response was influenced by activation status as well as maturation stage, the latter defined by different expression patterns of the surface markers CD27 and Mac-1.
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<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>β₂m</td>
<td>β₂-microglobulin</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DAP-10, -12</td>
<td>DNA activating protein of 10 or 12kD</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte monocyte stimulatory factor</td>
</tr>
<tr>
<td>HSCT</td>
<td>Haematopoietic stem cell transplantation</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>KARAP</td>
<td>Killer cell activating receptor-associated protein</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer cell immunoglobulin-like receptor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Ly49r</td>
<td>Ly49 receptor</td>
</tr>
<tr>
<td>MCMV</td>
<td>Murine cytomegalovirus</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKC</td>
<td>Natural killer gene complex</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>uNK</td>
<td>Uterine natural killer</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 GENERAL IMMUNOLOGY

The body is constantly under siege by various microbes, nonetheless these attacks rarely result in disease. To understand how the immune system can handle these attacks it is important to understand the two different arms involved in immune surveillance namely: innate immunity and adaptive immunity. Innate immunity is mediated by cells expressing germ line encoded receptors and act as an early defense able to recognize patterns common to many microbes while the adaptive immunity is mediated by T- and B- cells which have receptors encoded by genes that have undergone gene rearrangement. These receptors recognize specific antigenic epitopes, usually expressed on only one molecule of one microbe.

1.1.1 Innate immunity

Innate immunity is referred to as the first line of defense. It does not only involve different subsets of leukocytes but also the important epithelial cells which defend our body by building the skin and the mucosal lining. Some epithelial cells can also secrete antibacterial peptides and cytokines. Once the microbes have penetrated the epithelia the leukocytes start to respond. The first cell an invading microbe is likely to encounter is a macrophage. Macrophages phagocytoze the invading bacteria and are in parallel activated via pattern recognition receptors e.g. toll like receptors recognizing bacterial lipopolysaccharide (LPS). The macrophages start to produce cytokines and chemokines which attract other members of the immune system. The cytokines and other secreted mediators also affect endothelial cells in blood vessels, which are induced to express ligands allowing adhesion and extravasation of leukocytes. Some cytokines can act directly to inhibit viral replication, e.g. interferon-α or -β. Next type of cells to respond to an invading microbe are usually the neutrophilic granulocytes, which are recruited to the site of inflammation and help the macrophages to phagocytoze the invading microbes. Through secretion of cytokines such as IL-12 and IL-15, macrophages can also activate the natural killer (NK) cells. NK cells respond by producing IFN-γ which can inhibit viral replication, activate macrophage to phagocytoze and eliminate bacteria more efficiently and promote the upregulation of MHC class I and II. NK cells can also use perforin and granzyme to kill cells which show signs of infection by the expression of stress response induced molecules and/or reduced MHC class I expression.

Furthermore, not only leukocytes and local epithelia and endothelia participate, but also hepatocytes which produce soluble proteins from the complement family and “acute phase reactants”. Complement can act directly or via acute phase reactants (or via antibodies, appearing later in the immune response) to kill invading pathogens and further enhance the local vascular response to infection. These are all part of a chain of events which will lead to the activation of the adaptive immune response mediated by B- and T-lymphocytes after presentation of specific parts of the microbes by the major
histocompatibility complex (MHC) class I and II. The latter are present on so called professional antigen presenting cells (APCs). These cells are macrophages, DCs and B-lymphocytes. The antigen presentation to the B- and T-lymphocytes takes place after the antigen presenting cells have migrated to the lymph nodes.

### 1.1.2 Adaptive immunity

Once the APCs have interacted with the invading microbe and after engagement of pattern recognition receptors, like Toll like receptors and scavenger receptors, the APCs start to migrate to the lymph nodes via afferent lymphatic vessels where they present microbial proteins to T- and B-lymphocytes in the adaptive line of defense. The activation of T-lymphocytes will in turn lead to the generation of antigen specific effector cells, which can be CD4$^+$ T helper cells, CD8$^-$ killer cells, and antibody secreting B-lymphocytes. The activation of the B-lymphocytes and in many situations also the CD8$^+$ T-lymphocytes require stimulation from CD4$^+$ T helper cell via cytokines. This is a set of cytokines different from the early proinflammatory cytokines mentioned above. IL-2, IL-4, IL-5, IL-10 and IL-17 are examples of T-lymphocyte secreted cytokines.

Like all leukocytes, T- and B-lymphocytes are produced in the bone marrow. However, they differ from cells involved in the innate immunity in one important respect: they do not express germline encoded receptors to detect foreign antigens. Instead their receptors are generated during development of the cells via DNA rearrangements, combining different gene segments in a stochastic process leading to the expression of one specific receptor type per T- or B-lymphocyte and its clonal progeny. Since some of these randomly generated receptors are either useless or dangerous, there are processes for elimination of the clones that carry them. This clonal selection takes place mainly in the bone marrow for B-lymphocytes, and in the thymus for T-lymphocytes. In the induction of the adaptive response in the lymph node, it is the T- and B-lymphocyte clones with a relevant receptor for the invading microbe that are activated to proliferate, leading to clonal amplification of the relevant receptors. The clonal selection thus continues throughout the mature life span of these cells. The activation of a clone specific of B-lymphocytes will result in B-lymphocyte differentiation which in turn will lead to the development of plasma B-lymphocytes which produce and secrete a soluble form of the immunoglobulin receptor, the so called antibodies.

Activated CD8$^+$ T-lymphocytes and antibodies produced by activated plasma B-lymphocytes are transported in the blood to the site of inflammation directed by chemokines released in the inflamed tissue. The CD8$^+$ T-lymphocytes recognize the infected cells by the microbial peptides presented by MHC class I molecules. Antibodies specifically bind to the microbes, microbial products and infected cells. Through the Fc-part of the bound antibody the microbe will be recognized by phagocytic cells or the complement system resulting in the destruction of the microbe or the infected cell. CD4$^+$ T-lymphocytes enhance macrophage mediated destruction of pathogens. This phase of the immune response is thus characterized by intensive collaboration between the innate and the adaptive components.
1.1.3 MHC class I molecules

1.1.3.1 Major histocompatibility complex class I (MHC class I)

The primary function of MHC class I molecules is to present foreign peptides to CD8\(^+\) T-lymphocytes. However, MHC class I molecules can also be recognized by NK cell receptors. The MHC class I genes (or human leukocyte antigen, HLA in humans) are located on chromosome 17 in mice and on chromosome 6 in humans [1]. There are three MHC class I loci denoted H2-K, D and L in mice and HLA-A, B and C in humans [1]. MHC class I molecules are expressed on all cells with the exception for erythrocytes. Some cells, e.g. neurons express only low levels of MHC class I. MHC class I molecules present mainly peptides from internally expressed proteins which have been cleaved by proteasomes. The MHC class I heavy (or \(\alpha\)-) chain consists of three domains denoted \(\alpha_1\), \(\alpha_2\) and \(\alpha_3\) where \(\alpha_1\) and \(\alpha_2\) are the most polymorphic. The heavy chain (45kD) associates non-covalently with the non-polymorphic \(\beta_2\)-microglobulin (12kD) [2] and a 8-9 amino acid peptide, of self or non-self origin, to form a stable MHC class I complex [3-13]. The peptides are cleaved by the proteasome or other proteases and are transported to the ER via TAP where they are loaded onto MHC class I molecules [14-17]. Due to the high polymorphism, the binding grooves of MHC class I alleles can bind different sets of amino acids which results in a diverse peptide presentation [17]. Thus, it is hard for microbes to evade an immune response since at least some peptides derived from the microbes will most certainly bind to one or several of the MHC class I molecules expressed by the infected host and thus be presented to CD8\(^+\) T-lymphocytes. Mice and human also express non-classical MHC class Ib molecules. These molecules have low or no polymorphism; some have a closed groove and do not bind antigen, whereas other have an open groove specialized for binding of certain peptides or lipids. There are several MHC class Ib molecules which bind NK cell receptors: HLA-E, -F, -G, MIC, CD1 and ULBPs in humans; Qa-1,-2, -10, MILL, CD1, Rea-1 and H60 in mice [17].

1.2 NATURAL KILLER CELL BIOLOGY

NK cells were first discovered in the mid 70’s as granulolytic lymphocytes distinct from T- and B-lymphocytes. NK cells can without prior activation kill tumor cells [18-21]. A first clue to how the NK cells recognize their target cells came in the late 80s by Klas Kärre and co-workers, when they identified that the recognition of MHC class I by NK cells acted as a main inhibitor of NK cell mediated effector function [22]. Thus NK cells could recognize aberrant cells that had been transformed in such a way that they lacked or were severely impaired in their cell surface expression of MHC class I. This phenomena was later named the “Missing self hypothesis” [23]. NK cells have been shown to mediate several different effector functions, as well as immune-regulatory functions. Below I will shortly review NK cells in different contexts.
1.2.1 NK cell Receptors

1.2.1.1 Ly49 receptors

-The Ly49 gene localization and alleles

The Ly49 genes belong to the largest family of NK cell activating and inhibitory receptor genes known in mice. The Ly49 receptors are type II glycoproteins consisting of a homodimer linked with a disulfide bond. They belong to the C-type lectin-like family of receptors situated on chromosome 6 in the NK gene complex (NKC) stretching in a cluster of 620kb [24-28]. The most studied mouse strain is C57BL/6 where 16 Ly49 genes have been identified including pseudogenes (a-n, q and x) and 3 gene fragments (α, β and γ) [25]. a-j and q are active genes while k-n and v are pseudogenes [26, 29-32]. The Ly49 receptors (Ly49r) can exert inhibitory or activating function depending on the intracellular sequence of the receptor protein. Inhibitory Ly49r signal via immunoreceptor tyrosine-based inhibitory motif (ITIM) upon MHC class I recognition [33]. The number of Ly49 loci vary from strain to strain, and there are also allelic variants for many of the loci. Some of the Ly49r like Ly49V which is a pseudogene in C57BL/6 mice probably have functional capabilities in the 129/J strain where it can mediate inhibitory signals via an ITIM sequence [26, 34]. Table 1 displays a composed list of Ly49r expressed in C57BL/6, 129/J, CBA/J and NOD mice and their putative ligands (modified from Anderson et al [26]).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ly49</th>
<th>Putative ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>A</td>
<td>D^c, D^d, D^e</td>
</tr>
<tr>
<td>B6</td>
<td>B</td>
<td>?</td>
</tr>
<tr>
<td>B6</td>
<td>C</td>
<td>H-2^b, K^d, D^c, D^k</td>
</tr>
<tr>
<td>B6</td>
<td>D</td>
<td>D^d, D^e, D^p</td>
</tr>
<tr>
<td>B6</td>
<td>E</td>
<td>?</td>
</tr>
<tr>
<td>B6</td>
<td>F</td>
<td>?</td>
</tr>
<tr>
<td>B6</td>
<td>G</td>
<td>D^d, L^d</td>
</tr>
<tr>
<td>B6</td>
<td>H</td>
<td>D^c, D^d, D^e</td>
</tr>
<tr>
<td>B6</td>
<td>I</td>
<td>K^d</td>
</tr>
<tr>
<td>B6</td>
<td>J</td>
<td>?</td>
</tr>
<tr>
<td>CBA/J</td>
<td>L</td>
<td>H-2^h</td>
</tr>
<tr>
<td>NOD</td>
<td>M</td>
<td>?</td>
</tr>
<tr>
<td>129/J</td>
<td>O</td>
<td>D^c, D^d, D^e, L^d</td>
</tr>
<tr>
<td>NOD &amp; 129/J</td>
<td>P</td>
<td>D^d</td>
</tr>
<tr>
<td>B6</td>
<td>Q</td>
<td>?</td>
</tr>
<tr>
<td>129/J</td>
<td>R</td>
<td>D^3, D^k, L^d</td>
</tr>
<tr>
<td>129/J</td>
<td>S</td>
<td>?</td>
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</tr>
<tr>
<td>NOD</td>
<td>X</td>
<td>H-2^c, H-2^k</td>
</tr>
</tbody>
</table>

Table 1. List of Ly49 receptors expressed in different murine strains and their putative ligand. Green receptors are activating and red receptors are inhibitory.
- Ly49 and related genes in other species

Rats have been shown to express Ly49r in their NKC on chromosome 4 [35]. To date approximately 35 different alleles have been identified, including activating, inhibitory and pseudogenes [35, 36]. Much like the murine counterparts, some of the rat Ly49r downregulate the NK cell effector function by interacting with MHC class I [37]. One Ly49 gene (Ly49L) has been shown to be expressed in humans although it appears to be non-functional [38]. Other species with functional Ly49r are: Orangutan, baboon and cattle [39] However, humans, other primate species, cattle and pig express another family of activating and inhibitory MHC class I specific NK receptors, belonging to the immunoglobulin super family of receptors. These receptors are expressed as monomers consisting of two-three immunoglobulin domains. The genes are located on chromosome 19 and are designated killer immunoglobulin-like receptors (KIRs) [39-43]. Similar to the Ly49r found in rodents some of these receptors can mediate inhibitory signaling via ITIM in their cytoplasmic tail or activating signaling via associated immunoreceptor tyrosine-based activation motif (ITAM) bearing adaptor molecules [44-46]. Another similarity between the KIR and the Ly49 family of receptors, regardless of species, is that individuals (or inbred strains of laboratory animals) differ with the respect to the number of loci, as well as the alleles at each locus, adding up to considerable polymorphism of the population.

- Ly49 receptor signaling

  - Inhibitory signaling

As noted above the ITIM sequence is present in murine Ly49r, human KIR and in inhibitory NKG2A receptors in both species [47, 48]. The ITIM is located in the intracellular domain of the receptors recognized as I/S/T/LxYxxL/V where x denotes any amino acid. When the receptor is engaged with its corresponding ligand, the tyrosine is phosphorylated and tyrosine phosphatases such as SHP-1 are recruited to the ITIM via the SH2 domains where it is activated [47, 49]. Upon SHP-1 activation, SHP-1 can bind and dephosphorylate SLP-76 and Vav1, downstream of the signals to be transmitted from activating receptors [50-52], thus quenching the activation or keeping the cells in a balance hindering them from executing effector function (Figure 1). SHP-2 has also been shown to inhibit T-lymphocyte receptor signaling by binding SLP-76 [53], however, it is not yet clear if NK cells are regulated in the same fashion.

  - Activating signaling

While the Ly49 inhibitory receptors can mediate inhibition via phosphorylation of the ITIM and subsequent activation of signaling proteins, the activating Ly49D [54, 55] and Ly49H receptors [54, 56] lack an intracellular signaling domain. They need to signal via the adaptor molecules DAP12 [56], which express an immunoreceptor tyrosine-based activation motif (ITAM) [46]. The ITAM has a consensus motif of A/GxxTxx/L/I x6−8 Txx/L/I [57]. Upon receptor stimulation the ITAM tyrosine is phosphorylated resulting in activation of ZAP-70 and Syk tyrosine kinases via their SH2 domains [58, 59]. This results in NK cell activation and the initiation of
subsequent effector functions, unless the balance is shifted by quenching from the inhibitory pathways as discussed above.

- **Ly49 binding to MHC class I**

So far only two different Ly49-MHC class I interactions have been visualized by crystallography (Ly49C/H-2K\(^b\) and Ly49A/H-D\(^d\)). Both of these interactions mediate inhibitory signals. The first interaction studied in this way was the structure of Ly49A/H-D\(^d\) co-crystals, which showed that Ly49A can interact with two potential sites, designated site 1 and 2, of the MHC class I molecule [60]. In these co-crystals, Ly49A bound at site 1 with one Ly49 subunit to H-2D\(^d\) on one side of the MHC class I peptide binding platform while at site 2 the Ly49A bound with its homodimer into the cavity between \(\alpha 2\), \(\alpha 3\) and \(\beta 2m\) of H-2D\(^d\). Site directed mutagenesis has revealed that it is site 2 binding which is necessary for interaction between receptors and ligands in solution and for functional cellular interactions [61]. In studies by Dam and co-workers [62] the Ly49C receptors seemed to have a more closed conformation in its interaction with H-2K\(^b\), which was similar to the Ly49A interaction in site 2. They hypothesized that the Ly49r undergo dynamic changes between open and closed conformation. KIR bind to human MHC class I molecules in quite a different manner, by engaging part of the \(\alpha\)-helices and the peptide, in a similar way as T-cell receptor bind MHC molecules. One big difference between T-lymphocyte and NK cell MHC class I recognition is that T-lymphocytes recognize specific peptides displayed in the MHC class I binding grove, while NK cells are less discriminative. Some Ly49r bind independently of the peptides (e.g. Ly49A) while some are influenced by general features of the peptide sequence.

