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MAPPING OF THE SPECIFICITY IN MHC CLASS I
RECOGNITION BY NATURAL KILLER CELLS

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"Den vackraste känslan man kan känna är
känslan av ett mysterium. Det är källan till all
sann konst och all sann vetenskap. Den som
aldrig har känt detta kunde lika gärna varit död."

Albert Einstein

"Tankar när inte långt utan erfarenhet.
Erfarenhet när inte långt utan tankar."

Bodil Jönsson

To life and the future
Natural killer (NK) cells represent the third major lymphocyte subpopulation. They are distinguishable from B and T lymphocytes by their surface phenotype, cytokine profile and the ability to mediate spontaneous cytotoxicity, without prior sensitization, against certain tumor cells and virally infected cells. NK cells can mediate resistance against tumor growth and metastasis, as well as against certain viral and bacterial infections. They can also reject MHC mismatched bone marrow grafts, and they have recently been implicated in certain autoimmune conditions.

This thesis addresses the molecular specificity of NK cells, which express two functional types of receptors, activating and inhibitory. The activating receptors can recognize a variety of widely distributed cell surface molecules, including MHC class I molecules. Most of the inhibitory receptors characterized to date are specific for MHC class I molecules. Many normal cells express ligands for the activating receptors, and the specificity is therefore often ultimately determined by the inhibitory receptors. This can explain why NK cells kill MHC deficient tumor cells as well as nonautologous bone marrow cells lacking self MHC class I alleles as ligands for inhibitory receptors. The dominating MHC class I specific inhibitory receptors in the mouse are found within the Ly49 family, belonging to the larger superfamily of C-type lectin-like molecules. The Ly49 receptors are type II membrane glycoproteins expressed as disulfide-linked homodimers. At the start of the studies, the first receptor in this group (Ly49A) had just been identified; it was furthermore clear that tumor cells with total loss or incomplete expression of MHC class I molecules in relation to a particular host could be rescued from attack by NK cells of that host by restoring a complete MHC phenotype.

To address the allelic specificity of this MHC class I mediated protection, a series of mutant and transfected lymphoma cells were tested for the ability to escape rejection in SCID mice of H-2d type, devoid of T and B cells but with functional NK cells. It was not necessary to restore a complete self MHC phenotype in order to achieve protection from NK cells. One syngeneic allele, Dd, protected efficiently while another had no effect at all. Protection could be achieved also with an allogeneic class I molecule.

The next question was whether this allelic specificity could be mapped to a particular domain of the MHC class I molecule. This was addressed by exon shuffling between the Dd gene on one hand, and the Ld or Db gene on the other hand. The resulting chimeric genes were transfected to lymphoma cells, and studied with respect to their ability to confer protection against NK cell mediated rejection in vivo. This demonstrated that the allelic specificity resided in the α1/α2 domains, i.e. the membrane distal part of the MHC class I molecule that builds up the peptide-binding cleft. Further exon shuffling allowed a more precise mapping: the α2 domain of the Dd molecule was sufficient to confer protection in vivo as well as in vitro to rat NK cells transfected with the gene for the inhibitory Ly49A receptor. It was thus concluded that the allelic specificity (Dd) of the Ly49A receptor can be explained either by i) exclusive binding to the α2 domain, ii) simultaneous binding to conserved motifs in the α1 domain and to allele specific motifs in the α2 domain iii) binding to the α1 domain only, but to a conserved motif, strongly dependent on the Dd α2 domain.

This led to a search for structural motifs that differed between the Db and the Dd α2 domains, and could be tested for influence on Ly49A recognition. The search was initially based on molecular models, and eventually on the published structure of the Dd crystal. Two motifs, introduced or interfered with by site-directed mutagenesis, were found to markedly impair the Ly49A/Dd interaction: i) a hydrophobic ridge in the floor of the peptide binding cleft (absent in Dd, present in Db), created by amino acids at positions 73 and 156, ii) a solvent exposed loop (present in both alleles, but creating distinct motifs in each of them), formed by residues 102-109. The mutations introduced in this loop of the Dd molecule were studied by expression in constructs for MHC class I tetramer production, thus allowing direct assessment of the Ly49A binding to its ligand. The mutations in this loop did not only impair the Ly49A/Dd interaction; they also abrogated binding of the only monoclonal antibody that efficiently can interfere with Ly49A recognition of Dd.

These results are discussed in relation to the current literature on MHC class I ligands for NK cell receptors. This includes other studies on site-directed mutagenesis, on the role of the MHC bound peptide and on the MHC associated glycans, as well as the published cocrystal of the Ly49A/Dd complex, demonstrating two contact sites involving the α1 as well as the α2 domain of Dd.
Papers

This thesis is based on the following publications, which will be referred to by their roman numerals:


IV  Waldenström, M., Achour, A., Michaelsson, J., Rölle, A. and Kärre, K.: "The role of an exposed loop in the \( \alpha_2 \) domain in the mouse MHC class I H-2D\( ^d \) molecule for recognition by the monoclonal antibody 34-5-8S and the NK cell receptor Ly49A". Submitted.
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<th>Abbreviation</th>
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<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
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<td>BCR</td>
<td>B cell receptor</td>
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<td>BM</td>
<td>Bone marrow</td>
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<td>CRD</td>
<td>Carbohydrate recognition domain</td>
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<td>ER</td>
<td>Endoplasmatic reticulum</td>
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<td>H-2</td>
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<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
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<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
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<tr>
<td>KIR</td>
<td>Killer cell immunoglobulin-like inhibitory receptor</td>
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<td>mAb</td>
<td>Monoclonal antibody</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>NCR</td>
<td>Natural cytotoxicity receptor</td>
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<td>NK</td>
<td>Natural killer</td>
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<td>NKC</td>
<td>NK gene complex</td>
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<td>TAP</td>
<td>Transporter associated with antigen processing</td>
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Introduction

Natural killer (NK) cells and their role in the immune defence

NK cell biology
NK cells are considered to be the third major lymphocyte subpopulation. They were discovered independently 1975 at the Karolinska Institute and at the US National Institute of Health, by Kiessling et al. and Herberman et al. [1-3]. NK cells account for about 5-15% of circulating lymphocytes in the blood. They are also present in the spleen but are rare in other organs such as lymph nodes, thymus and bone marrow (BM) [2, 4]. In the mouse, they constitute 3-5% of the spleen cells. They are distinguishable from B and T lymphocytes by their surface phenotype, cytokine profile and the ability to mediate spontaneous cytotoxicity, without prior sensitization, against certain tumor cells and virally infected cells.

There are many excellent reviews on NK cell biology, e.g. [5-8]. The following description is based on these four reviews unless otherwise noted.

An NK cell may be defined as a sIg⁻, TCR-CD3⁻ lymphocyte that can mediate natural killing against certain targets (including also target that lack MHC class I expression or express non-self MHC class I molecules). The NK cells can be identified by expression of CD56 in the human and NK1.1 in the mouse, in combination with the criterion that the cell must also be CD3 negative. They express overlapping subsets of activating and inhibiting receptors but do not, as B and T cells, rearrange their receptors; nor do they require MHC class I expression for target cell lysis. They do not require the thymus for maturation.

Many studies of the in vivo function of NK cells are based on comparisons of normal mice and mice depleted of NK cells. This is usually achieved by treatment with a monoclonal antibody directed against NK1.1, expressed by all NK cells in some mouse strains. NK cell depleted mice are more susceptible to growth of certain tumor transplants. The NK cells appear particularly efficient at eliminating blood borne tumor cells, thereby preventing hematogenous metastasis. In humans, IL-2 activated blood lymphocytes (so called LAK cells) known to contain mainly activated NK cells, can cause reduction of tumor metastases after reinfusion to patients [9].
NK cell depleted mice also become susceptible to certain, but not all virus infections [10]. Local activation of NK cells is seen early after virus inoculation, and the NK cells are thought to play a role early in this phase, before the adaptive responses are fully efficient. This is corroborated by anecdotal observations in patients with impaired NK cell function. The most well studied case of a patient with a total and selective NK cell defect was a young woman, who came down with severe forms of several herpes virus infections (Varicella-, Cytomegalo- and Herpes Simplex Virus) [11]. Each infection was eventually resolved as the B and T cell responses developed. It should be noted that some viruses, such as LCMV, do induce an early NK cell activation in mice, but this response does not seem important at all for protection against the infection. NK cells can also contribute to resistance against certain bacteria and parasites (e.g. Listeria monocytogenes [12] and Toxoplasma gondii [10]).

In contrast to normal mice, which reject BM grafts in MHC mismatched (i.e. in allogeneic as well as in F₁ antiparental host/graft) combinations even after T cell reactivity has been abrogated by high dose irradiation, NK depleted mice fail to do so. This capacity to reject MHC mismatched cells is seen also with transplanted lymphocytes and hematopoetic tumors.

More recently, NK cells have been implicated also in autoimmune disease, acting either directly by killing autologous cells [13], or by cytokine regulation of adaptive immunity [14, 15]. There is also evidence that NK cells can regulate myelopoiesis.

The activity of NK cells is controlled through a delicate balance between positive and negative receptor signaling [16]. This controls the specificity of NK cells, a key issue in this thesis. The nature of these receptors will therefore be discussed in detail in a later section. NK cells exert their main effect through direct killing of targets cells by exocytosis of lysosomal granules containing perforin (pore-forming protein), granzymes (serine proteases) and other enzymes, as well as by cytokine secretion. NK cells can also in certain situations kill via the Fas/Fasligand interaction. The direct, perforin dependent killing effect is responsible for the rejection of at least some tumor cells in vivo. The role of this effector mechanism is less clear in bone marrow rejection by NK cells; the outcome of a series of experiments addressing this issue depended on the animal colony where perforin defective mice were kept (M.
Bennet, personal communication). In the resistance against virus infections, NK cells may use direct killing as well as cytokine secretion, even against the same virus. For example in cytomegalovirus infection, resistance against virus replication is perforin dependent in the spleen, but IFN-γ dependent in the liver [17]. NK cells are in some situations critical for controlling the type of adaptive response, e.g. T_h1 versus T_h2 dominance. This control is exerted via cytokine secretion by NK cells. Apart from IFN-γ, NK cells can also produce TNF-α, GM-CSF and IL-3.