**Figure 1.** Schematic figure of signaling by activating and inhibitory Ly49 receptors and the subsequent downstream signaling events.
(e.g. Ly49C), without relation to the origin of the peptide (e.g. self versus non-self) [63, 64]. This influence of the peptide may seem paradoxical, given that Ly49C does not contact the peptide, but rather bind the MHC class I molecule beneath the antigen binding groove, contacting also the α3 domain and β2-microglobulin. A likely explanation is that peptide binding induces conformational changes in the MHC molecule transmitted through the floor of the antigen binding groove.

- Ly49 receptor expression and specificity

The Ly49r alleles are expressed on NK cells in a partly stochastic order [65] so that each receptor is expressed in a given proportion of NK cells. This given proportion varies between different receptors. The expression of each receptor is largely independent of the expression of other receptors. Accordingly, each NK cell can express either no, one or any combination of more than one receptor. The probability for expression of multiple receptors follows the “product rule”: if the frequency of NK cells expressing Ly49x is x% and the frequency for Ly49y is y%, then the frequency of double positive Ly49 xy cells is x multiplied by y. [66]. For example, Ly49C is expressed in about 40% of NK cells, while Ly49A is expressed in about 15% of NK cells; accordingly 0.40 x 0.15 = 0.06 (6%) of NK cells are double positive for these two receptors. Furthermore, usually only one out of the Ly49 alleles are expressed. However, this may be a reflection of the “product rule”, rather than a mechanism for allelic exclusion [67]; both alleles can be expressed at the same time in a NK clone [68]. The mechanisms determining whether a given receptor gene should be expressed or not are unclear. The Ly49r share this general “variegated” expression pattern with the human KIR.

- Ly49 surface expression – influence of host MHC class I genes

As shown in figure 1 each Ly49r interacts preferentially with one or a few different alleles of the MHC class I genes. One might assume that each inhibitory Ly49r would be co-expressed with their corresponding ligand in order to ensure that the NK cells are inhibited and do not kill healthy cells. However, the genes for Ly49r and MHC class I are located on different chromosomes and are therefore not co-inherited [1, 24]. It nevertheless appears that the Ly49 expression pattern is somatically influenced by host MHC. First, there is a marginal reduction in the size of NK subsets expressing multiple receptors for a given ligand when that ligand is present in the host [69]. Secondly, there is a reduced cell surface expression level of Ly49r on each NK cell when the corresponding MHC ligand(s) is present in the host, e.g. Ly49C expression is downregulated in H-2b mice in comparison to H-2d or β2m-/- mice [69-71]. However, there is no corresponding difference at the Ly49A mRNA level [68]. One clue to the paradox of down regulation of self-inhibitory receptors was addressed by Doucey et al when they demonstrated that not only could Ly49A interact with Dβ molecules in trans with other cells, Ly49A could also bind with Dβ in cis, with the NK cells own Dβ molecules. The cis interaction by Ly49A with Dβ limits the interaction with Dβ on target cells and thus the inhibition is decreased [72]. This finding can also in part explain why β2m-/- NK cells express higher levels of all known Ly49r since they almost completely lack the expression of MHC class I and hence no or limited cis interaction can occur. The downregulation observed might in fact not be true down-regulation but
rather masking of the Ly49 making them unreachable by antibody binding. However, this is not the only explanation. There are now two explanations for the downregulation of the Ly49r on the protein level is 1) the binding of Ly49r with its cognate MHC on the cells own membrane (\textit{cis}) [73], 2) the interaction of Ly49r with MHC on other cells (\textit{trans}) [73, 74].

\subsection*{1.2.1.2 CD94/NKG2 family of receptors}

The CD94/NKG2 receptors are dimers linked with disulfide bonds. They belong to the C-type lectin like family of receptors and are expressed in the NKC in both mice and humans. These genes are located on chromosome 6 and 12, respectively. CD94/NKG2 receptors can either form inhibitory heterodimers (CD94/NKG2A and -B), activating heterodimers (CD94/NKG2C, -E and –H) or an activating homodimer NKG2D [75-82]. CD94/NKG2A interacts with the non classical MHC Qa-1 in mice and HLA-E in humans. These homologous non-classical MHC class I molecules present mainly leader sequences from some MHC class I proteins [76, 77, 80, 83-87]. Failure to express the right MHC class I leader sequences can thus lead to reduced expression of Qa-1 or HLA-E, which can result in reduced inhibitory signaling via NKG2A. NKG2C and -E receptors promote activating signals upon ligand engagement with the non-classical MHC class I molecules HLA-E and Qa-1 [84, 88], while NKG2D interacts with the ligands MICA, -B, ULBPs in humans, or retinoic acid early inducible 1 (Rae-1) and H60 minor histocompatibility gene in mice [89-91]. These ligands are induced by cellular stress and the DNA damage response, common to many infected and transformed cells.

In humans NKG2D can only be expressed in one isoform containing a long cytoplasmic tail (NKG2D-L). However, the murine gene can splice into two different isoforms resulting in either NKG2D-L or NKG2D-S (S, for short cytoplasmic tail). Similar to Ly49D and –H, NKG2D cannot mediate activating signals by itself but need to associate with adaptor proteins to do this. NKG2D-L associates with DAP10 while NKG2D-S can associate with both DAP10 and DAP12 [92, 93]. DAP10 which lacks an ITAM can phosphorylate a tyrosine in its cytoplasmic tail resulting in a downstream signaling independent of members from the Syk family protein tyrosine kinases. This eventually leads to NK cell cytotoxic function [94].

\subsection*{1.2.1.3 NKR-P1 family receptors}

- \textit{Nkrp1} gene family

The \textit{Nkrp1} genes are located on chromosome 6 in the mouse and belong to the C-type lectin-like receptors. So far seven genes have been identified (\textit{a, g, c, b, d, f} and \textit{e}) [95, 96]. Both activating and inhibitor NKR-P1s have been identified e.g. NKR-P1C associates with an adaptor molecule which mediate activating signals via its ITAM sequence [57, 97]. Furthermore, the inhibitory NKR-P1B signals via ITIM sequences much like the inhibitory Ly49r [98]. The NKR-P1 ligands are as the NKR-P1s C-type
lectin-like in structure and are members of the osteoclast inhibitory lectins (Ocil) or the C-type lectin-related (Clr) gene family and are located between the NKR-P1 genes [96, 99, 100]. Similar to the MHC class I molecules the Ocil/Clrs are frequently downregulated in tumors enabling “missing self” recognition [100]. The fact that the NKR-P1s and the Ocil/Clrs are genetically linked secures that the receptors and the corresponding ligands can be co-inherited enabling a safe immunoregulation by these receptors.

1.2.1.4 2B4 family of receptors

- 2B4 expression and function on NK cells

The NK cell receptor 2B4 belongs to the immunoglobulin superfamily which is located on chromosome 1 in mice [101, 102] and chromosome 1 in humans [103]. 2B4 has been suggested to be an activating NK cell receptor since the blocking of 2B4 increased NK specific lysis of YAC-1 targets much like the blocking of the activating receptor NK1.1 [102]. However, one can interpret these data to mean that blocking of 2B4 prevents inhibitory signaling upon target cell interaction and thus that the 2B4 is inhibitory. In recent studies, a B16 melanoma transfected with the ligand for 2B4 (CD48) induced less metastasis when inoculated in wildtype mice than in C57BL/6 mice deficient in 2B4, while CD48⁺ and CD48⁻ B16 melanomas were rejected equally well in the 2B4 deficient mice. This suggests an inhibitory effect of 2B4 on NK cells [104]. Two studies have demonstrated that 2B4 can be expressed in two different splice variants, one with short cytoplasmic tail (2B4-S) and one with long cytoplasmic tail (2B4-L) [105]. 2B4-L has immuno-receptor based tyrosine switch motif (ITSM) in its intracellular part [101] and has been shown to associate with SHP-2 in the rat NK cell line RNK-16 transfected with 2B4 [106] suggesting inhibitory function. However, RNK-16 transfected with 2B4-L demonstrated activating effector functions.

As mentioned earlier 2B4 is also expressed on human NK cells. However, here 2B4 seem to have an activating role [107]. Further studies need to be performed in order to fully understand the effect of 2B4 signaling on NK cell effector function and its biological significance.

1.2.1.5 Natural cytotoxicity receptors

Human NK cells can also express a series of activating immunoglobulin-like receptors termed natural cytotoxicity receptors (NCRs), NKp30, NKp44 and NKp46 (NKp46 also has a murine homologue [108]), [109-111]. There are some suggested ligands for NKp30, -44 and -46, but these have been questioned and further research is necessary in order to obtain a conclusive picture [112-116]. NKp30 and NKp46 associate with the adaptor molecule CD3ζ [59, 110, 111, 117-119] in order to mediate activating signals, while NKp44 associates with the adaptor molecule DAP12 [110]. NKp46 may be the
first marker expressed by all (and nothing but) mature NK cells. NKp30 appear to have an important role in NK-DC interactions.

1.2.2 Effector functions and cytokine regulation

NK cells are in a resting state in the blood and in peripheral tissues. Upon an infection the NK cells are activated by type I interferons [120-122] resulting in increased NK cell activity [123]. Furthermore, NK cells can be cultured in vitro in IL-2 which augments NK cells cytotoxicity and induce them to proliferate [123]. IL-2 also activates NK cells in vivo. IL-12 and IL-15 have also been demonstrated to activate NK cells which results in increased cytotoxicity, in IFN-γ, TNF-α, and GM-CSF production, in ADCC and in proliferation [124-127]. When NK cells interact with target cells a tight junction or “immunological synapse” (NK-IS) is formed between the target cell and the NK cell. The first NK cell synapse to be described was the inhibitory synapse, i.e. when NK cells interact with healthy cells and detach without killing them (iNK-IS) [128]. Later the NK cell activating synapse was described and was termed the cytotoxic NK cell immune synapse (cNK-IS). Upon synapse formation NK cells will either start to engage effector function or leave the target cell depending on the strength of signals transferred via NK cell activating and inhibitory receptors [128, 129]. When the activating signals predominate the NK cells will start to reorient their secretory apparatus towards the target cell and degranulate [130, 131], resulting in the release of several different effector proteins. The three most important effector cytotoxic effector proteins are: Granzyme A and –B [132] and Perforin [133-135]. Perforin will disrupt the membrane of the target cell making pore forming holes [135]. The pores enable Granzyme A and -B to enter the cytoplasm of the target cell engaging the cell to go into apoptosis [136-138]. Another consequence of activation is secretion of IFN-γ, which can act directly on viral replication but is also important for the upregulation of MHC class I and II and activation of macrophages [139-141]. Thus, during an infection NK cells are recruited to the inflamed site and start to produce IFN-γ. In turn, the infected cells will upregulate MHC class I and II which will enable greater peptide presentation for CD8⁺ and CD4⁺ T-lymphocytes. Infected or phagocytizing macrophages become more efficient at eliminating pathogens under the influence of the IFN-γ secreted by NK cells.

1.2.3 NK cell subsets

1.2.3.1 Human NK cell subsets

As noted above, one can divide the NK cell population into subsets with different specificity (but otherwise similar function) according to their expression of Ly49r (or in humans, KIR). There is another way of defining subsets according to general function, maturation or tissue localization that is becoming gradually more important. For historical and other reasons, it is pertinent to introduce this research from the point of human NK cells, which are defined as CD3⁻ and CD56⁺ [142]. Human NK cells where
in the late 1980s divided into two distinct populations characterized by the expression of the cell surface marker CD56 [142-145]. It was further demonstrated that the CD56\textsuperscript{bright} NK cells where the predominant producers of IFN-γ, TNF-β, GM-CSF, IL-10 and IL-13 [146], while CD56\textsuperscript{dim} NK cells exerted more efficient cytotoxic capabilities [147]. The CD56\textsuperscript{bright} NK cells usually express no or only low levels of NK cell inhibitory KIRs but higher levels of the inhibitory receptor NKG2A. While all CD56\textsuperscript{bright} NK cells are NKG2A\textsuperscript{+} only half of the CD56\textsuperscript{dim} NK cells are so [148, 149]. CD56\textsuperscript{bright} NK cells are predominately found in human secondary lymphatic organs where they can stimulate DCs and T-lymphocytes, promoting adaptive immune responses, while CD56\textsuperscript{dim} NK cell are found in peripheral blood where they can exert their effector function by direct engagement and subsequent killing of aberrant cells. CD56\textsuperscript{dim} NK cells also express higher levels of the Fc binding receptor CD16, enabling NK cells to bind and kill cells which have been coated with antibodies (antibody dependent cytotoxicity, ADCC) [147]. There are additional subsets of human NK cells relating to certain physiological or pathological conditions. In pregnancy the major lymphocyte population in the placenta during the first trimester is an NK subset characterized as CD56\textsuperscript{bright} CD16\textsuperscript{−} but strongly positive for KIR (this will be discussed in more detail below) During an HIV infection, a CD56\textsuperscript{−} NK population emerges in many patients.

1.2.3.2 NK cell development and subsets

NK cells originate from a common lymphoid progenitor (CLP) which gives rise also to T- and B-lymphocytes [150, 151]. The CLP further differentiates into a T/NK progenitor (T/NKP) which can only mature into T or NK cells [152-156]. The earliest committed NK progenitors (NKP) express the IL-2 and IL-15 receptor common β subunit (CD122) [155, 157]. The differentiation from NKP to mature NK cells have been proposed to compromise at least 5 different stages [158, 159]. The first stage is as mentioned above the CD122\textsuperscript{+} T/NKP cells. The second stage involves acquisition of different receptors e.g. NKR-P1C (NK1.1), CD94/NKG2, NKG2D, integrin α\textsubscript{v}hi, integrin α\textsubscript{v}lo, Mac-1lo and CD43lo [158]. In the third stage the NK cells acquire Ly49r and c-Kit [158]. In the fourth stage the NK cells downregulate integrin α\textsubscript{v} and acquire DX5 and KLRG1 followed by the expansion of the NK cells, still in the bone marrow [158, 160]. The NK cell can at this stage produce low levels of IFN-γ and their cytotoxicity function is also modest. The expansion of NK cells at this stage seems reasonable since the functional NK cell repertoire of Ly49r and CD94/NKG2 can be used in order to sense the MHC class I environment and hence assist in making the NK cell tolerant or educated before the migration from the bone marrow to peripheral tissues. At the last stage (V) the NK cells upregulate Mac-1 and CD43 while the integrin α\textsubscript{v} expression is absent. The NK cells are now fully functional and can mediate all effector functions.

As described above human NK cells can be divided into two distinct subsets determined by their CD56 expression (CD56\textsuperscript{bright} and CD56\textsuperscript{dim}). However, mice do not express a homologue of human CD56. Murine NK cells are phenotypically characterized as NK1.1\textsuperscript{+}/DX5\textsuperscript{+}, CD3\textsuperscript{−}. Resent data by Hayakawa et al [161] suggest
that murine NK cells can be divided into three functionally distinct subsets with different characteristics determined by the expression of CD27 and Mac-1 (Mac-1<sup>hi</sup> CD27<sup>lo</sup>, Mac-1<sup>hi</sup> CD27<sup>hi</sup>, Mac-1<sup>lo</sup>CD27<sup>hi</sup>). These NK cells have distinct functional properties. The CD27<sup>lo</sup> NK cells express higher levels of inhibitory Ly49C/I receptors than the CD27<sup>hi</sup> NK cells, indicating that the CD27<sup>lo</sup> NK cells maybe more prone to be inhibited by self-MHC class I molecules. However, the CD27<sup>hi</sup> cells have lower expression of the inhibitory receptor NKG2A. Indeed, the CD27<sup>hi</sup> show an increased killing capacity against the prototypic NK cell targets YAC-1 and RMA-Rae-1β (RMA transfected to express the ligand for the activating receptor NKG2D). Furthermore, the CD27<sup>hi</sup> cells were more prone to produce IFN-γ when stimulated with IL-12, IL-18 or with IL-12 in a combination with IL-18, or by DCs alone, while the CD27<sup>lo</sup> NK cells could only produce IFN-γ in a combination of IL-12 and IL-18 or by DCs, but still at lower levels than the CD27<sup>hi</sup> NK cells. This study for the first time demonstrated that different mature NK cell subsets can have different effector functions in mice. An intriguing study by Vosshenrich et al have demonstrated that mice express a subset of NK cells (CD127<sup>+</sup> GATA-3<sup>hi</sup>) which seems to have thymic origin [162]. What is most interesting with these cells is that their phenotype is clearly different from bone marrow derived NK cells. The CD127 NK cells expressed lower levels of the inhibitory Ly49A, -C/I and –G2 receptors. Furthermore, they lacked CD16 and had a decreased cytotoxicity but elevated cytokine production. These NK cells phenotypically were very similar to the human CD56<sup>bright</sup>CD16<sup>-</sup> NK cells. The human CD56<sup>bright</sup>CD16<sup>-</sup> NK cells expressed CD127 and had higher levels of GATA-3 than CD56<sup>bright</sup>CD16<sup>+</sup> NK cells which also lacked the expression of CD127, indicating the existence of similar populations in mice and humans. This finding is of great importance since the knowledge and use of murine counterparts of the human NK cell populations will allow for studies in mice, required to assess NK cell functions during different pathological conditions which would be difficult to study in systematic way in humans.

### 1.2.4 NK cells in infection

#### 1.2.4.1 Viral infections

There is evidence from both experimental and clinical studies that NK cells can contribute to resistance to infections [163]. One of the most studied infection models for NK cells is based on murine cytomegalovirus (MCMV) infection in mice. Upon MCMV infection the NK cells are stimulated by DCs that produce IFN-α/β and IL-12 [164-166]. These cytokines induce the NK cells to proliferate and to produce IFN-γ which inhibits viral replication [167], activates macrophages [168] and upregulates of MHC class I and II molecules [169]. Murine NK cells are able to assist in the clearance of MCMV by the production of IFN-γ in the liver and by mediating cytotoxicity by granule release in the spleen [170]. In C57BL/6 mice the NK cells have a specific activating receptor Ly49H which can bind the viral glycoprotein m157 [171] and there by lyse infected cells. Several mouse strains lack Ly49H, and are also more susceptible to MCMV, and so are mice which are defective in the signaling via Ly49H associated adaptor molecule DAP-12 [172]. In some strains inhibitory Ly49r have been identified
which bind m157 suggesting that m157 from the beginning evolved as a viral escape mechanism from NK cells.

During CMV infection in humans, CMV evades the NK cell response by active downregulation of CD155, the ligand for NK cell activating receptors DNAM-1 and TACTILE [173]. The virus can also hamper the expression of ligands for the activating receptor NKG2D in the infected cells [174]. Furthermore, individuals which have defective NK cells have been shown to suffer from severe herpes infections, including CMV [163].

1.2.4.2 NK cells in bacterial infections

One model for studying NK cells during bacterial infection is based on murine listeriosis. Early production of IFN-γ by NK in response to IL-12 and TNF-α, as well as, cross presentation of IL-15 by DCs is essential for the effective clearing of Listeria infection [175-179]. However, reports have also demonstrated that the presence of NK cells can slow down the clearance of Listeria monocytogenes [180] and that IFN-γ can be produced by lymphocytes other than NK cells during early L.monocytogenes infection [181, 182]. Further studies need to be conducted in order to elucidate the contribution of NK cells during this model.

Studies on Shigella flexneri in mice have demonstrated that NK cells have a role in clearance of this infection. Mice deficient in RAG2 (and thus deficient in T- and B-lymphocytes) show decreased bacterial titers in comparison to mice deficient in both the RAG2 and the common γ-chain (which lack both T-, B- and NK cells) [183].

1.2.4.3 NK cell function during parasitic infections

NK cells can also play an important role in the clearance of parasitic infection. Mice infected with Trypanosoma cruzi showed increased parasitic titers and decreased survival if they were depleted from NK cells [184, 185]. T.cruzi infects macrophages which in response to the infection produce IL-12, which activates NK cells [186-188]. Furthermore, IFN-γ production by NK cells is important in the resistance to T.cruzi infection [189]. Human NK cells are important in the production of IFN-γ in response to Plasmodium falciparum infected erythrocytes [190]. An interesting observation was that both CD56dim and CD56bright responded with significant IFN-γ production to the infected erythrocytes. The IFN-γ response by NK cells also depends on macrophage IL-18 production in response to the infection [191].

Altogether, the above mentioned examples illustrate that NK cells have an important role in the innate resistance to several different types of infection. Production of IFN-γ appears to be essential in many of the models. However, more studies are needed in order to fully understand the complexity of NK cell contribution in different infections.
1.2.5 NK cells during pregnancy

One interesting aspect of NK cells is that they are present in the uterus (uNK cells) in both humans and mice and that they infiltrate the decidua during pregnancy [192]. The NK cells represent about 70% of all immune cells in the decidua during the first trimester and in humans these NK cells are phenotypically characterized as CD56<sup>bright</sup> CD16<sup>−</sup> KIR<sup>+</sup> [193]. The existence of NK cells in the decidua is interesting since fetal cells not only express maternal MHC class I alleles but also paternal MHC class I alleles which should render them susceptible to immunological attack by T-lymphocytes. However, fetal cells cannot be killed by uNK cells, although the later retain their ability to kill NK sensitive tumor cells [194-196]. The failure of human NK cells to kill a throphoblast cell line [197] cannot be explained by defective NK cell receptor expression since the decidual NK cells have surface expression of the activating receptors NKp30, NKp44, NKp46, 2B4, LFA-1 and NKG2C, -D and –E (NKG2E determined as mRNA transcripts). Furthermore, these NK cells can form an immune synapse with tumor cell lines 721.221 and K562 [198-200]. The decidual NK cells are also superior to their peripheral blood counterpart in producing the cytokines GM-CSF, MIP-1α, CFS1. Peripheral blood NK cells that were stimulated with an antibody specific for the activating receptor KIR2DL4 induced secretion of IFN-γ, TNF-α, IL-1β and IL-8 which are all proangeogenic mediators [201]. KIR2DL4 is expressed by decidual NK cells [200] suggesting that NK cells could have regulatory function on angiogenesis during early pregnancy. One theory suggests that NK cells contribute to the remodeling of the spiral arteries in the uterus, required to maintain adequate blood supply to the fetus. If this process is perturbed, complications such as preeclampsia can develop. Interestingly, there is an association between certain maternal KIR haplotypes and fetal HLA-C combinations and the risk for preeclampsia [202]. The mechanism of NK cell tolerance in the decidua has, however, not been understood. Further studies on this paradox can give essential clues into how NK cell tolerance can be maintained in other cellular compartments.

1.3 IMMUNOLOGICAL TOLERANCE

1.3.1 General immunological tolerance

1.3.1.1 The role of MHC class molecules in the induction of tolerance by immune cells

The body is under constant surveillance by the cells of the immune system. However, the immune cells (T-, B-, and NK cells) need mechanisms to distinguish between healthy and aberrant cells in order to not become autoreactive, but to only kill or mediate effector functions against aberrant cells. As earlier mentioned, T-lymphocyte tolerance is mainly controlled in the thymus. To ensure that only self-tolerant T-lymphocytes leave the thymus the T-lymphocytes interact with both MHC class I and II molecules which express self-peptides. T-lymphocytes carrying receptors with too high or too low affinity to self peptides/MHC complexes are clonally deleted. This selection
process should guarantee that only those T-lymphocytes that are tolerant towards self-peptides will migrate to the blood and other tissues. However, due to several factors the selection by the thymus cannot be fully accurate and there is some leakage of potentially autoreactive cells. Such cells can, however, become hyporesponsive or even be deleted in extrathymic tissues, in processes summarized as “peripheral tolerance”. Such tolerance can for example be induced when T-lymphocytes recognize MHC/self peptide complexes (=“signal 1”) on cells that are not professional APCs (i.e. failing to provide the co-stimulatory “signal 2” in the form of CD80, CD86 or CD40, recognized by CD28 or CD40L receptors on the T-lymphocyte). The “signal 1 without signal 2” interaction renders the T-lymphocytes anergic; it cannot become activated by another MHC/peptide encounter, not even if co-stimulation is provided. The interaction can even lead to apoptosis of the T-lymphocyte. Furthermore, autoreactive T-lymphocytes can be deleted in the periphery by the expression of FAS (binding FAS-L on other cells), and they can also be suppressed by different types of regulatory T-lymphocytes [203]. There are intensive ongoing studies aiming at defining the molecular correlates of the anergic or suppressed state in T-lymphocytes.

B-lymphocytes are made tolerant in the bone marrow through several processes. Binding of high avidity antigens by the B-lymphocyte receptor results in internalization of the receptor and a stop in developmental progression. The autoreactive B-lymphocyte undergoes apoptosis unless it is rescued by a process called “receptor editing”. This involves additional recombination at a light chain locus, allowing the B-lymphocyte a ‘second chance” with a novel receptor based on this new recombined light chain gene. B-lymphocytes can also be anergized by low avidity or soluble antigens. Similar to T-lymphocytes, autoreactive B-lymphocytes can escape the selection in the bone marrow. However, B-lymphocytes usually need to be activated by T-helper cells, hence B-lymphocyte autoreactivity requires both a self-recognizing B-lymphocyte receptor and a autoreactive T-helper cell which binds to another epitope of the antigen in question, and can be presented by the MHC class II molecules of the B-lymphocyte [204].

1.3.2 NK cell tolerance

1.3.2.1 Why is a mechanism for NK cell tolerance necessary?

NK cells do not, as T- or B-lymphocytes, express receptors encoded by genes that are randomly rearranged during development, and allowing the cell to recognize one specific antigen. NK cells are instead equipped with a vast range of both activating and inhibitory germline encoded receptors. Some of these receptors are activating, others are inhibitory. A couple of these receptors recognize MHC class I molecules, others recognize non MHC class I ligands. Many of the inhibitory receptors are specific for one or a subset of MHC class I alleles, and the rules for expression of Ly49r (and KIR) allow that certain NK cells may lack inhibitory receptor for the MHC of their host. To ensure that NK cells are tolerant to self there must therefore exist mechanisms to delete certain dangerous NK cells or ensure that the signaling does not progress in NK cell effector function when NK cells interact with healthy cells. How is that tolerance
achieved? One possibility would be based on the principle that MHC class I molecules would determine the frequency of different Ly49r subsets. This could occur through positive selection for NK cells expressing at least one receptor for self-MHC, or clonal deletion of all NK cells failing to do so. NK cell tolerance would thus be based on analogous principles to those involved in selection of T- and B-lymphocytes. However, the expression of a given MHC class I gene has only a marginal influence on the frequency of NK cells expressing the corresponding Ly49r, and if anything this influence acts in the opposite direction (e.g. there are somewhat less NK cells with receptors for the MHC in question). The dominant hypothesis now instead assumes that tolerance is achieved by altering the functional capacity of each NK cell, so that NK cells without self-MHC receptors become hyporesponsive and cannot attack normal cells. Since MHC control of NK cell education and tolerance is central for two papers in this thesis, the previous key studies in this field will be reviewed in some detail below.

1.3.2.2 MHC class I genes influence education and tolerance of NK cells

Early studies on “F1 hybrid resistance” established that natural resistance mediated by NK cells is controlled by MHC linked genes. However, the dominating model for interpretation of this observation generally assumed that this control was not exerted by the MHC class I genes themselves, but rather by postulated MHC linked “Hybrid histocompatibility (Hb)” genes. The first formal evidence that a host MHC class I gene can control NK specificity was published by Höglund et al [205]. Transgenic introduction of D\textsuperscript{d} in C57BL/6 (B6) mice (K\textsuperscript{b}D\textsuperscript{b}) conveyed NK cell mediated rejection of an otherwise resistant tumor RMA (K\textsuperscript{b}D\textsuperscript{b}). The conclusion was that NK cells could sense the lack of D\textsuperscript{d} as missing self and kill the tumor cells. Further evidence for the education by MHC class I genes came with a publication by Öhlen et al [206], demonstrating that D\textsuperscript{d} transgenic B6 mice could reject bone marrow grafts from B6 mice, but not from D\textsuperscript{d} transgenic B6 mice. F\textsubscript{1} hybrids between D\textsuperscript{d} transgenic and non-transgenic B6 also rejected B6 bone marrow grafts though the rejection was weaker [206]. The D\textsuperscript{d} transgene was also sufficient to protect marrow from rejection by B10.D2 (H-2\textsuperscript{d}) recipients or by (B6xB10.D2) F\textsubscript{1} hybrids. This demonstrated that the MHC class I gene could control NK cell mediated rejection at the level of the recipient, as well as at the level of the donor/graft, according to the rules predicted by the missing self model. The paper also presented data showing that introduction of the D\textsuperscript{d} gene in the donor/graft was sufficient to induce rejection by non-transgenic recipients. This allorejection rather followed the classical laws of transplantation, and had not been predicted by the missing self model.

In two later publications in 1991 [207, 208] Höglund et al and Liao et al both demonstrated that activated NK cells from B6 (H-2\textsuperscript{b}) mice could effectively kill ConA blasts from B6 mice that had been targeted for the β\textsubscript{2}-microglobulin (β\textsubscript{2}m) gene (β\textsubscript{2}m association with the MHC class I heavy chain is essential for MHC class I expression). These findings demonstrated that NK cells could not only kill tumor cells lacking MHC class I but also normal cells with this phenotype. Furthermore, the NK cells of the β\textsubscript{2}m targeted mice were unable to kill the β\textsubscript{2}m deficient ConA blasts. Furthermore, the study
by Liao et al, as well as the study by Öhlen et al [206] showed that β2m deficiency controlled NK mediated bone marrow graft rejection at the level of the recipient and donor/graft in the same way as it controlled NK mediated killing of ConA blasts in vitro. Taken together, these studies demonstrated that NK cells need to interact with MHC class I in order to achieve competence for missing self recognition during development and to be able to distinguish between different MHC class I alleles.

Höglund et al also addressed the question of where MHC class I molecules need to be expressed in order to educate NK cells. They used chimeric mice constructed by irradiation of B6 mice followed by reconstitution with β2m−/− bone marrow [207]. NK cells retrieved from these mice still lacked the ability to kill β2m−/− ConA blasts demonstrating that it is sufficient with a β2m−/+ haematopoietic compartment to maintain NK cell tolerance to this phenotype (or conversely, that MHC class I/β2m expression in the marrow is necessary to develop NK cells competent for missing self rejection). In contrast, the expression of MHC class I on cells in the non-haematopoietic compartment was sufficient to mediate selection of functional β2m−/− CD8+ T-lymphocytes, e.g. bone marrow expression of MHC class I was not necessary for positive selection of T-lymphocytes. The role of the non-haematopoietic cells was first addressed in a publication by Wu et al [209]. They irradiated β2m−/+ or β2m−/− mice and then reconstituted with bone marrow from β2m−/− mice or with a 50:50 mix of β2m−/− with β2m−/+ cells. When β2m−/− mice were reconstituted with β2m−/+ haematopoietic cells and then challenged with β2m−/− grafts, these were accepted. This meant that non-haematopoietic β2m−/− cells can induce tolerance to β2m−/+ cells. However, there was a slight percentage of β2m−/− haematopoietic cells in the chimeras that had only been given β2m−/+ haematopoietic cells, which could then possibly influence the tolerance of the β2m−/+ cells. But the B6 mice that were given a 50:50 mix of cells rejected β2m−/− grafts slightly better than β2m−/− mice that were given the same mix. These results clearly demonstrate that even though there was a slight contamination of the β2m−/− haematopoietic cells in the chimeras the effects of these cells could be disregarded.