NK cells cannot only produce cytokines. They can also, as all other cells of the immune system, respond to several cytokines: they are activated by IL-2, IL-12, IFN-α, IFN-β and TNF-α. Resting NK cells express the intermediate-affinity IL-2 receptor βγ, which may explain why NK cells are the main responders when nonsensitized lymphocytes are exposed to IL-2. IL-12 and TNF-α activates NK cells to produce IFN-γ, which is crucial in controlling an infection before the action of T cells. NK cells can be downregulated by TGF-β.

Several chemokines can influence the mobility as well as cytotoxic activity of NK cells. For example, MIP-1α appears to be critical to attract NK cells to infectious foci in containing Cytomegalovirus [18].

NK cells are bone marrow derived [19]. It seems like NK cells arise from a common lymphoid progenitor. Mutation in the Ikaros gene, required for the initiation of lymphocyte lineage differentiation, resulted in complete deficiency of B, T and NK cells [20]. NK cells more closely resemble T cells than B cells, suggesting that NK and T cells may arise from an immediate common progenitor cell. However NK cells are normal in SCID and RAG-1 or RAG-2 mice. These mice have mutations in the recombinase machinery resulting in B and T cell-deficiency. Moreover, athymic nude mice appears to have normal NK cells, suggesting that NK cell development in thymus independent. Cytokines that influence NK cell development include IL-7, IL-12, IL15 and IL-18 [21].

NK cells share several cell surface markers with T cells as CD2, Thy-1, CDζ and CD69 (on activated cells). In the human, thymocytes have been shown to develop into NK cells in vitro [22]. In the mouse, a recent in vitro system was established
where bone marrow derived cells differentiate into NK cells [21]. In a normal mouse, NK cells are considered to be fully mature after three weeks, on the basis of activity levels and patterns of receptor expression [23].

**Innate immunity**

NK cells are important in the first line of defense against invading pathogens. This phase is called the innate response; it has a critical role in controlling infections the first days or week until the adaptive immune response can act in full effect. In most cases the innate host defenses prevent infection from being established, but when evaded or overwhelmed an adaptive response is required. The initial response to a foreign pathogen acts within minutes of infection. It uses pre-existing or rapidly inducible (within hours of infection) cellular and soluble proteins. These cellular components provides an immediate defence against microorganisms by directing the engulfment and breakdown of microbial cells. Plasma proteins, such as the complement cascade and the mannose binding protein, identify carbohydrates of microbial cell walls. In this phase of the response, the complement pathway is activated without antibodies. The most important cells of the innate response are neutrophils, macrophages and NK cells. Macrophages and neutrophils are primarily phagocytic cells and through their cell surface receptors, e.g. for complement, mannose, lipopolysaccharide, they ingest pathogens directly or engulf coated particles, which they then destroy in intracellular vesicles. Activation of these phagocytic receptors triggers the phagocytes to produce inflammatory mediators as IL-1, IL-6, IL-8, IL-12, TNF-α, oxygen radicals, peroxides, nitric oxide, prostaglandines and leukotrienes. Together this leads to the recruitment of more phagocytes and effector mechanisms to the site of infection.

Another important function of these mediators is to induce the expression of adhesion molecules on endothelial cells of the local blood vessels, which bind to the surface of circulating monocytes and neutrophils. This greatly increases the rate of migration of these cells out of the blood and into the tissues. The cells and molecules of the innate immune system also play a crucial part in the initiation and subsequent direction of the adaptive immune response. The dendritic cells have a key role in this respect [24]. They can recognize some pathogen associated molecular patterns directly, e.g. via Toll-receptors [25]. As a consequence of this and the influence of
cytokines relaxed during early induced responses, they are induced to migrate to local lymph nodes. They are also induced to mature from mainly a pinocytosing phenotype to an antigen presenting phenotype with high expression of MHC and costimulatory molecules, and to secrete cytokines. Cytokines produced during the early phase of an immune response have an important role in shaping the development of the adaptive immunity. IL-12 is produced by macrophages and dendritic cells, and IFN-γ is produced by NK cells in the early phases of responses to viruses and some intracellular bacteria. These two cytokines favour the differentiation of T_{H1} cells (see below).

**Adaptive immunity**

The adaptive immune response is built up by the systems of T (cellular) and B (humoral) lymphocytes. Each cell in those two systems expresses a distinct antigen receptor that has arisen by somatic gene rearrangement. Upon exposure to antigens of an infectious organism, presented by MHC molecules on dendritic cells that have migrated to the lymph node, the T and B cells that specifically recognize the antigen are activated and start to proliferate. The presentation machinery will be discussed in the next section. The frequency of T and B cell cells specific for a particular antigen is initially too low for a rapid protective response, perhaps 1 in 10,000-100,000. During the ensuing 1-2 weeks, the T cell clones replicate about 1000 times and mature into cells that defend the host by killing virally infected cells, or by activating macrophages and B cells. The activated B cell clones undergo similar processes and eventually secrete antibodies. Upon a second exposure of the same microorganism, the response is rapid due to expanded, antigen-specific memory cells remaining from the first exposure. The CD8 T cells mostly develop into cytotoxic cells and the CD4 T cells into cytokine secreting “helper” cells. The latter activate macrophages and killer cells, or stimulate B cells to divide, to undergo further switching rearrangements and to eventually secrete antibodies.