The tissue requirements of MHC class I expression with respect to NK cell education have also been studied in transgenic B6 mice expressing Dd under the metallothionein promoter. The Dd gene was expressed at low levels in liver, small intestine and the testis, and could be fully induced by supplementing the drinking water with zink [210]. Some leakage occurred from the promoter, and small amounts of Dd could also be detected in thymus and kidney. The interesting finding was although CD8+ T-lymphocytes were educated by Dd in the sense that they were tolerant to Dd expressing cells, NK cells were not educated, i.e. they could not reject grafts from B6 mice lacking Dd. NK cells also retained their ability to reject Dd+ bone marrow, possibly by Ly49D+ NK cells. It was thus not sufficient to express an MHC class I gene in certain organs in the periphery in order to influence NK cell education, consistent with the previous observations in chimeric mice with a β2m−/− haematopoietic system and normal MHC class I expression in the periphery. However, it cannot be excluded that the continuous interaction with Dd expressing cells might influence the education of the resident hepatic NK cells.
1.3.2.3 Models for cellular mechanisms of education

From the previous section we can conclude that the interaction of NK cells with MHC class I molecules during development and/or the mature life span can alter the specificity of NK cell responses. As already briefly introduced above there are at least two general types of models for how the NK system is shaped by the MHC class I molecules of the host.

The first is based on the notion that MHC class I molecules affect the distribution of frequencies of different Ly49r subsets, i.e. “the Ly49 repertoire”. This could occur through positive selection (survival and/or proliferation) or negative selection (death) of NK cells with predetermined Ly49r expression, or a combination of the two. Host MHC might also influence the Ly49 repertoire by altering the expression of these receptors on each NK cell according to whether they find matching ligands in the host. For example, the NK cell could be induced to switch on an additional inhibitory receptor if no match is recorded, until the NK cell has at least one inhibitory receptor for a self-MHC class I molecule. Common for these general type of models is that they would act to ensure that NK cells with inhibitory receptors for a given MHC ligand should be more common or even dominate the repertoire in mice expressing this ligand.

The second type of general model is based on the notion that MHC class I molecules can shape the specificity of the system without changing the frequency of Ly49r subsets, by instead altering the activation status of each individual NK cell. In such models, NK cells that lack inhibitory self receptors are not deleted or underrepresented, but rather retained as (partly) anergic. Below I will discuss each of the different models in more depth. It should be stressed that these models are not mutually exclusive.

1.3.2.4 The “at least one” model

The “at least one” model was proposed by Valiante and co-workers when they presented data on the NK cell receptor repertoires on human NK cells from two blood donors which were different in their HLA class I type and in their KIR repertoire [211]. Each out of 100 NK cell clones maintained in culture on IL-2 expressed at least one inhibitory receptor (KIR or NKG2A) which inhibited the killing of autologous cells. This finding supported a model based on MHC gene control of KIR/NKG2A repertoire, such that NK cells lacking at least one self inhibitory receptor would be excluded from the NK cell repertoire. These inhibitory receptors would not necessarily have to be KIR but could also include inhibitory receptors which interact with non-classical MHC class Ib molecules (HLA-E), e.g. NKG2A. Furthermore, other receptors which are not exclusively expressed on NK cells have been shown to mediate NK cell tolerance without interacting with MHC class I. These receptors include 2B4 and CEACAM-1[212-214].

The first studies of the Ly49 repertoire in mice did not lend support to models based on positive selection of NK cells with Ly49r for self-MHC. These studies predicted increased frequencies of NK cells with Ly49r specific for self-MHC, and the evidence
rather indicated the opposite [69]. NK cells with Ly49r specific for self-MHC tended to be underrepresented (compared to mice with deficient MHC class I expression), although this was statistically significant only for cells expressing more than one self-MHC receptor. This rather suggested a negative selection against cells receiving to much inhibitory signals. However, the data did not disprove the “at least one model”, since the knowledge (and available panel for antibody reagents) on Ly49r was incomplete. It could not be excluded that additional self receptors secured tolerance. Based on these studies two selection models were proposed by how NK cells with at least one inhibitory receptor could be developed. 1) The “sequential activation” model, which states that an NK cell turn on the gene expression of one inhibitory receptor at a time and that when the NK cell have at least one inhibitory receptor against one self-MHC class I complex, the NK cell will mature and become fully functional. 2) The “two-step selection” model, which states that any given immature NK cell will express a random set of inhibitory receptors, and that NK cells that express no or too many inhibitory receptors for self-MHC class I will be deleted and only NK cells which express one inhibitory receptor will mature [66]. However, since different inhibitory receptors have different affinity for self-MHC class I molecules several “weak” inhibitory receptors could be expressed by one NK cell if they can mount the same inhibitory signals as one “strong” inhibitory receptor. These two models were tested using mathematical modeling [215] but no conclusive data could be drawn due to incomplete knowledge about the affinity of each inhibitory receptor-MHC class I interaction.

1.3.2.5 NK cells in mice with mosaic MHC class I expression; a first clue to anergy as a tolerizing mechanism

Johansson and co workers studied NK cells from MHC mosaic (DL6) derived from a founder strain where a α3L/a1γd hybrid gene (hereafter denoted α12Dd) had been introduced on B6 (KbDd) backround. Although the transgene was inherited as a single autosomal co-dominant gene, the expression in each offspring varied such that between 10 and 80% of the cells expressed α12Dd in addition to KbDd. These mice were NK cell tolerant in vivo to cells expressing only KbDd, and in vitro cultured NK cells of both phenotypes, i.e. KbDd and α12DdKbDd NK cells were also tolerant to KbDd target cells [216]. However, when these two phenotypes of NK cells were separated and cultured for 4 days with IL-2 in vitro, the α12DdKbDd NK cells became reactive towards KbDd cells, i.e. they could now recognize the lack of Dd. This demonstrated that the potentially autoreactive cells had not been deleted but rather maintained in a non-reactive, anergic state. The tolerization could be broken if the NK cells where only separated for 6 hours (personal communication with M. Johansson and P. Höglund). This suggests that continuous interaction with Dd negative cells is crucial in order to maintain tolerance. Further studies also demonstrated that the potentially autoreactive NK cells in such mice had not been rendered tolerant by the acquisition of Ly49r recognizing the other self-MHC molecules, i.e. Kb or Dd. This suggested that MHC class I may be able to shape the responsive state of each individual NK cell and that the ones failing to display inhibitory receptors against self-MHC class I become anergic.
However, there was no way to conclusively prove the existence of anergic NK cells at the single cell level at the time of this study.

1.3.2.6  Mice and humans have NK cells which lack the expression of all known inhibitory receptors against self-MHC class I molecules

The first study to demonstrate NK cells in MHC class I+ mice which did not express any known inhibitory receptor for self-MHC class I came from Fernandez and co-workers [217]. They studied murine NK cells from B6 mice and could identify 10-13% of NK cells which did not express any of the Ly49C or -I inhibitory receptors, neither the NKG2A receptor with potential to recognize Qa-1 loaded with Db leader sequence as “self” in these mice. Phenotypic characterization of these NK cells showed that they had an impaired function and were not able to kill β2m− lymphoblasts, YAC-1 or RMA cells transfected with NKG2D ligands Rae-1γ or H60, neither could the CI/NKG2A− NK cells produce substantial amounts of IFN-γ. One possible mechanism for the anergy could be that signaling pathways for activating receptors are dampened. NKG2D, NKR-P1C and Ly49D cross-linking was performed which showed dampened IFN-γ responses by the CI/NKG2A− in comparison to CI/NKG2A+ NK cells. This study thus provided definite evidence for the persistence of NK cells without self-MHC receptors in an anergic state.

At the same time Kim and co-workers [218] demonstrated that Ly49C+ NK cells from β2m− mice were relatively deficient (compared to the corresponding subset in mice expressing Kb, the ligand for Ly49C) in producing IFN-γ when they were stimulated with monoclonal antibodies against NK1.1, NKR-P1C and KLR-B1C. Furthermore, they demonstrated that signaling through the inhibitory Ly49 receptors are necessary to render the NK cells non-anergic: NK cells with a defective ITIM sequence and thus no inhibitory signaling also developed an anergic phenotype, even if they matured in Kb expressing mice. Thus, NK cells need an interaction with their inhibitory Ly49r in order to develop functional missing self reactivity. Kim et al also studied NK cell populations expressing only Ly49 inhibitory receptors which are not necessary for NK cell recognition in the host, such as Ly49A+ NK cells in B6 mice. These NK cells also displayed anergy when cross-linked with monoclonal antibodies against NK.1.1 Furthermore, they generated a transgenic mouse which only expressed a single-chain trimer that consisted of the H-2Kb heavy chain β2m and a peptide on back crossed β2m−; Kb−; Db− triple knockout mice. Hence, the only Ly49r which could interact with Kb on these mice is Ly49C. Interestingly, the Ly49C+ NK cells in these transgenic mice could mount a functional response measured as IFN-γ production when cross-linked with NK1.1, indicating that NK cells need to be able to interact with at least one self-MHC class I molecule in order to become fully functional. Studies with similar assays at the single cell level in humans by Anfossi et al have now also demonstrated the existence of human NK cells which lack all known inhibitory receptors for self-MHC class I molecules, and which remain anergic [219].

These four studies provide strong evidence that NK cells do not need to express inhibitory receptors to survive and that these NK cells become anergic. Furthermore,
the studies of mosaic mice by Johansson et al suggest that this anergic state may be reversible. Several questions arise. How can one reconcile the discrepancy between these latter studies and the early studies of human NK clones supporting the “at least one model”? How can NK cells tune their intracellular signaling in order to become tolerant to MHC class I molecules and what is the functional capacity of different MHC class I molecules in the education of NK cells? These questions will be addressed later.

1.3.2.7 Licensing vs Disarming

The studies reviewed above support that one major mechanism for NK cell tolerance is based on altering the functional capacity of individual NK cells on the basis of how their Ly49r match or do not match the MHC ligands of the host. Two different hypotheses regarding the process to achieve this education have been presented. 1) The first hypothesis by Kim, Yokoyama et al states that NK cells are non-responsive to normal cells by default, and need to be activated by the interaction with self-MHC class I molecules in order to become responsive. Hence, NK cells which fail to interact with self-MHC during development remain hyporesponsive, and those that interact with self-MHC become “licensed to kill” (“licensing model”) [218]. Kim et al further demonstrated that the inhibitory signaling is necessary in order to achieve education, since NK cells expressing Ly49r for self-MHC although with a defect in the Ly49r ITIM resulting in absent signaling, all became hyporesponsive. 2) The hypothesis put forward by Raulet et al [220] postulates that all NK cells develop into a responsive state from the beginning; some of them are then “disarmed” via input from activating and inhibitory stimulation to become tolerant, such that failure to interact with self-MHC class I and dominance of activating signals during development would make the NK cell hyporesponsive. Both of these hypotheses are based on the idea that NK cells need to be educated from their surrounding MHC environment in order to be fully functional in their effector function, but they differ in the proposed process leading to the responsive or hyporesponsive state.

1.3.2.8 How do the licensing and disarming models fit with data observed in mosaic DL6 mice?

The licensing model of Kim et al [218] postulates that NK cells encountering their inhibitory ligands become competent to attack normal cells. If this were a dominant phenomenon, one would expect that a fair number of NK cells in the mosaic DL6 mice reviewed above, whether they were of K^bD^b or α1,2D^dK^bD^b phenotype, would encounter educating cells of the α1,2D^dK^bD^b phenotype. If education is based on NK cells interacting with more than one educating cell, one might even expect that all NK cells should encounter cells of the α1,2D^dK^bD^b phenotype. This should make a proportion of (or all) NK cells “licenced” to attack and kill cells deficient for D^d ligands. We would expect to observe such killing in vitro, and we would expect that the mosaic pattern in vivo should gradually shift to dominance of cells with the α1,2D^dK^bD^b, as the K^bD^b expressing cells were gradually eliminated.
However, this was not observed. The mosaic pattern was stable, and no NK cell mediated killing of cells expressing only K\textsuperscript{b}D\textsuperscript{b} was observed. This is more consistent with the “disarming model”, in which the ligand deficient cells dominantly decide the outcome of education. The mosaic mice therefore favor the simple “disarming” over the simple “licensing” model, and in addition opens up for a more complex model, whereby education may be reversible (i.e. a combination of “licensing” and “disarming”). This is based on the observation that the missing D\textsuperscript{d} recognition by α\textsubscript{1,2}D\textsuperscript{d}K\textsuperscript{b}D\textsuperscript{b} Ly49A\textsuperscript{+} NK cells could be restored once they have been cultured alone, separated from K\textsuperscript{b}D\textsuperscript{b} cells for four days \textit{in vitro} [216].

1.3.2.9 Influence of host MHC class I on cellular expression levels of Ly49 receptors

Since Ly49r expression is necessary for the NK cells to become fully functional in their missing self recognition it is challenging to think that MHC class I molecules in their turn can alter the surface expression of Ly49r and thus fine tune the NK cells to receive a perfectly balanced signaling via inhibitory and activating receptors. This question was addressed by Sykes \textit{et al} who made chimeric mice mixing bone marrow cells from B10 (H-2\textsuperscript{b}, D\textsuperscript{d}-) mice and B10.A (H-2\textsuperscript{a}, D\textsuperscript{d+}) mice and then injecting them to irradiated B10 mice. The B10 cells downregulated their expression of Ly49A (Ly-49) which they interpreted as an education of the B10 cells by the B10.A haematopoetic cells [221].

Based on the results by Sykes \textit{et al} demonstrating that NK cell Ly49 inhibitory receptors were downregulated [221], as well as, later studies by Blazar \textit{et al} [222] on Ly49C/Ir positive NK cells, Sykes hypothesized that NK cells were educated depending on the affinity of the NK Ly49 and its cognate ligand. This affinity/NK subset model implied that Ly49r with high affinity to self ligands would be downregulated while Ly49r with intermediate affinity would still be present. However, one common misinterpretation at the time was that NK cells completely downregulated their Ly49A upon interaction with its ligand MHC class I H-2D\textsuperscript{d}. This misinterpretation was probably due to the suboptimal efficiency of antibody reagents, fluorochromes and/or instruments used at the time. Later studies have demonstrated that Ly49A does not completely disappear from the cell surface of NK cells when D\textsuperscript{d} ligands are present in the host. The paradox of how NK cells in a D\textsuperscript{d} mouse could recognize target cells when they downregulate Ly49A was tackled by Olsson \textit{et al} [71] in studies of a transgenic mouse which expressed the ligand for Ly49A, D\textsuperscript{d}, on a H-2\textsuperscript{b}/B6 background. Olsson \textit{et al} demonstrated that the percentage of NK cells that expressed Ly49A was similar to those in B6 mice but, the intensity of Ly49A staining per cell was 50-80% lower in the transgenic mice. The Ly49A\textsuperscript{+} NK cells from the transgenic mice were inhibited according to “missing self” rules when exposed to Balb/C (H-2\textsuperscript{d}) targets, but killed a tumor cell line SP2/0, which expressed H-2D\textsuperscript{d} albeit at lower levels. The NK cells from the non-transgenic B6 mice, with high Ly49A expression, failed to kill both types of target cells. Olsson \textit{et al} hypothesized that the NK cells were “calibrated” depending on the amount of ligands they were exposed to during development. Since the transgenic mice had been exposed to “normal” levels of D\textsuperscript{d}, the
NK cells would have downregulated their Ly49A levels in order to tune the balance so that they would not be over-inhibited. On the other hand, the NK cells from B6 mice would not have been exposed to D\(^d\) and hence these NK cells retained high levels of Ly49A. As a consequence, the latter NK cells failed to sense reduced surface levels on the SP2/0 tumor cells, while the Ly49A\(^+\) NK cells from transgenic mice were competent to do this, making them more “useful”. Olsson et al termed their hypothesis the “receptor calibration model”. This model postulating that NK cells calibrate their expression levels to an absolute “useful” level according to the levels of ligands they are exposed to during education has been challenged on several grounds. Held et al studied mice expressing Ly49A receptors at different levels, and found that the receptor levels were not downregulated to the same absolute level [223]. Rather, the staining was reduced by 50-70% irrespective of the starting level, suggesting that downregulation was a simple consequence of receptor-ligand interactions in vivo, without purpose to reach a certain balanced level. This was supported by Manilay et al in studies of mixed haematopoetic chimera mice, demonstrating that the down-regulation of Ly49A varied according to the number of D\(^d\) ligand positive cells, arguing against calibration towards a certain level of MHC class I ligand per educating cell [224]. The concept of calibration in order to achieve “useful” inhibitory input is therefore questionable. However, the phenomenon of Ly49r down-regulation in response to host self-MHC ligands has been widely confirmed, and can be used as a tool to track NK – MHC ligand interactions in vivo, as will be developed further in one of the studies in this thesis. It is also clear that such Ly49r down-regulation is associated with functional alterations, e.g. reduced inhibitory input to the NK cell, as originally reported by Olsson et al [71].

1.4 NK CELLS IN TUMOR THERAPY

NK cells were first discovered due to their ability to kill tumor cells in vitro, and there has ever since been an interest for the use of NK cells in tumor therapy. There are several different strategies for the use of NK cells in tumor therapy, some of which will be reviewed below as a background to paper II of this thesis.

1.4.1 NK cell activation via cytokines

During the 1980’s, it was demonstrated that NK cells could be activated and induced to proliferate by the use of Interleukin-2 (IL-2) [225]. With the production of recombinant human IL-2 (rIL-2) [226, 227] it became obvious to test if rIL-2 could have a stimulatory effect in vivo on NK cells. Initial studies demonstrated that mice which had been given rIL-2 intravenously showed increased ex vivo NK cell killing against the lymphoma cell line YAC-1 [228]. Early studies in humans demonstrated that stimulated peripheral blood lymphocytes from patients bearing solid tumors had a lower production of IL-2 than lymphocytes from normal subjects. This dysfunction was correlated with lower NK cell activity. This suggested that IL-2 could be used as a stimulator to increase NK cell activity [229]. Furthermore, patients suffering from AIDS were restored in their relatively low NK cell activity when treated with rIL-2
The first trial based on administration of rIL-2 for treatment of patients with malignant disease showed no effect on tumor regression, nor did the NK cell activity increase [231]. However, later studies demonstrated that patients with cancer undergoing rIL-2 therapy had an increased cytotoxic activity of NK cells but not of T-lymphocytes [232, 233]. The first study demonstrating some anti-tumor effects due to administration of rIL-2 alone was done by Walle et al on renal cancer patients [234]. The effect of NK cell activation by in vivo administered rIL-2 using different protocols (with and without other treatment modalities) is still under evaluation. It should also be noted that T-lymphocytes express IL-2 receptors, and this may influence the overall response in patients treated with IL-2.

An alternative strategy is to expand autologous NK cells in vitro and then later administer these cells to the patient, with or without IL-2 treatment. Such studies were pioneered by Rosenberg’s laboratory, first in mice, then in patients. Autologous peripheral blood or spleen mononuclear cells were expanded with IL-2 into so called lymphokine activated killer (LAK) cells, with strong cytotoxic potential towards tumor targets. The LAK cells were initially described as a novel lymphocyte population. Later studies revealed that their slightly unusual characteristics could be explained by the fact that the IL-2 stimulated cultures contained both activated T- and NK cells. The clinical studies demonstrated beneficial effects of a combination of LAK and rIL-2 in some patients [235, 236]. However, the number of responding patients was low, and the side effects of the treatment were considerable, the patients had to be monitored in intensive care units during the IL-2 treatment. This treatment was therefore not practical to implement in larger scale. Today, twenty years later, with the experiences gained in adoptive transfer and cytokine administration protocols, as well as the more advanced knowledge on molecular aspects of NK cell recognition and regulation, time may be ripe to try more sophisticated protocols for NK cell transfer. A recent study by Miller et al have now demonstrated that patients with acute myeloid leukemia can tolerate adoptive transfer of allogeneic NK cells in combination with IL-2. The donor-recipient combinations could be classified according to whether the transferred NK cells would be alloreactive based on KIR ligand mismatch (discussed further in next section) [237]. No particular side effects were found to be associated to alloreactive NK cell transfer, and there was even an indication that this might be beneficial for disease development, although caution must be applied in the interpretation of this small pilot study.

1.4.2 Allogeneic haematopoietic stem cell transplantation (HSCT)

Through the pioneering studies on haploidentical haematopoietic stem cell transplants in Perugia by Ruggeri, Velardi and collaborators, a new strategy to optimize NK cell effects through genetic matching has emerged [238-240]. Haploidentical transplants are based on using the mother, father or a half-matched sibling as a donor in situations where no fully matched donor is available. Such transplants are matched for only one of the MHC haplotypes, and there are therefore several concerns, due to the high risk for rejection or the possibility that the graft becomes reactive towards donor tissues, i.e. graft versus host disease (GVHD). GVHD occurs when mature donor T-lymphocytes are primed on recipient DCs. To avoid rejection, the recipients therefore get an
aggressive conditioning treatment and a high dose of stem cells. To avoid GVHD, T-lymphocytes are depleted from the graft using much more stringent protocols than those used for matched transplants. However, GVHD can be beneficial for the recipient since low GVHD can be accompanied with a graft versus tumor (GVT) effect which contributes to the clearance of tumors. Removal of T-lymphocytes may therefore increase the risk for leukemia relapse. There is now evidence that one can use NK cells to achieve a GVT effect in the haploid identical setting, based on combining donor and recipient according to KIR-ligand incompatibility with the aim of generating an NK allo- (“missing self”) reactivity.

There are three types possible donor-recipient combinations in the context of HLA-KIR ligand compatibility. The donor and the recipient always share one set of KIR ligands (usually HLA-C or B encoded), since they are matched for one haplotype, but different possibilities arise for the other HLA haplotype. First, the donor can lack the KIR ligands of the recipient which in theory could give rise to host versus graft (HVG) reactivity and NK cell mediated rejection of the graft. Secondly, the recipient can lack KIR ligands of the donor which in theory could result in graft versus host (GVH) where the donor NK cells would attack the recipient’s cells. Thirdly, there can be a full match where donor NK cells will express all the KIR ligands of the recipient and vice versa; no effects due to NK allo (“missing self”) reactivity would be expected. In studies of patients transplanted for acute myeloid leukemia (AML) or acute lymphatic leukemia (ALL), the AML (but not the ALL) patients which received haploidentical grafts with at least one KIR-ligand mismatch had a lower frequency of relapses than patients receiving KIR-ligand matched grafts [238-240]. The donor NK cells showed functional missing self reactivity in the post transplantation period. NK cells retrieved from recipients could kill recipient pre-transplant lymphocyte blasts, as well as leukemia cells. However, NK cells where tolerant towards recipient lymphocyte blasts three months post transplantation [238]. Furthermore, there was no increased risk for GVHD associated with KIR ligand mismatch. There was rather evidence for the opposite: donor recipient combinations with KIR ligand mismatch had a somewhat reduced risk for GVHD. In parallel studies of Ly49-ligand incompatibility in mice, Ruggeri et al confirmed this phenomenon and in addition provided a possible explanation: the NK cells seem to exert a protective effect against T-lymphocyte mediated GVHD by eliminating recipient DCs, known to be important for activation of donor T-lymphocytes [239]. As will be discussed more in detail below, NK cells have been demonstrated to kill immature DCs [241].

Altogether, NK cells may thus have a double beneficial effect for the outcome of HSCT, eliminating leukemic cells in a GVT reaction and eliminating DCs, preventing a GVH reaction. It should be noted that there are several retrospective studies where these beneficial effects of KIR ligand incompatibility between donor and recipient could not be confirmed [242]. This may be a question of the clinical protocols used, since many of these studies were based on matched (for HLA-A and B, but not C), unrelated donors. In this situation, there is less aggressive recipient conditioning, and also lower dose of transplanted stem cells and less stringent T-lymphocyte depletion for the graft. The beneficial effects of KIR ligand incompatibility has, however, been seen in some studies using similar protocols to the one used in Perugia, and several questions can be raised. What determines whether allo (“missing self”) reactivity will
play a role after HSCT? How is NK cell tolerance to recipient ligand phenotype established under these conditions and can this be delayed to achieve even better GVT effects? This in turn requires understanding of the basic process by which MHC ligands educate NK cells (studied in paper I of this thesis). Can the Ly49r/KIR-ligand incompatibility be mimicked to achieve therapeutic effects in a completely autologous situation, e.g. by blocking the inhibitory receptor on NK cells in vivo (studied in paper II of this thesis)? Can the KIR-ligand incompatibility effect be used also in non-myeloablative therapeutic strategies, e.g. for activated lymphocyte infusions (see discussion of studies by Miller et al in previous section).

1.4.3 Regulation of NK cells by manipulation of receptor input

As discussed in the previous section, the use of KIR ligand incompatibility in allogeneic SCTs introduces an interesting possibility to enhance NK cell mediated elimination of recipient tumor cells. However, there are certain risks involved in using allogeneic SCTs, so is it possible to manipulate the cells in order mimic the HLA-KIR mismatch in the autologous setting? One alternative is to block one or several inhibitory receptors on NK cells. Studies by Koh et al [243] demonstrated that blocking of an NK cell inhibitory Ly49 receptor by a F(ab) antibody fragment, induced NK cell killing of leukemia cells in vitro. In vivo treatment with the same reagent reduced malignancy development after transplantation of the same leukemia cells [243]. A risk with the use of autologous BMT in leukemia patients is that there might be tumor cells present in the autologous graft which will give rise to tumor relapse post transplantation. Koh et al has furthermore demonstrated that by blocking NK cell inhibitory receptors, tumor cells can be killed in an autologous graft prior to transplantation without reducing haematopoietic reconstitution. This resulted in decreased tumor relapse and the effect could be further increased with the use of allogeneic NK cells [244, 245]. These findings open up for new solutions for treatment of leukemia and possibly also other types of malignancies. However, several important questions remained unanswered. Does the in vivo treatment with antibodies against inhibitory receptors really lead to directly enhanced, NK cell mediated elimination of tumor cells? Does the treatment induce NK cells to become autoreactive towards normal autologous cells since the blocking of inhibitory receptors would mimic missing self recognition in vivo? How does prolonged blockade of an inhibitory receptor affect the NK cell activity? This question is important in relation to the different education models discussed above – since they all are based on the notion that the input through inhibitory receptors have an important role for the responsiveness (or anergy) development of NK cells. These different questions were addressed in paper II of this thesis, and will be further discussed below.

1.4.4 Antibody dependent cell mediated cytotoxicity (ADCC)

There are now several antibodies to tumor antigens used in clinical routine for treatment of malignancies. Relatively little is known about the mechanisms behind the efficacy of these treatments. There are at least three possibilities. Antibodies may 1)
recruit and activate the complement cascade leading to tumor cell cytolysis. 2) Activate intracellular pathways promoting apoptosis of tumor cells. 3) Recruit effector cells that can mediate ADCC. NK cells are among the latter cells, since they can use their Fc binding receptor CD16 to recognize antibody coated cells [246]. The CD16 receptor is a strong activating receptor, and triggering via this receptor alone is sufficient to induce the whole process leading to target cell directed release of perforin and granzymes [247]. Macrophages, as well as eosinophilic and neutrophilic granulocytes can also mediate ADCC. The relative role played by the three mechanisms mentioned above is not known for any antibody used in therapy, since it is difficult to study this. The issue is important, however; if NK cell mediated ADCC is a major mechanism, it would be possible to enhance it further by combination with other modalities, e.g. IL-2 or blocking of inhibitory receptors.

1.4.5 Designed NK cells

Recent studies in NK cell biology have shown that the NK cell-line NK-92 engineered to express a receptor against ERBB2 (antigen present on breast cancer cells) can be used in order to kill tumor cells expressing this antigen [248, 249]. What is unique with NK-92 is that it is a tumor cell line with full cytotoxic capacity, which can be expanded indefinitely in culture. After irradiation, the cells of this line will start to die within 72 hours. This opens for a clinical setting where in vitro expanded NK-92 cells are adoptively transferred to patients with malignancies. Before transfer, the cell line can be engineered in vitro to express the appropriate activating and inhibitory receptors and to express the right HLA molecules to minimize the risk for rejection and GVHD. In an optimistic scenario, oncologists would have a vast album of NK-92 and other, similar lines, which could be used in order to treat different types of cancer in patients with different HLA phenotypes.

1.5 NK AND DC INTERACTIONS

NK cells have been shown to influence the adaptive immune responses not only by interacting directly with T- and B-lymphocytes but also by interacting with dendritic cells (DCs). NK cell-DC interactions may affect adaptive immune responses in two ways: 1) through DC stimulation of IFN-\(\gamma\) production by NK cells and 2) NK cell-mediated lysis of DC. Both of these mechanisms will be discussed.

NK cell activation by DC was first demonstrated by Fernandez et al [250]. In this study, the authors demonstrated that the activated DCs can prime resting NK cells to promote NK cell cytotoxic activity and production of IFN-\(\gamma\). The critical role of DCs in activating NK cells in vivo has recently also been shown by Lucas et al [179]. Using mice that had the CD11c promoter under the control of diphtheria toxin receptor, they demonstrated no NK cell activation in mice where DCs were depleted. Furthermore, IFN-\(\gamma\) production by NK cells was shown to be an important factor in priming the T-lymphocyte response in vivo [251]. In studies with human NK cells and DCs, the cross talk between NK cells and DCs was dependent on the NK:DC ratio [241, 252, 253].
While NK cells may be stimulated by mature DCs, NK cells were also found to be able to stimulate maturation of DCs. Another important cytokine for NK cell activation is IL-2, which can be produced by DCs after bacterial stimulation in the early stage of an infection, enabling the production of IFN-γ by the NK cells [254]. Thus, NK:DC interactions are important not only for the activation of NK cells but also for shaping the immune responses.

NK cells may control immune responses also by eliminating DCs. Initial studies by Chambers et al [255], demonstrated that NK cells could lyse immature DCs in vitro. Similar observations were reported for human NK cells and DCs [256-258]. In these studies, DCs stimulated with LPS were protected from NK cell mediated-lysis. Furthermore, infection of DCs with viruses or bacteria could also protect the DCs [256, 259]. However, as mentioned above, these DCs could stimulate naïve NK cells. The NK receptor responsible for mediating the killing of iDCs in humans, as well as activation of the DCs by TNF-α and IFN-γ, is NKp30 [253, 260]. In the mouse, the receptor involved in NK cell mediated-killing of DCs has not yet to been identified. Studies by Della Chiesa et al [259] demonstrated that the human NK cells responsible for mediating iDC killing belong to the NKG2A KIR phenotype. These data correlate well with the NKG2A ligand expression of iDC, showing low levels of the non-classical MHC molecule HLA-E [259, 261]. NK cell mediated elimination of DCs was also observed in vivo. However, unlike with the in vitro observations, lysis of DCs was not dependent on perforin but was dependent on TRAIL. CTL responses from mice immunized with peptide loaded DCs that had been depleted of NK cells or deficient in TRAIL were increased compared to control mice. Furthermore, anti-tumor responses in mice depleted of NK cells or deficient in TRAIL were greater than in control mice. These data suggested that NK cell elimination of DCs could also affect adaptive immune responses by limiting the number of DCs that could present peptide.

In conclusion, the NK cell – DC interaction can shape the outcome of the adaptive immune response by regulating the antigen presentation displayed to T-lymphocytes, both in terms of the amount of antigen presented (dependent on the number of DC) and the quality of DC signals associated with the presentation (dependent on the maturation and activation stage of the DC). This may be an important feature to minimize the T-lymphocyte surveillance and enabling the mDCs to migrate to the secondary lymphatic organs for antigen presentation.
2 AIMS OF THE THESIS

As presented in the introduction in the previous pages, there is now considerable knowledge about the effector mechanisms of NK cells and many of the receptors that control these. However, the mechanisms which keep the natural killer cells tolerant towards healthy cells have been debated for a long time and are currently subject for intensive discussions. These concern not only how the NK cells manage to become tolerant but also the general MHC guided education process that determine the development of different subsets as defined by inhibitory receptor expression. The overall knowledge about these functions is important to understand not only how NK cells make decisions vis-à-vis different target cells, but also to understand how NK cells can shape the immune response by interacting with healthy cells like dendritic cells (DCs) during an infection. Furthermore, if knowledge of these different aspects of NK biology can be dissected in well defined murine experimental models, this may lead to useful tools and concepts to interpret and manipulate the human NK system.

In paper I of this thesis, led by my collaborators Sofia Johansson and Petter Höglund, we set out to utilize mice expressing different single MHC class I genes in order to identify the impact that different MHC class I alleles have on the education of missing self recognition by the NK cells. We posed the following questions: 1) Do different MHC class I alleles educate equally well for missing self recognition, i.e. the rejection of cells lacking the allele in question? 2) Do different combinations of MHC class I alleles change the influence of a given allele? 3) Is it possible to find cellular correlates (e.g. in number of mature NK cells, NK cells with certain activating receptors) of MHC guided education? Can one for example predict the influence of a certain MHC class I allele by studying the Ly49 inhibitory receptor expression?