CD8^{+} T cells kill mainly via the release of cytoplasmic granules containing perforin and granzymes as described for NK cells. They can also kill via Fas ligand/Fas interactions. Naive CD4^{+} T cells can, upon activation, develop into either T_{H1} or T_{H2} cells. These subsets of CD4^{+} T cells differ in their cytokine profile and hence in their function. The T_{H1} type T cells secrete IL-2 and IFN-γ, and can thereby activate CD8^{+}
T cells and infected macrophages to release cytokines inducing lysosomal fusion followed by bactericidal activity. Another important mechanism involves the Fas/Fas ligand interaction, leading to apoptosis of macrophages who have lost their ability to become activated. The Th2 type T cells use cytokines to activate B cells to produce certain isotypes of antibodies, mainly for combat of extracellular pathogens. The main Th2 cytokines are IL-4, IL-5, IL-6 and IL-10. Because the decision to differentiate into Th1 versus Th2 cells occurs early in the immune response, the cytokines produced by the cells of the innate immune system play a crucial part in shaping the following adaptive immune response. There is recent evidence that NK cells can influence the functional T cell response after immunization with autoantigen, thereby influencing the pathogenic response in myastenia gravis [14, 15]

The innate and adaptive systems can interact also in the later phases of an immune response. Antibodies produced by B cells can cause neutralization (binding of antibody results in blocking of pathogenic properties), independently of other components, but many effector mechanisms of antibodies require cells of the innate system. Opsonization, binding of antibody resulting in recognition by phagocytic receptors of granulocytes and macrophages, is one example. NK cells express the Fc receptor FcγRIII (CD16) which recognizes the IgG1 and IgG3 subclasses and triggers cytotoxic attack by the NK cell on antibody-coated targets. This combined innate-adaptive mechanism is called antibody-dependent cell-mediated cytotoxicity (ADCC) and shows many similarities to killing described for NK cells (see above). A third example is the activation of macrophages by Th1 cells discussed above.

The two adaptive systems also synergize: activated B cells whose antigen specific receptors have undergone “affinity maturation”, i.e. have been selected for high affinity for antigen after mutations, present antigen to T cells in the later phase of the response. The T-help is thus focused on the B cells with increased specificity as the response develops.

Understanding the adaptive immune response and its interactions with innate mechanisms is important for the development of tools to control allergies, autoimmune disease, organ graft rejection, vaccination and responses against cancer cells.
MHC class I molecules

Genes
The major histocompatibility complex (MHC) (encoding MHC molecules) extends over almost $4 \times 10^6$ base pairs which corresponds to 0.1% of the total genome [26]. The identification of this gene complex is based on the concept of histocompatibility [27]. When skin grafts are transplanted between individuals, the transplant is usually destroyed by the recipient’s immune system because the cells in the donor tissue express molecules on their surfaces that are different from those expressed by the recipient. This is termed histoincompatibility. If the graft does not express foreign molecules, it is accepted; it is then considered histocompatible with the host. More than 40 different loci control skin graft rejection in the mouse. The second locus identified historically, H-2 located on chromosome 17, was found to be by far the most important locus. This was evident from the rapid destruction of grafts after transplantation over the H-2 barrier. It soon became clear that the genetically defined locus represented a complex of several genes, and H-2 was thus termed the major histocompatibility locus. In humans, the MHC is called HLA (Human Leucocyte Antigen) and is located on chromosome 6.

The MHC molecules from a different individual act as foreign antigens, in the same way as molecules of an invading virus or bacterium, and as such stimulate the recipient’s lymphocytes. The MHC encoded molecules thus determine whether the tissue will be immunologically compatible with the recipient, and they were termed histocompatibility antigens. Naturally occurring MHC molecules are termed allogeneic in relation to a particular host if they differ from the MHC molecules of that host; if not, they are termed syngeneic. We know today that the function of MHC molecules is to transport and present short peptide antigens to T cells, which will recognize the MHC molecules as “altered self” if the presented peptide is foreign. The strong recognition of allogeneic MHC molecules in histoincompatibility reactions can be interpreted as a side effect of the physiological function, i.e. the allogeneic MHC molecule is perceived as an altered altered MHC molecule by T-cells. The genes in the MHC that encode peptide presenting molecules mainly fall within two groups: MHC class I and MHC class II genes. Both groups encode proteins expressed on the cell surface, but with different tissue distribution and function.
class I genes are divided into class Ia and Ib. Class Ia genes are in the mouse encoded by the H-2K and H-2D loci; some strains also encompass an H-2L locus. In humans the corresponding loci are called HLA-A, -B, and -C. The MHC class Ia genes are polygenic, i.e. there are several MHC loci in a given individual, encoding molecules with different specificity in terms of the presented antigens. Each of the loci encoding the classical class I and II are also polymorphic; one out of many different but related genes, alleles, are expressed in that particular locus (or loci; one from the mother, one from the father) in a given individual. The combined effects of a polygenic and polymorphic system make it possible to present many different antigens to the immune system, within the individual, an to an even greater extent, within the population.

The class Ib genes encode the non-classical class I molecules, including HLA-G and -E and MICA in humans and Qa-1^b in mice. These are in general nonpolymorphic, or at least less polymorphic than the class Ia molecules. There are also MHC class I-like genes encoded outside the MHC, i.e. the CD1 family.

The molecules encoded by the class II genes are H-2IA and IE in mice, and HLA-DR, DQ and DP in humans. Genes encoding molecules that do not present antigen, but are nevertheless involved in antigen presentation, are also located within the class II region. These include the two genes for the subunits of transporter associated with antigen processing (TAP) and the LMP genes, which encode components of the proteasome.

There are also MHC class III genes. These encode some of the proteins in the complement system and cytokines such as TNF-α and TNF-β (lymphotoxin).

Finally some words about the rat MHC, since this has also been studied in relation to NK cell specificity. It is designated RT1 and is located on chromosome 20. The genetic organisation resembles mouse and human MHC. The genes corresponding to classical class Ia genes are localized in a region called RT1-A in the rat. Some haplotypes express only a single locus, whereas other haplotypes express two loci. These genes display a clear sequence difference from mouse classical class I.
molecules. There are also nonclassical class I genes in the RT1.C region, including at least one which appears to encode an activating MHC ligand for NK cells [28].

Structure

Today we know a lot about both the structure and function of MHC molecules. Since my thesis deals with the MHC class I molecules and their interaction with NK cells, more precisely an NK cell inhibitory receptor, I will use the rest of this section to describe these molecules. Since the publication of the HLA-A2 crystal structure in 1987, many additional structures of the MHC molecules have been solved. The MHC class I molecule is a complex of the MHC encoded heavy chain, β₂-microglobulin (encoded by a gene on chromosome 2 in the mouse and 15 in the human) and an 8-11 amino acid long peptide (Figure 1). The heavy chain (approximately 44 kDa) consists of three extracellular domains (denoted α₁, α₂ and α₃, each 80 amino acids), a transmembrane part and a cytoplasmic domain. The β₂-microglobulin of 12 kDa is non-covalently linked mainly to α₃ residues.

Figure 1. A schematic view of the Dd complex. Adapted from [29].
The $\alpha_1$ and $\alpha_2$ domains interact to form a platform consisting of an eight-stranded, $\beta$-pleated sheet supporting two parallel strands of $\alpha$-helices creating the antigen binding groove. The 8-10 amino acid long peptide fits into the cavity bounded by the $\alpha_1$ and $\alpha_2$ domains. Although MHC class I molecules bind many different peptides, each allele binds only a subset of available peptides. The polymorphic residues lining the antigen binding groove creates pockets, which complement a small number of specific amino acids from the antigenic peptide. These positions in the peptide, called anchors, contribute to the restrictions as to which peptides can bind. The combination of preferences at different pockets along the cleft produces a sequence motif that is useful to predict whether a particular peptide will bind to a particular MHC molecule [30-33]. The non-polymorphic region of the $\alpha_3$ domain contains the binding site for CD8 [34].

TCR binding to HLA-A2 has been studied by site directed mutagenesis as well as by cocrystallization of soluble forms of receptor and ligand [35, 36]. The studies indicate that the TCR binds the MHC complex diagonally across the peptide binding groove. The TCR contacts peptide as well as MHC residues of the $\alpha$-helices.

The binding of NK cell receptors to MHC class I molecules are relevant to this thesis and will therefore be discussed in detail in “Results and discussion”.

**Function**

There are two functionally different ways of presenting antigens to the immune system; in the context of either MHC class I or MHC class II molecules. All nucleated cells express MHC class I molecules whereas the MHC class II molecules are expressed predominantly on professional antigen presenting cells (macrophages, B cells, dendritic cells and thymic epithelial cells).

MHC class I molecules present peptides from intracellularly-derived proteins present in the cytosol, i.e. viral proteins, some bacterial or endogenous proteins (reviewed in [37] and [38]). The peptides are generated by the proteasome, that degrades proteins continuously to be replaced by newly synthezised proteins. The peptides are transported into the lumen of the endoplasmatic reticulum (ER) by the ATP-dependent peptide transporter; the TAP-1/TAP-2 complex, which is located in the ER.
membrane. This complex prefers peptides of 8 or more amino acids with hydrophobic or basic residues at the carboxy terminus, one reason for the presence of non-polymorphic residues at the C-terminal end of the peptide binding groove of MHC class I molecules. Mutation in either the TAP-1 or the TAP-2 gene can abolish peptide transport. In the ER, there are newly synthesized MHC class I heavy chains and β2-microglobulin molecules waiting to be assembled. The last component to be associated in the formation of the complex is the peptide. A number of molecules such as calnexin and tapasin assist in stabilizing the MHC class I molecule and the loading of peptide.

Correctly folded MHC class I molecules are transported through the Golgi complex to the cell surface where they can be recognized by CD8⁺ T cells. An immune response will be elicited when the CD8⁺ T cells recognize a foreign peptide in the MHC class I molecules. Activation of naive T cells require that the antigen presenting cell is professional, i.e. expresses costimulatory molecules such as B7.1.