In paper II of this thesis, our general aim was to find out whether, once tolerance was established, it was possible to break it by blockade of inhibitory receptors in such a way that tumor rejection was induced or enhanced, while tolerance was maintained towards healthy cells. More specifically, the following questions were addressed: 1) Is it possible to induce missing self reactivity in B6 mice towards their normal MHC class I expressing haematopoetic cells, by blocking the Ly49C/I inhibitory receptors that are partly responsible for maintaining NK cell tolerance with F(ab')2 fragments against these receptors? 2) Is it possible by a similar approach to increase killing against the MHC class I expressing tumor cell line RMA without breaking the tolerance towards healthy cells? 3) What are the effects of long term blockade of an inhibitory NK cell receptor? Is it for example possible to influence the NK education, proposed to be dependent on input via inhibitory receptors?

The focus was then set on NK cell inhibitory receptors in interaction with DCs, in studies led by my colleagues Catrine Persson and Benedict Chambers. In paper III, our aim was to study the mechanisms which are involved in regulation of DC susceptibility to NK cells, in particular the influence of different maturation stages of DCs. The following questions were addressed: 1) Can NK cells discriminate between mature and
naïve DCs? 2) Does Qa-1 on the mature DC population have a protective role against NKG2A+ NK cells?

The effector function exerted by NK cells involves degranulation mediated cytotoxicity via perforin and granzymes, as well as cytokine production. These effector functions have been suggested to be exerted by different NK cell subpopulations in humans. However, it has not been possible to classify murine NK cells in a similar way. In paper IV, our general aim was to study whether the two main effector functions are coordinately regulated, in particular with respect to subpopulations defined by the cell surface markers CD27 and Mac-1. These markers have been proposed to distinguish different maturation stages of murine NK cells. The following questions were addressed: 1) Can all murine NK cells exert both cytotoxicity (through degranulation) and cytokine production? 2) Is degranulation and cytokine production regulated differently under different conditions of target cells encounter? and 3) Is it possible to distinguish the NK cell effector response based on the cell surface markers CD27 and Mac-1?
3 RESULTS AND DISCUSSIONS

3.1 EDUCATING IMPACT OF DIFFERENT MHC CLASS I ALLELES  
(PAPER I)

3.1.1 Rational behind the study

As mentioned in the introduction, several studies published more than 15 years ago demonstrated that NK cells were educated by host MHC class I molecules themselves. The first evidence came from the introduction of the D\textsuperscript{d} allele into C57BL/6 mice, which changed the NK cell repertoire and induced NK cells to a missing self response against D\textsuperscript{d} cells [205, 206]. Furthermore, NK cells from C57BL/6 mice were shown to reject bone marrow from β\textsubscript{2}m\textsuperscript{−/−} mice, demonstrating that the presence of self-MHC class I induced the capacity of NK cells to reject normal cells lacking all MHC class I alleles [262]. These studies also demonstrated that NK cells from β\textsubscript{2}m\textsuperscript{−/−} mice could not reject ConA blast or bone marrow cells from β\textsubscript{2}m\textsuperscript{−/−}, indicating that β\textsubscript{2}m\textsuperscript{−/−} NK cells were anergic [208]. One interesting observation was that NK cells from mice expressing L\textsubscript{d} on the C57BL/6 background were unable to reject C57BL/6 bone marrow [263], despite a missing self barrier between this recipient and donor. Rejection of β\textsubscript{2}m\textsuperscript{−/−} cells was unaffected, indicating that the NK cells in these transgenic mice were not anergic. These data contrasted with the situation in D\textsuperscript{8} mice (H-2\textsuperscript{b} + D\textsuperscript{d}), which rejected bone marrow from C57BL/6 donors. From this comparison, it seemed as if different MHC class I molecules differ in their capacity to educate NK cells.

Since mice express multiple MHC class I genes and several Ly49 receptors, the individual impact of every MHC class I-Ly49r interaction and subsequent activation/inhibition of the NK cell is difficult to delineate. In order to study the mechanisms behind NK cell education further, we have therefore in studies led by Petter Höglund developed a more reductionistic system based on mice expressing single MHC class I molecules. As a general test system, these mice where injected i.v. with donor cells labeled with 5,6-carboxyfluorecein diacetate succinimidyl ester (CFSE), making the labeled cells fluorescent. Rejection of the injected cells was determined 18 hours post inoculation. This assay measures NK cell rejection in a more quantitative manner than other in vivo rejection assays, adding yet another important parameter to the analysis.

In the following study, we asked the following questions: 1) Do different MHC class I alleles convey a different strength in NK cell education towards missing self recognition, i.e. against cells lacking the expression of these alleles? 2) Does the expression of several different MHC class I alleles influence the impact of NK cell education by individual alleles? 3) Is it possible to predict the educating impact of a set of MHC class I alleles by studying the expression of Ly49 receptors?
3.1.2 Different MHC class I alleles can convey different strength in education

To study the influence of different MHC class I alleles on NK cells, mice expressing either K\textsuperscript{b}, D\textsuperscript{b}, L\textsuperscript{d} or D\textsuperscript{d} as a single MHC gene where injected with MHC\textsuperscript{-/-} cells together with syngeneic cells. Thus, there was an internal control for the rejection of the MHC\textsuperscript{-/-} cells which could be quantified. We found that all MHC class I alleles, including L\textsuperscript{d}, could educate for missing self recognition. However, the rejection strength against MHC\textsuperscript{-/-} cells showed consistent differences between the strains. The rejection was strongest in mice expressing D\textsuperscript{d}. The rejection strength could be grouped in the following order: D\textsuperscript{d}>K\textsuperscript{b}>L\textsuperscript{d}>D\textsuperscript{b}. These results demonstrate that the NK cell education to reject MHC class I deficient cells depends on which MHC class I allele the NK cell interacts with during the development (Figure 2). We defined the term “educating impact” of a MHC class I gene as the strength by which mice with this particular allele educate the NK cells in missing self-rejection of MHC class I deficient cells.

![Graph showing the rejection of MHC class I-/- splenocytes by different single MHC class I mice. The rejection was determined by using an internal control (syngeneic splenocytes) to measure the rejection efficacy. Figure modified from Johansson et al, Journal of Experimental Medicine, 2005, 201, 1145-55.](image)

3.1.3 Introduction of MHC class I gene with weak educating impact does not affect the educating impact of a gene with strong educating impact

We further tested if the influence of alleles with different educating impact in single MHC class I mice could be altered when they were co-expressed. We therefore used C57BL/6 mice (K\textsuperscript{b}D\textsuperscript{b}) which have a combination of one allele with strong educating impact and one allele with weak educating impact. C57BL/6 where then challenged with K\textsuperscript{b} or D\textsuperscript{b} single grafts. The presence of an additional MHC class I allele did not influence the strength of the educating impact of the other. This conclusion was drawn
on the basis of the result that grafted cells lacking D\(^b\) only were as poorly rejected in B6 mice as in single D\(^d\) mice, despite the presence of the K\(^b\) allele during education. Conversely, the educating impact of K\(^b\) was not weakened by the simultaneous presence of D\(^b\). Hence, the educating impact of these two alleles appeared independent from one and another.

### 3.1.4 D\(^d\) and K\(^b\) educating impact is retained when these genes are co-expressed together with D\(^b\) or L\(^d\)

D\(^d\) was the allele with the strongest educating impact of the four alleles tested. Previous studies have demonstrated that D\(^d\) transgenic B6 mice (D8 mice expressing K\(^b\), D\(^b\) and D\(^b\)) will reject grafts which lack D\(^d\). To test if MHC class I molecules with strong educating impact (D\(^d\)) could weaken the influence of the other alleles with strong educating impact in the same mouse (K\(^b\)), we grafted K\(^b\)D\(^b\), D\(^b\)D\(^d\) and K\(^b\)D\(^d\) cells to D8 mice. Interestingly, the strong educating impact was retained for both D\(^d\) and K\(^b\) while the educating impact decreased for D\(^b\). These data demonstrate that alleles with strong educating impact do not attenuate the educating impact of other strong alleles. However, the educating impact of weak alleles can be further weakened when co-expressed with alleles conveying a strong educating impact.

### 3.1.5 The educating impact by L\(^d\) is attenuated only when co-expressed with both K\(^b\) and D\(^b\)

Together with D\(^b\), L\(^d\) demonstrated a weak but significant educating impact in the rejection of MHC\(^{-}\) cells. Previous studies by our group have demonstrated that K\(^b\)D\(^b\) grafts transplanted to K\(^b\)D\(^b\)L\(^d\) mice are not rejected [263], demonstrating that the L\(^d\) was not capable of educating missing self recognition on this background. With our experimental system, we were able to study this question further. We first demonstrated that K\(^b\)D\(^b\) grafts transplanted to K\(^b\)D\(^b\)L\(^d\) hosts were poorly rejected, corroborating our previous result [263], while L\(^d\) expressed as a single MHC class I allele was a good educator. To elucidate which allele on the B6 background that was responsible for attenuation of the educating impact of L\(^d\), we studied different combinations where L\(^d\) was the only missing allele in the graft. Hence, we grafted K\(^b\)D\(^b\) cells to K\(^b\)D\(^b\)L\(^d\) mice, K\(^b\) cells to K\(^b\)L\(^d\) mice and D\(^b\) grafts to D\(^b\)L\(^d\) mice. Surprisingly, there was a considerable rejection in the two latter, but not in the first combination. In other words, there was no influence of the educating impact of either K\(^b\) alone or D\(^b\) alone, but the combination of the two strongly reduced the educating impact of L\(^d\).
3.1.6  MHC class I educating impact does not correlate with altered expression of NK cell activating receptors, maturation markers or KLRG1

Next, we attempted to address a cellular correlate to education. In theory, the different educating impact of the MHC class I molecules in single MHC expressing mice could be due to an influence of the total number of NK cells, the efficiency by which each NK cell attacked MHC class I deficient cells or a combination of these two. To address this question, we measured expression of a panel of different NK cell surface markers. We were unable to detect any major differences between the four single MHC mice (or between them and MHC$^{-/-}$ mice). Parameters investigated were the total number of NK cells, the frequencies of NK cells expressing different surface markers, and the levels of surface expression of each of these, including the activation receptors NKG2D, NK1.1, 2B4, CD16 or Ly49D. The same pattern was observed for the maturation marker CD11b (Mac-1). The receptor KLRG1 has been proposed as a marker for NK cell activation and their competence for functional missing self reactivity [264]. No differences could be detected between the single MHC mice, however, all single MHC mice had higher frequencies of KLRG1$^+$ NK cells then the MHC$^{-/-}$ mice, as previously reported for B6 mice [264]. We could thus not define any correlate to educating impact within the number of NK cells with known activating receptors or with cell surface expression levels of the latter.

3.1.7  Frequencies of NK cells with downregulated Ly49r or NKG2A do not correlate with the educating impact of individual MHC alleles

As discussed above, the NK cell Ly49 inhibitory receptors expression levels at the cell surface are regulated by the MHC class I alleles expressed in the mice [68, 265]. Since mice expressing multiple MHC class I

![Figure 3](image-url)  

Figure 3. Expression pattern of different Ly49 receptors in the four different single MHC mice expressed as % MFI of the receptor expression in MHC$^{-/-}$ mice. Figure modified from Johansson et al, Journal of Experimental Medicine, 2005, 201, 1145-55.
alleles have been used in the studies of Ly49r expression it has been difficult to exactly determine what impact each of the different MHC class I alleles have on specific Ly49r. We therefore studied the cell surface expression levels of inhibitory Ly49r in the single MHC mice and normalized the values against MHC$^{-/-}$ mice. Our result demonstrated that each MHC class I allele indeed shaped the cell surface levels of inhibitory Ly49r in a specific pattern (Figure 3). We reasoned that each NK cell with at least one downregulated receptor had been influenced by the MHC molecules of the host, i.e. that it had been potentially educated. The total number of NK cells with at least one downregulated receptor could therefore be considered to reflect the total number of educated NK cells, and might be used as a determinant to predict the educating impact. To analyze this we could not simply add the number of cells for each receptor studied. Since some cells express more than one receptor, this would overestimate the number of educated NK cells. We compensated for this by using the “product rule”, allowing us to exclude counting NK cells that downregulated more than one Ly49r twice (for the calculation of the “product rule” see the chapter on Ly49 receptors). We also included the expression of the inhibitory receptor NKG2A, which has a ligand in all single MHC mice except for K$^b$ single mice. There was no correlation between the educating impact and the numbers of NK cells with any downregulated Ly49 receptor, regardless of whether co-expression of NKG2A was taken in account, and no difference in the frequencies of NKG2A$^+$ NK cells were seen between the mice. This suggests that the educating impact of an MHC class I gene does not simply reflect the number of NK cells that have been educated by Ly49 interaction with the corresponding gene product.

3.1.8 Ly49r surface expression levels in combination with number of NK cells with downregulated Ly49r can be correlated with the educating impact of individual MHC alleles

Some Ly49-MHC interactions might influence the NK cell stronger if affinity and interaction time affects the strength of the resulting signaling. In the calculation above, we had considered each Ly49 receptor downregulation as equal. We reasoned that the degree by which each Ly49 receptor was downregulated may reflect the affinity and the interaction strength between the Ly49 receptor in question and the MHC class I allele expressed. As shown in figure 3, the degree of downregulation for a given Ly49 receptor varied substantially between different single MHC mice. It was thus possible that the degree of downregulation reflects the educating impact of the Ly49-MHC class I interaction in question. In each of the MHC class I single strains, we therefore calculated the degree of downregulation for each individual Ly49 receptor and multiplied it with the frequency of NK cells with this particular receptor. This was done for all downregulated receptors and was then summarized. When we compared this weighted sum of downregulation with the educating impact for each MHC class I allele, we found a correlation: D$^b$ and L$^d$ with low educating impact had low weighted sums (8.5 and 18.8), while alleles with strong educating impact (D$^d$ and K$^b$) had high weighted sums (29.8 and 33.4). Our study therefore suggested that the MHC class I alleles differ in the number of NK cells that they educate, as well as the degree by
which each NK cell is educated. Together these two factors would determine the efficiency of rejection of MHC−/− cells in mice expressing that MHC molecule, i.e. the educating impact. One important feature of this model is that the education may not simply function as an on-off switch, but rather act in a quantitative fashion in the individual NK cell. This has later been developed and tested further by Petter Höglund and other colleagues in the group.

3.1.9 How do our results fit with existing models?

In the introduction, I have discussed the two major hypotheses on how NK cells can be tolerized by altered functional status of each single cell rather than altered frequency distribution of different Ly49r subsets; the “Licensing” model [218] and the “Disarming” model [217]. Both of these models imply that NK cells need to interact with inhibitory, as well as activating receptors to achieve tolerance. Neither of the above mentioned hypotheses has taken into account the quantitative effects of different alleles in the educating process of NK cells. The “licensing” model states that an NK cell needs an interaction between its inhibitory receptor(s) and the cognate ligand(s) in order to be able to fully mature and display competent missing self recognition. However, if the strength of the Ly49-MHC class I interaction in the “licensing” model can vary quantitatively (depending on numbers of receptors and ligands and the affinity between them), the result of a low avidity interaction of the Ly49 receptors and their cognate ligands would result in an NK cell which have low reactivity. Hence, it would be less responsive, in terms of how fast it kills cells or how much cytokine it produces. As to the “disarming”, this model implies that each NK cell need to interact with both inhibitory and activating receptors in order to be fully competent in “missing self” recognition, and NK cells which fail to interact with both inhibitory and activating receptors would become anergic. Using similar quantitative reasoning as the one applied for the “licensing” model, the disarming model would imply that NK cells can become more or less disarmed, e.g. a low avidity interaction with the inhibitory Ly49 receptors and their cognate ligand(s) would make the NK cells less responsive but not completely anergic.