As to the non-classical class I molecules, the mouse Qa-1β and human HLA-E molecules are specialized in presenting peptides derived from the signal sequence of classical MHC class I molecules. Paradoxically, their loading, transport and cell surface expression are TAP dependent. Since the signal sequence is cleaved off within the ER, one would not expect that TAP mediated transport of these peptides to be necessary. The Qa-1β and HLA-E molecules serve as ligands for certain NK cell receptors discussed below.

The non-polymorphic CD1 molecules, associated with β2-microglobulin, present lipid and glycolipid antigens. The CD1 molecules are relevant for a subset of T cells, usually referred to as NK/T cells, due to their expression of the NK1.1 marker [39].

Other bacteria and some parasites are engulfed into endosomes, by phagocytic cells such as macrophages. Their antigens are presented by MHC class II molecules to CD4⁺ T cells. The MHC class II molecules on B cells can also bind free antigen.
NK cell receptors

NK cells express two functional types of receptors; activating receptors and inhibitory receptors. The activating receptors, which can recognize a variety of widely distributed cell surface molecules, including MHC class I molecules, will be discussed further below. Most of the inhibitory receptors characterized to date are specific for MHC class I molecules. There are two major structural classes of inhibitory NK cell receptors: those belonging to the immunoglobulin (Ig) superfamily and those belonging to the C-type lectin-like family. There are at least two subgroups of the latter, the Ly49 family and the CD94/NKG2 group of receptors. Murine NK cells use receptors almost exclusively within the C-type lectin superfamily. Human NK cells use receptors in the Ig superfamily as well as in the C-type lectin superfamily, although in the latter case only receptors in the CD94/NKG2 group. Thus, Ly49 receptors are used exclusively in mice.

Inhibitory receptors and their role in NK specificity

Many normal cells appear to express ligands for the activating receptors, and the specificity of NK cells is therefore often, but not always, determined by the inhibitory receptors. That means that the lysis is often a consequence of something missing on the target cells, which is the basis of the concept of missing-self recognition, discussed further below. The inhibitory receptors in different species will be reviewed below. Here, I will discuss introduce some common features of the inhibitory receptors in different structural families (and species) that may help to understand how the specificity of each NK cell and the total repertoire is formed.

All the inhibitory receptors contain a so called ITIM (immunoreceptor tyrosine-based inhibitory motif) in their cytoplasmic tail (discussed further below), used to transmit the inhibitory signal after recognition of the ligand (reviewed [5]). Each receptor can bind MHC class I, but with a broader specificity than TCRs. This means that it binds a group of different MHC class I alleles; in some cases, shared motifs explaining this crossreactivity have been defined. Conversely, there are MHC class I alleles that can be recognized by more than one receptor. Furthermore, each NK cell can express several different receptors. The process that determines these clonal or subset expression patterns of receptors is not clarified. However, the consequence is that
the NK cell repertoire achieves its specificity and diversity by the expression of functional receptors in overlapping subpopulations. Today the NK cell subsets in both rodents and humans can be defined on the basis of expression of inhibitory receptors specific for self-molecules. To add to the complexity of the situation, receptors belonging to both the Ig superfamily and the C-type lectin-like family include related isoforms that can activate NK cells.

There is to date only one inhibitory receptor that is not MHC class I specific, p75/AIRM1 [40]. It is expressed on human NK cells and on cells of the myelomonocytic lineage and it belongs to the Ig-like superfamily. The ligand for this receptor is still unknown, but p75/AIRM1 appears to mediate a sialic acid-dependent ligand recognition [40, 41].

This thesis addresses the specificity of MHC class I inhibitory receptors, which is discussed mainly in relation to the protective effect of the class I molecule in the cytotoxic effector/target interaction. It should however be noted that the contacts between receptors and ligands may have other consequences, and may be important also in other phases of NK cell development. NK cells are tolerant to the cells of the host phenotype, i.e. they appear be educated with respect to the MHC class I molecules of the host. This process is largely unknown, but it seems likely that it somehow involves interaction between inhibitory receptors and their class I ligands. Different models based on selection of clones or adaptation by “sequential receptor expression” are discussed [42]. Most models predict that the expression of a given receptor should be influenced by the presence of the ligand in the host. This is indeed so, but the nature of these changes are not fully understood. When a ligand (e.g. D^D) is introduced, the number of NK cells with one of the corresponding receptors (Ly49A) is marginally reduced [42], and the expression of the same receptor per cell is considerably reduced [43]. The latter finding has been interpreted within a calibration model for NK cells [43]. NK cell transfer studies have shown that this modulation of the number of receptors per cell is induced by continuous ligand contacts, where external ligands (on other cells, in trans) as well as internal ligands (on the NK cell itself, in cis) have an influence [44]. Surprisingly, the continuous Ly49/MHC class I ligand contacts have another consequence: the ligand is taken up by the NK cell membrane, in a selective way (i.e. only NK cells with a given Ly49
Activating receptors
While most of the inhibitory receptors recognize MHC class I molecules, the activating receptors include Fc receptors, receptors for costimulatory and adhesion molecules as well as receptors for MHC class I molecules. Some recently identified “natural cytotoxicity receptors” (NCRs) that appear to play a major role in the induction of natural killing recognize widely distributed, but otherwise unknown ligands on normal and tumor cells [45].

The most extensively studied activating receptor is the low-affinity receptor for IgG; CD16 (FcγRIII), expressed on most human and mouse NK cells, activated monocytes and a subset of T cells. Upon CD16-mediated activation, NK cells secrete cytokines, mediate ADCC and may undergo apoptosis [16]. CD16 is not covalently associated with the γ subunit of the receptor for IgE (FcεRI-γ) in mouse NK cells and with FcεRI-γ or the ζ subunit of the TCR complex in human NK cells. The signalling pathways induced by CD16 stimulation are similar to those activated on T cells by TCR triggering. Upon CD16 ligation by soluble or membrane-bound antibodies, the tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) in the ζ and γ chain of CD16 by tyrosine-kinases initiates a cascade of events that in turn results in the activation of three main signaling pathways. First, the phospholipases C γ1- and γ2-dependent pathway, that mainly controls the transcription of cytokine genes (through NF-AT and NF-kB). Second, the activation of the ras-MAP kinase pathway, mainly involved in granule exocytosis and cytokine release. Third, the PI-3K dependent pathway, mainly regulating the granule release. The signaling events leading to the activation of these pathways have been extensively studied showing a high degree of cross-talk between them [46]. The action of the CD16 receptor requires specific antibodies that mediate the specificity. While the function is a nice example of interaction between innate and adaptive immune components, it can not be responsible for the direct triggering of NK cells by target cells recognized without antibodies.
Another common receptor for the mouse, rat and human is 2B4, expressed by all NK cells and a subset of T cells. In the mouse, 2B4 exists in two isoforms due to alternative splicing, both as a stimulatory and an inhibitory receptor [47, 48]. In humans, only the activating isoform has been described so far [49]. The relative proportion and distribution of the two isoforms in the NK repertoire is unknown. The 2B4 activates NK mediated cytotoxicity in both humans and mouse when either crosslinked with mAbs or engaged with its specific ligand CD48, that is expressed on all hematopoietic cells [50]. The 2B4 receptor has been suggested to cooperate with the activating NCRs in NK cell triggering, and it is controversial whether it is a coreceptor or whether it can trigger NK cells by itself [51]. The signaling pathway(s) of activated by 2B4 are still poorly identified, but it has been described that an adaptor molecule called SAP binds to tyrosine-phosphorylated 2B4 in human NK cells and can function as a regulator of 2B4 function [49]. Interestingly, in patients with the X-linked lymphoproliferative disease (XLP), characterized by loss of function mutations in the SAP molecule and in the inability to control EBV infection, it also appears that the activating function of 2B4 is lost [52]. These data suggest a potential critical role for 2B4 in the regulation of an efficient anti-EBV response.

A third common receptor family expressed in several species is the NKR-P1 family. These receptors are type II integral membrane proteins with an extracellular C-type lectin domain. In both mice and rats there are at least three homologous genes (NKR-P1A, B and C) that map to a cluster designated the NK gene complex (NKC) [53]. NKR-P1A and C are stimulatory whereas B may be inhibitory. The NKR-P1A was first cloned in rat and was later recognized to be a homologue to the mouse NKR-P1C, encoding the mouse NK1.1 antigen, recognized by mAb PK136 [54-56]). Their ligands remain unknown. The status as triggering receptors (A and C) is based on the observation that their crosslinking with antibodies leads to cytotoxicity and cytokine release.

A fourth emerging group of receptors are the NCRs. This group so far includes NKp46, NKp44 and NKp30. These are non-MHC-restricted triggering receptors that appear to play a critical role in the induction of NK-cell mediated cytotoxicity. NKp44 is expressed exclusively by activated NK cells [57], while NKp46 is expressed both by resting and activated NK cells [58, 59]. NKp46 recognizes a ligand that is
expressed both by normal and tumor cells and that is conserved between the mouse and the human [60]. Interestingly, an NKp46 homologue has been cloned in the mouse and it is the only NCR described in other species so far [61]. NKp30, like NKp46, is expressed by all NK cells and it is the major receptor responsible for killing some tumor target cells, for which NKp46 or NKp44 do not appear to play a significant role [62].

The NKG2D acts as an activating receptor on human NK cells, where it recognizes a stress-inducible non-classical MHC molecule (MICA) that is frequently expressed on tumor cells [63, 64]. In the mouse, the homologous NKG2D receptor instead recognizes molecules encoded by the retinoic acid early inducible (RAE-1) and H60 minor histocompatibility antigen genes. These molecules are absent or expressed at low levels on normal, adult tissues. However, they are constitutively expressed on some tumors and upregulated by retinoic acid [65].