In our study, we demonstrated that one MHC class I allele is sufficient to educate the NK cells in the “missing self” recognition. However, the different MHC class I alleles show considerable differences in their ability to educate. In our model this can in part be explained by the fact that MHC class I alleles can interact with many different Ly49 receptors (for example, Dd has been shown to interact with both Ly49A, Ly49C and Ly49G2). Hence, depending on the affinity between the MHC class I and the given Ly49 receptor, the NK cells would be tuned with more or less inhibitory signals. In this model, an MHC allele which can bind many Ly49 alleles with high affinity would thus impose a stronger imprint in terms of Ly49 downregulation on the NK cells and educating impact, compared with an Ly49 receptor which binds fewer MHC class I alleles with weaker affinity. High educating impact could thus be associated with a higher number of educated NK cells, as well as with a higher responsiveness of each NK cell.
Thus our data can fit both the “licensing” and “disarming” models, in both cases adding a quantitative touch to the process. However, as discussed in the introduction, previous studies by Johansson and co workers [216] indicated that NK cells in mice with chimeric expression of their MHC class I alleles were tolerant in vivo but lost tolerance after separation of MHC ligand expressing and non-expressing cells and culture in vitro. As already discussed, this observation fits the “disarming” model better than the “licensing” model, and in addition, suggests that tolerance is reversible.

Apart from the implications for understanding education and tolerance, understanding the role of single receptor-ligand interactions could have important practical implications. There are some viruses and tumors that downregulate MHC class I molecules of one allele, thus avoiding T cell-mediated killing. However, they might still express other alleles of MHC class I which inhibits NK cells and which are not as fully potent in mediating T cell-mediated killing. Thus, it is important to test whether different MHC class I alleles have different influence on the education of missing self reactivity on the NK cells.

3.2 BLOCKING OF NK CELL INHIBITORY RECEPTORS TO INDUCE TUMOR CELL KILLING (PAPER II)

3.2.1 Manipulating NK cells perform missing self recognition of autologous cells

Certain human leukemia’s e.g. acute myeloid leukemia (AML) is associated with high mortality and there is a need for development of alternative treatment regimes. The current treatments involve the use of allogeneic HSCT (from related HLA matched donors, or unrelated partly HLA matched donors, or haploidentical donors with one HLA haplotype matched). As discussed above, the introduction of haploidentical transplants has shown some promising results relating to KIR ligand mismatching and possible NK cell effects elicited by missing self recognition. However, there is still high mortality due to infections in haploidentical HSCT, and HLA matched transplants can also elicit GVH reactivity due to minor histocompatibility antigens. Hence, alternative strategies are of great interest, and the question is if NK cells, which apparently can mediate some AML effects, can be harnessed in new approaches.

One approach already discussed in the introductory part is to block the inhibitory receptors on the NK cells with antibodies or antibody fragments, in order to mimic missing self recognition, analogous to what occurs with KIR ligand mismatched haploidentical transplants. Koh et al have demonstrated that NK cell inhibitory receptors Ly49C/I, which recognize the MHC alleles H-2b, could be blocked in vivo, leading to an increased natural resistance towards leukemia development after grafting of a MHC class I expressing leukemia cell line [243]. Such blocking of NK cell inhibitory receptors opens up for a new and interesting way to manipulate NK cells to achieve enhanced anti tumor activity. However, the study by Koh et al left several questions unanswered. It was not clear whether the treatment worked by directly
inducing NK cells to kill leukemia cells. Furthermore, the possible effects of inhibitory receptor blockade on rejection of normal cells were not investigated, nor were the long term effects of the treatment. Would this lead to hyporesponsiveness of NK cells, or allow persistent augmentation of NK activity, and if so, would that lead to autoimmunity? Since many tumor cells express higher levels of activating NK cell ligands than healthy cells, we hypothesized that there would be a “therapeutic window” where one could partly block the inhibitory MHC class I receptors on NK cells in such a manner that it would induce killing of tumor cells but not the killing of normal autologous cells.

3.2.2 In vivo model for studies of rejection of tumor and normal cells

In an attempt to address these questions we set up an in vivo model where we used F(ab’)2 fragments against the Ly49C/I inhibitory receptors, binding to H-2Kb and the most important of the Ly49r for inhibiting NK cell activation in H-2b mice. The F(ab’)2 fragments lack the Fc part of the antibody which should allow the Ly49C/I+ NK cells to bind the antibody without being subject for depletion by macrophages and neutrophilic granulocytes. As a tumor model, we chose the RMA lymphoma, known to be relatively NK resistant, partly due to high MHC class I expression, since the TAP/MHC class I deficient variant line RMA-S is highly NK susceptible. We injected B6 mice i.p. or i.v. with the F(ab’)2 fragments in a pre-titrated dose that was sufficient to block up to 90% of the Ly49C/I receptors. At different time points after antibody inoculation, usually 24 hours, we challenged the mice by i.v. inoculation of cells labeled with CFSE, usually a mixture of two different “target” populations (e.g. RMA lymphoma cells and normal spleen cells, identified by different fluorescence intensity due to the use of different CFSE concentrations in the labeling procedure). The mice were sacrificed at different time points, usually 48 hours after inoculation of cells, and different organs were removed so that the survival of inoculated cells could be estimated by counting of the fluorescently labeled cells in flow cytometry. Previous studies with this assay have shown that different numbers of fluorescently labeled cells in the spleen reflects surviving cells in the animal as a whole, rather than differential organ distribution; a reduced number in the spleen is associated with reduced number also in the blood, liver and lungs (paper I, [266], and Kärre et al unpublished observations). To control for the role of NK cells, most experiments included groups of mice inoculated with anti NK1.1 antibody.

3.2.3 In vitro blocking of Ly49C/I can break tolerance against RMA and B6 ConA lymphoblasts

Before we started the in vivo rejection experiments we checked that the F(ab’)2 fragment could block Ly49C/I efficiently in vitro and in vivo. This was done by adding a fluorochrome conjugated, complete version of the same anti Ly49C/I antibody (5E6) to cells that had been exposed to the F(ab’)2 reagent. We were able to block the NK cell Ly49C/I receptors by up 80% using our standard protocol including one washing step
before addition of the complete antibody conjugate, and this could be increased to 90% by omitting the washing step. The failure to block completely may be due to a high off-rate for the F(\(ab')\)\(_2\) reagent. In vitro blocking of Ly49C/I receptors on cytokine activated NK cells resulted in increased killing of RMA cells in vitro, as well as in killing of syngeneic B6 lymphoblasts obtained by culture of spleen cells with ConA. This indicated that blockade at these levels with the F(\(ab')\)\(_2\) reagent had functional consequences leading to NK cell reactivity against both tumor and normal cells, at least under conditions where the NK cells, as well as the normal target cells had an activated phenotype. The F(\(ab')\)\(_2\) reagent could block Ly49C/I expression also after in vivo inoculation. The kinetics of this process were rather slow, at least when the F(\(ab')\)\(_2\) were inoculated i.p: optimal blockade (80-90%) occurred 3 days after injection whereafter it slowly diminished.

3.2.4 Blockade of inhibitory receptors induces NK mediated killing of tumor cells while tolerance to healthy cells is robust

As described in paper II, we were able to induce NK mediated killing of RMA cells by blockade of Ly49C/I receptors. The number of surviving tumor cells in the spleen was diminished by 40 - 80% by the treatment. In contrast, we never observed any reduced survival of normal spleen cells, in a series of more than 25 independent experiments, even when the kinetics were chosen so that the normal cells were exposed to NK cells during the maximal Ly49C/I blockade. This pattern differed from the in vitro data where normal lymphoblasts induced by ConA stimulation were killed by cytokine activated, Ly49C/I blocked NK cells. It was possible that the ConA stimulation led to up-regulation of NK cell activating ligands on otherwise healthy cells, thus tipping the balance in favour of killing. We therefore challenged our mice with ConA stimulated spleen cells. However, no autoreactivity could be detected against ConA stimulated cells in vivo, indicating that polyclonal activation of T-cells during inflammation does not induce NK cell mediated cytotoxicity against these cells undergoing vast proliferation, not even if the major inhibitory Ly49 receptor is blocked. We went on to test also bone marrow cells containing more proliferating cells, as well as immature cells with reduced MHC class I expression. Also these cells showed the same survival in mice treated with Ly49C/I blockade.

We concluded from these observations that tolerance towards normal cells is quite robust, and that it is possible to induce killing of tumor cells without affecting normal cells. Why was it impossible to break the tolerance towards normal cells? First, it should be noted that the killing of tumor cells induced by the Ly49C/I blockade was not extremely efficient, and certainly not what one would expect from fully fledged, “missing self” based rejection. This is evident from comparison with our data on animals inoculated with RMA-S cells, with severely reduced MHC class I expression. In untreated mice, the survival of these cells was always reduced by more than 95% compared to RMA-cells. The difference in rejection efficiency (RMA in Ly49C/I blocked mice vs RMA-S in untreated mice) may be due to several factors, i.e. the incomplete (never more than 90%) blockade of Ly49C/I, or the existence of additional inhibitory receptors (discussed further below). This implies that even the breaking of
tolerance towards tumor cells was incomplete, opening the possibility that this allowed for a “therapeutic interval” where normal cells were unaffected, perhaps due to their lower expression of activating ligands. It should be noted that also β2m−, and thus cell surface MHC class I deficient, but otherwise normal spleen cells showed at least 95% reduced survival in untreated animals. This shows that the robust tolerance to normal cells was not just an artefact of our assay, since normal cells could be efficiently rejected under conditions of “complete missing self recognition”. As to the discrepancy towards the in vitro results, where Ly49C/I blockade induced killing of normal lymphoblasts, it should be noted that these were obtained with cytokine activated NK cells. Perhaps the combination of target cell activation and effector cell activation involved more activating receptors as well as more activating ligands, tipping the balance in favor of activation and breaking of tolerance. We have recently observed that the effect of Ly49C/I blockade on rejection of RMA cells can be augmented considerably by parallel treatment of mice with IL-2 (Vahlne and Tadepally, unpublished observations), and this may be important to explore further also in relation to tolerance towards normal cells.

Given the different possibilities discussed in the previous paragraph, it appears important for further studies to identify the activating ligands and receptors that are crucial for killing of normal vs tumor cells, and develop reagents and methods to quantify their expression. It will also be important to address the role of other inhibitory receptors. The NK cell tolerance in B6 mice is maintained by Ly49C/I but presumably also by the inhibitory receptor NKG2A. Even though the Ly49C/I receptors were blocked, NK cells could maintain tolerance against healthy cells by NKG2A inhibition, overriding the activating signals. As a first step to investigate this possibility, we used a mouse B6 strain that has been gene targeted for the MHC class I gene Db, thus expressing only Kb. Unlike many other MHC class I genes, including Db, Kb does not have a leader sequence that can stabilize Qa-1 molecules. These, Kb single mice should therefore lack Qa-1 expression and their cells should not be able to induce inhibition via NKG2A recognition. However, when spleen cells from Kb single mice where inoculated into B6 mice which had been administrated with F(ab')2 fragments against Ly49C/I, we still could not observe any rejection. This indicates that NKG2A may not be solely responsible for the maintenance of tolerance of healthy cells after Ly49C/I blockade. To test this in a more critical manner, it will be important to try double blockade of Ly49C/I and NKG2A in future experiments.

3.2.5 Diminished β2m− rejection but not of RMA-S by C57BL/6 mice treated with the 5E6 F(ab')2

To ensure that missing self recognition and NK cell effector function was normal in the tested mice we used β2m− spleen cells and RMA-S cells as controls in most of our in vivo experiments. As already noted above, both of these MHC class I deficient cell types were always efficiently rejected in normal mice. However, one unexpected and potentially interesting finding was that the mice treated with Ly49C/I blockade consistently rejected β2m− spleen cells less efficiently than control mice. This observation is of interest first because it could imply that NK cells would reject also
MHC class I deficient tumor cells less efficiently after inhibitory receptor blockade. This would make a potential treatment protocol counterproductive in individuals with this tumor phenotype. However, RMA-S rejection was not influenced by the 5E6 F(ab')² fragment treatment. Secondly, one may speculate about the mechanism behind the reduced rejection of normal β²m⁻ spleen cells. One possible explanation for this finding is that upon binding of the 5E6 F(ab')² fragment to Ly49C/I, the receptors signal weakly through the inhibitory pathway giving rise to less pronounced killing. However, since the tumor express higher levels of activating ligands, this inhibition would not be sufficient to override the activation and hence would not affect tumor rejection. Another possible explanation could be that due to weaker inhibition, the NK cells take longer time during interaction and synapse formation with normal, MHC class I expressing cells in the environment. Even though they do not eventually kill these cells, the lower inhibitory input due to Ly49C/I blockade may make them more “hesitant”, resulting in longer time before they decide not to kill. This would lead to a “cold target competition” between normal cells in the mouse and the inoculated β²m⁻ spleen cells, and eventually to increased survival of the latter. Further studies are needed to make any final statements about this surprising finding of reduced killing of β²m⁻ spleens cells in mice treated with Ly49C/I blockade.

3.2.6 Repetitive administration of 5E6 F(ab')²: maintained tumor cell killing, but no detectable signs of autoreactivity

Since we had used a short term assay in order to assess the effect of Ly49C/I blockade on NK cell mediated activity, it was important also to study mice subjected to long term treatment. We first applied the protocol to maintain blockade for slightly more than two weeks. There were several possible outcomes of this experiment: 1) Loss of tolerance also to normal cells. 2) Sustained loss of tolerance to tumor but not to normal cells. 3) Anergy (due to “disarming” or failure to “license” maturing NK cells. 4) Deletion of the Ly49C/I subset due to lack of input during maturation. We observed sustained loss of tolerance to tumor cells but not to normal cells, (alternative 2) indicating that maintained blockade may be beneficial in a therapeutic setting. It is interesting to consider these results in relation to the discussion on tolerance and education above, where it was emphasized that the inhibitory input during maturation is essential for education of an NK cell. One might therefore have expected that the Ly49C/I⁺ subset would show some abnormality in terms of education upon continuous long term blockade. It may be argued that the blockade should have been maintained for a longer time period in order to see such effects – two weeks is not sufficient to renew the whole NK population. The reduced efficiency in rejection of β²m⁻ spleen cells as discussed above was also observed after two weeks of blockade, but if this is a consequence of “disarming” due to less inhibitory input, it must occur very rapidly, since it was consistently observed in experiments conducted over 72 hours. The possibility of such a rapid educating effect may seem farfetched, but it is interesting to note that the effect of Ly49C/I blockade in this respect resembled an MHC phenotype with very low “educating impact”. Deeper exploration of this may open novel ways of addressing the induction and maintenance of NK cell education.
To further address the issue of autoreactivity, the blocking protocol was applied in a long term experiment, where B6 mice received the treatment twice a week for 13 weeks. After 13 weeks a necropsy was performed on all mice and more than 50 different organs and tissues were examined. No signs of autoreactivity could be detected, and there were no abnormalities in terms of differential blood cell counts.

3.3 NK CELL REGULATION OF DC’S VIA THE INHIBITORY RECEPTOR NKG2A (PAPER III)

As mentioned above, DCs are required for NK cell activation but NK cells can also kill of immature DCs. Hence, NK cells have been proposed to be important in the regulation of the adaptive immune response due to DC killing. The maturation stage of the DC is important since immature DCs are more sensitive to NK cell-mediated than DC infected with viruses or bacteria or DCs treated with TLR binding ligands such as CpG or LPS.

In this study we investigated the potential protective effect of the inhibitory ligand Qa-1\textsuperscript{b} on DC killing mediated by NK cells. Qa-1\textsuperscript{b} is a non-classical MHC class I molecule that binds peptides derived from the leader sequences of proteins and in particular the Qdm peptide derived from H-2D\textsuperscript{b}. The receptor for Qa-1\textsuperscript{b} on NK cells is the NKG2A-CD94 complex. We examined the ability of NK cells and NKG2A subsets of NK cells to kill of stimulated DCs both \textit{in vitro} and \textit{in vivo}. From these studies, we found that NKG2A expression on NK cells determines the ability of NK cells to discriminate between mature and immature DCs.

3.3.1 IFN-\gamma and LPS stimulation of DCs protects against NK cell mediated killing both in vivo and in vitro

Maturing DCs with IFN-\gamma or LPS resulted in increased expression of classical MHC class I molecules as well as the non-classical molecule Qa-1\textsuperscript{b}. Using conventional \textit{in vitro} cytotoxicity assays, we confirmed previous findings that mature DCs are less sensitive to NK cell mediated lysis. We also employed an in vivo assay, in which DCs were labeled ex vivo with \textsuperscript{51}Cr and then injected i.v. into mice. After 16 hours, the lungs from these animals were removed and examined for radioactivity. In this case, increased detection of radioactivity is an indicator of cell survival. Using the \textit{in vivo} assay, we also observed that mature DCs were protected from NK cells \textit{in vivo}.

3.3.2 Decreased killing of DCs by NKG2A\textsuperscript{+} NK cells

Since IFN-\gamma and LPS stimulated DCs had an increased surface expression of Qa-1\textsuperscript{b}, we hypothesized that Qa-1\textsuperscript{b} could protect the DCs from killing mediated by NKG2A\textsuperscript{+} NK cells, which make up approximately 50% of all NK cells. We therefore sorted NK cells into their NKG2A\textsuperscript{+} or NKG2A\textsuperscript{-} subsets and tested there killing against immature or
mature DCs (IFN-γ or LPS) in an *in vitro*. From these assays, we found that NKG2A⁺ NK cells had reduced killing of mature DCs while the NKG2A⁻ NK cells could still kill mature DCs.

### 3.3.3 Stable expression of Qa-1ᵇ protects DCs from NK cell mediated killing

Qa-1ᵇ expression is dependent on the Qdm peptide derived from the leader sequence of H-2Dᵇ molecules. We therefore challenged NK cells with DCs derived from mice lacking part or all H-2 molecules. B6.Kᵇ/ᵇ⁻ mice are C57BL/6 mice, which lack the expression of Kᵇ, but still have expression of Dᵇ. Mature DCs from these mice were protected from NK cell mediated lysis both *in vitro* and *in vivo* to same level as wild type mice. However, mature DCs from B6.Kᵇ/ᵇ⁻ x Dᵇ/ᵇ⁻ mice were killed by NK cells both *in vitro* and *in vivo*. Since these mice also have reduced expression of Qa-1ᵇ, we hypothesized that the increased killing was due not to lack of Db itself but lack of Qdm to stabilize Qa-1ᵇ.

Since the expression of Qa-1ᵇ and the Qa-1ᵇ/Qdm peptide assembly is partially dependent on TAP, we tested the killing of mature DCs from B6.TAP⁻/⁻ mice which lack expression of classical MHC class I molecules as well as Qa-1. DCs from these mice were incubated overnight with IFN-γ and Qdm or control peptide (Qdm-C4). While NKG2A⁺ NK cells killed mature TAP⁻/⁻ DCs loaded with Qdm or control peptide equally well, NKG2A⁻ NK cells were inhibited from killing the DCs loaded with Qdm peptide. This demonstrated that stable Qa-1ᵇ expression was required for protecting DCs from being lysed by NK cells. Finally, stabilizing TAP⁻/⁻ DCs with the Qdm peptide increased the survival of DCs when injected into wild type mice. In fact, the level of protection was equal to that seen for mice that had been depleted of NK cells.

### 3.3.4 Conclusions and future studies

In this study, we have demonstrated that mature DCs are protected from NK cell-mediated by increased expression of not only classical MHC class I molecules but also the non-classical MHC class I molecule Qa-1. Furthermore, stabilization of Qa-1ᵇ can protect the DC from NK cell mediated lysis. This may mean that using DCs loaded with peptides that stabilize Qa-1 or its human equivalent HLA-E may be more effective in immunizations as less of the cells will be eliminated by NK cells.

Our findings might have implications for the potential benefits in clinical settings were HSCT is conducted. Donor NK cells have been proposed to mediate less GVHD by killing of recipient DCs, leading to less priming of the donor T-cells. Blocking of NKG2A on donor NK cells could thus improve the killing of recipient mature DCs resulting in less activation of donor T-cells and hence less GVHD.
3.4 REGULATION OF NK CELL EFFECTOR FUNCTIONS (PAPER IV)

3.4.1 Rational behind the study

Two major effector functions in NK cells are cytotoxicity and cytokine release. Cytotoxicity is at least in part mediated via exocytosis of secretory lysosomes containing perforin and granzymes (a process here referred to as degranulation). It is not fully elucidated if these effector functions are coordinately regulated or not. Human NK cells can be divided into two different subpopulations based on their cell surface expression of CD56. CD56bright NK cells are more efficient producers of cytokines while CD56dim NK cells are efficient killer cells and degranulate upon target cell interaction. Recent data have suggested that the murine NK cells can be divided into two subsets depending on their surface expression of CD27 and Mac-1 [161]. One interesting possibility is that these murine subsets correspond to the human NK cell subsets defined by the degree of CD56 expression. Furthermore, in experimental settings NK cells have demonstrated an ability to eliminate small tumor challenges and blood borne tumor cells. However, NK cells show poor effects on tumor masses which have reached a certain size, indicating that NK cells may be inhibited in their effector function when the ratio of tumor cells versus NK effector cells becomes too high. In the study in paper IV we set out to study the regulation of NK cell effector functions in naïve NK cells and NK cells pre-activated with the IFN-α/β-inducer tilorone. We investigated the functional properties of NK cell subsets based on the expression of CD27 and Mac-1 and further studied if NK cells can be differently regulated depending on the amount of tumor cells encountered by the NK cells.

3.4.2 Method used

A recent methodological advance in the research on cytotoxic cells allows the study of NK cell (or T-lymphocyte) degranulation with the use of specific targeting of a lysosomal vesicle membrane bound protein, CD107a [267-270]. In the course of NK cell degranulation, perforins and granzymes are released via secretory lysosomes (also known as cytolytic granules) in the NK cell:target cell synapse, with the subsequent initiation of apoptosis of the target cell. When the granules are released their membranes fuse with the NK cell membrane and CD107a is brought to the NK cell surface (Figure 4). Once CD107a reaches the surface it is possible to tag the CD107a with a fluorescently labeled monoclonal antibody. Thus NK cells which have been degranulating can be tagged on a single cell level and subsequently investigated by the use of FACS analysis. The advantage of this method is that it allows the investigator to identify, enumerate and interrogate additional functions of the cells that have actually engaged in degranulation based effector function. We have set up an in vitro model to investigate mouse NK cell degranulation and IFN-γ production, as well as effector function of the CD27 and Mac-1 populations.
3.4.3 Influence of cytokine pre-activation of NK cells on effector responses to stimulation of activating receptors.

Stimulation of resting NK cells (i.e., isolated from normal non-treated mice and tested without in vitro activation) with YAC-1 tumor cells or plate bound antibodies against NKG2D resulted in poor degranulation responses and no IFN-γ responses. Effector functions mediated by resting human NK cells have been studied by Bryceson et al [247, 271]. They concluded that the stimulation via a single activating receptor may lead to degranulation in resting NK cells. However, in order to perform fully functional degranulation, reorienting the secretory apparatus and releasing the granules in the NK:target cell synapse, it was often necessary to stimulate via several activating receptors in parallel [271]. The only single receptor stimulation leading to fully functional degranulation was the one elicited through CD16. Our study of mouse cells showed that the pre-activation of murine NK cells in vivo by the interferon inducing agent tilorone was sufficient to enable NK cells to degranulate after stimulation via NKG2D or NK1.1. The use of tilorone activated NK cells would mimic the activation via IFNα/β in response to a viral infection in vivo. NK cells might thus be “primed” during such conditions to be able to react via one single type of activating receptor. However, further studies are required to investigate whether this enables fully functional degranulation including reorientation of the secretory apparatus.
3.4.4 Differentially regulated degranulation and IFN-γ production dependent on tumor cell densities

In our in vitro model, using the prototypic NK cell targets YAC-1 lymphoma cells, we demonstrated that a higher fraction of NK cells produced IFN-γ when the tumor cell numbers were high, while NK cells that encountered low numbers of tumor cells were more prone to degranulation. Degranulation seemed to be inhibited at high target cell densities. We cannot formally exclude trivial explanations based on tumor cell factors interfering with the assay used for measuring degranulation, however, our control experiments argue against such explanations, and allow explanations based on effects on NK cell function. Antibody-mediated stimulation of activating receptors resulted in similar increases in responses for both effector functions at higher antibody concentrations. This argued against that the degree of stimulation of activating receptors could explain the differential responses to changes in target cell numbers. Furthermore, NK cell interactions with other types of tumor cells, expressing inhibitory ligands of other types or at other levels, resulted in similar effector responses. Thus, the degree of inhibitory receptor stimulation is unlikely to explain these results. Other unknown target cell factors may play a role in dampening NK cell degranulation at high target cell numbers.

These findings may provide an in vitro correlate to the observation that NK cells usually fail to mediate tumor resistance in vivo when tumor burdens are large. Our results may also be discussed in relation to observations in the course of murine cytomegalovirus infections (MCMV). MCMV infects both hepatocytes and splenocytes however there is a higher infection rate in hepatocytes then splenocytes. NK cells inhibit viral replication in the liver mainly by producing IFN-γ while NK cells in the spleen do this mainly by perforin (i.e. degranulation dependent mechanisms). The relevance of our data for these biological situations are speculative at this stage. However, we can safely conclude from our data that IFN-γ production and degranulation are not coordinately regulated and that caution should be used when interpreting data based on one type of assay or NK function: low IFN-γ production is not equivalent with low degranulating capabilities and vice versa.

3.4.5 CD27 and Mac-1 effector functions

The search for murine NK cell subsets corresponding to the human CD56 bright/dim populations have lead to the suggestion that CD27 and Mac-1 can be used as markers for a similar classification of murine NK cells. Hayakawa et al [161] showed that murine NK cells can be divided into two mature NK cell populations, Mac-1 high CD27 high and Mac-1 high CD27 low. They suggested that the former might correspond to the human CD56 bright NK cells since both murine CD27 high and human CD56 bright NK cells can mediate cytokine responses such as IFN-γ production upon stimulation. However, the murine CD27 high NK cell subset also displays functional cytotoxicity against tumor cells in vitro, a feature that does not correlate well with those of the human CD56 bright
NK cells. The Mac-1\textsuperscript{high} CD27\textsuperscript{high} NK cell population exhibited more potent responses upon stimulation by target cells, as well as by IL-12 and IL-18, than the Mac-1\textsuperscript{high} CD27\textsuperscript{low} NK cell subset. The activation of the Mac-1\textsuperscript{high} CD27\textsuperscript{high} NK cells resulted in both killing of target cells and IFN-\gamma production. One suggested explanation for these findings was lower levels of inhibitory receptors on the Mac-1\textsuperscript{high} CD27\textsuperscript{high} cells than in the Mac-1\textsuperscript{high} CD27\textsuperscript{low} population. Hence the higher levels of inhibitory receptors expressed by the Mac-1\textsuperscript{high} CD27\textsuperscript{low} subpopulation would induce stronger inhibition making these NK cells less sensitive to missing self recognition. Since CD27 has been suggested to be a marker for immature NK cells one could hypothesize that as the NK cells mature they downregulate CD27. Hayakawa et al nicely demonstrated in their study that the Mac-1\textsuperscript{high} CD27\textsuperscript{low} cells were less efficient in turnover measured as BrdU incorporation in response to stimuli than the Mac-1\textsuperscript{high} CD27\textsuperscript{high}, which further would strengthen hypothesis that the Mac-1\textsuperscript{high} CD27\textsuperscript{high} subpopulation is less mature.

In order to further dissect the NK populations defined by CD27 and Mac-1 markers, we used the assay based on detecting CD107a to study degranulation and IFN-\gamma production by these different subsets. Similar to Hayakawa et al we observed that the Mac-1\textsuperscript{hi} CD27\textsuperscript{hi} NK cell population was the most efficiently responding population, in terms of degranulation, as well as IFN-\gamma production. However, in contrast to Hayakawa et al we demonstrated that the Mac-1\textsuperscript{hi} CD27\textsuperscript{lo} population also could respond to target cell stimulation by degranulation and IFN-\gamma production. In fact, we observed that a greater proportion of the Mac-1\textsuperscript{hi} CD27\textsuperscript{hi} NK cells were better in producing IFN-\gamma than the Mac-1\textsuperscript{hi} CD27\textsuperscript{hi} NK cells, that is a greater fraction of the CD27\textsuperscript{low} NK cells had the ability to produce IFN-\gamma than in any other of the subpopulations studied. On the basis of our findings, we suggest that the NK cells can be distinguished based on their maturation stage: the least mature NK cells (Mac-1\textsuperscript{low} CD27\textsuperscript{high}) respond more efficiently with respect to degranulation, and as the NK cells become fully mature acquiring the Mac-1\textsuperscript{high} CD27\textsuperscript{low}, phenotype they become more efficient in cytokine production. It should be noted that in our studies we use mice stimulated with the IFN-\beta inducer tilorone; hence the activation level of our NK cells were probably higher than in the Hayakawa study were they used NK cells purified from unstimulated mice.

Both our study and the Hayakawa study have however demonstrated that murine NK cell subsets can differ in their ability to perform effector functions determined as degranulation, cytotoxicity and cytokine production. These findings may contribute to the further exploration of the relevance of different human NK cell subsets and maturation stages, and functional correlates \textit{in vivo}. 
4 CONCLUDING REMARKS

In this thesis I and my colleagues have studied different aspects of NK cell regulation, from the education of NK cells in missing self recognition to the activation of the NK cells with different stimuli resulting in the degranulation and IFN-γ production of these cells.

Different MHC class I alleles exerted different educating impact for missing self recognition (the strength by which NK cells reject cells missing the relevant MHC gene). Furthermore, the observations suggested some rules for how this impact is affected when two or more MHC class I alleles are expressed together. The data also suggested that the educating impact of each MHC gene depends on the number of educated NK cells as well as the efficacy state that each NK cell has been educated to by the gene. The latter aspect is particularly interesting, since it suggests that NK cell education does not operate as a simple on-off switch to achieve “licensed/armed” vs “nonlicensed/disarmed”. In future studies, it will be important to critically test this in single cell assays and also to understand the molecular mechanisms behind such NK cell education. It will also be important to understand how NK cells are educated during haematopoetic reconstitution: clinical studies suggest that allo-(missing self)-reactive NK cells can exert beneficial effects by eliminating recipient malignant cells and possibly also GvH inducing DCs, although this can occur only during a short window in time before the NK cells develop tolerance towards recipient cells.

A second set of studies demonstrated that it is possible, once tolerance has been established by MHC guided education, to break it by antibody blockade of inhibitory Ly49 receptors in such a way that tumor rejection is induced or enhanced, while the tolerance is maintained towards healthy cells. This finding opens up for the use of blocking NK cell inhibitory receptors in the treatment of malignant disease. Future studies should address whether the blockade can be combined with other modalities to achieve synergistic effects on NK cells, such as cytokines or antibodies (to elicit ADCC) [249]. It will also be important to study whether blockade during a longer period, covering the regeneration time of the NK cell population, interferes with education which might lead to hyporesponsive NK cells.

When DCs mature after being stimulated by e.g. microbes they upregulate the expression of the surface protein Qa-1. We demonstrated the interaction of the inhibitory NK cell receptor NKG2A and the ligand Qa-1 on the mature DCs is important to maintain NK cell tolerance to DCs in vivo.

Last by studying the NK cells on a single cell level we demonstrated that depending on the maturation stage of the NK cells, determined as the cell surface expression of CD27 and Mac-1, they are differently effective in exerting their effector functions such as cytotoxicity and cytokine production.

The advances in the generation, the engineering and the production of antibody-based reagents will allow for further manipulation of NK cells. NK cell mediated ADCC
should be further investigated, alone, as well as in combination with cytokines or inhibitory receptor blockade with the improved knowledge of NK cell receptors, several potential markers can be tested for manipulating NK cell effector functions. Clinical trials based on blocking inhibitory KIR have just started. In this context, one interesting additional receptor to target in blocking-studies is NKG2A. The benefit of blocking NKG2A is that this receptor is expressed by a high percentage of NK cells in all individuals. Hence, the same blocking antibody could be used in all HLA phenotypes. NKG2A is co-expressed on some T-lymphocytes and it will thus be important to exclude that the blockade of NKG2A renders T-lymphocytes autoreactive. It must be kept in mind that NKG2A blockade may induce NK cell mediated elimination of DCs \textit{in vivo}. This can be a risk, since the induction of adaptive responses may be hampered. On the other hand, this may be an advantage in certain settings, e.g. in prevention of GvH in haematopoetic transplantation.
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