**Mouse NK cell receptors with MHC class I specificity**

In the mouse, so far mainly receptors belonging to the C-type lectin family have been well characterized (Figure 2A). The Ly49 family are type II membrane glycoproteins that are expressed as disulfide-linked homodimers and recognize MHC class I molecules. They display allelic polymorphism and there is evidence for alternative splicing of transcripts [66-69]. There are several cDNAs identified; Ly49A-P [70]. The most well characterized are Ly49A, C, G2 as inhibitory receptors, and Ly49 D and H as activating receptors. The Ly49 inhibitory receptors contain in their cytoplasmic domains ITIMs which upon stimulation are phosphorylated and able to recruit SHP-1 phosphatase to attenuate intracellular signals. In their extracellular domains there is a membrane proximal stalk region and a membrane distal “carbohydrate recognition domain” (CRD). The latter is composed of approximately 125 amino acids and accounts for 60% of the extracellular portion of the molecule. Domain swapping experiments have demonstrated that all anti-Ly49 antibodies tested are reactive with epitopes found within the CRD. They appear to lack the conserved Ca²⁺-binding site found in the Ca²⁺-dependent lectins of this structural family. In Ly49A, the putative Ca²⁺ dependent carbohydrate-binding loop (residues 224-231) is shorter and displays a different conformation. However, in the C-type lectins, in addition to its direct role in carbohydrate interaction, Ca²⁺ is integral to the structure and stabilizes the
surrounding loops that are essential for ligand binding [71, 72]. On the basis of these special properties of the CRD in Ly49 molecules, it is questioned whether the latter are indeed carbohydrate recognizing lectins, even if they belong to the same superfamily.

The first Ly49 gene was described by Chan and Takei [73] as a potential T-cell receptor candidate. Karlhofer, Yokoyama and colleagues provided the first evidence that the molecule now termed Ly49A functions as MHC class I-recognizing receptor on NK cells [74].

As depicted in Figure 2A, each Ly49 molecule can recognize a set of class I alleles. It is important to note that all groups do not agree on the exact specificity of the receptors. The discrepancy may be due to the several different techniques that have been used to measure the interaction. There are at least four principal approaches, with variations within each of them.

1. **Functional cytotoxicity assays.**
   These assays are based on comparing NK susceptibility of a target cell with and without transfection of a particular MHC class I gene.

2. **Cell to cell binding assays.**
   These assays are based on the binding between cells, on one hand with or without expression of MHC class I genes, and on the other hand, with or without expression of Ly49 genes. This is usually based on transfection of single genes in at least one of the cell populations: e.g. Takei et al. transfected Ly49 genes transiently to COS cells and studied binding to different tumor cell lines (51Cr-labeled) expressing different endogenous MHC class I molecules [75].

3. **Assays based on soluble molecules binding to cells.**
   These assays can utilize either MHC class I ligands or Ly49 receptors as soluble molecules. Kane et al. were the first to demonstrate a physical binding between D\(^d\) and Ly49A [76]. Affinity purified MHC class I molecules were immobilized on a plastic well and incubated with murine T cell lymphoma EL-4 cells transfected with the Ly49A gene. The binding could be inhibited with mAb against either D\(^d\) or Ly49A.
Figure 2A. Murine MHC class I specific inhibitory receptors on NK cells. The role of Ld as a ligand for Ly49G2 is controversial. There is also accumulating evidence that Db can act as a weak ligand for Ly49A.

Figure 2B. Human MHC class I specific inhibitory receptors on NK cells.
Another assay in this group is based on tetramerized biotinylated MHC class I molecules, which are used to stain reporter cells transfected with Ly49 genes, or freshly derived NK cells [77, 78] (paper IV).

4. Assays based on soluble molecules binding to each other.
These assays use a biochemical approach to trace the binding between soluble molecules, such as surface plasmon resonance, isolation of receptor-ligand complexes by immunoprecipitation or gel filtration, and ultimately crystallization. For example, Natarajan et al. estimated the affinity between Ly49A and Dd to be from 6 to 26 °C using surface plasmon resonance [79].

In general, the first and to a somewhat lesser degree, the second type of assays are dependent on several variable parameters (in addition to receptor-ligand binding), including; expression levels of receptor-ligand genes, the presence of other receptor-ligands that might interfere, clonal variations in other molecules that influence general adhesion, lytic susceptibility etc. The third and forth type of assays are more likely to reflect the receptor-ligand binding, since they are less dependent on other parameters. However, they are not without pitfalls. For example, soluble molecules may allow interactions that do not occur when the molecules are attached to a cell membrane, and molecular contacts in crystals may be imposed by the lattice formation.

The genes encoding NK receptors in the C-type lectin superfamily map to the NKC and is located on chromosome 6 in the mouse, chromosome 4 in the rat and chromosome 12 in the human. Its different subregions contain the Ly49 family (in the mouse), the NKRP-1 family, the CD69, theCD94 and the NKG2 genes [53, 80, 81].

The cytoplasmic domains of the Ly49 receptors are all similar in length. However, the activating Ly49 receptors (D and H) lack ITIMs. Instead, they associate with the ITAM-containing adaptor molecule DAP12, through a charged interaction in the transmembrane domains. Upon ligand recognition, they can stimulate transmembrane signaling initiated by tyrosine phosphorylation events [82]. Ly49D
recognizes D\text{d}, and an unknown ligand on CHO cells. The ligand of Ly49H is unknown.

The other group of C-type lectin receptors expressed by mouse NK cells consists of heterodimers of CD94/NKG2A, C or D. The ligand for CD94/NKG2A and CD94/NKG2C is the class Ib molecule Qa-1\text{b} (Figure 2A), recognizing certain MHC class I signal sequence derived peptides presented by Qa-1\text{b} [83-86].

The CD94 and and the four NKG2 subunits differ in their cytoplasmic tail. The CD94 has a short cytoplasmic tail, while the inhibiting NKG2A has a long cytoplasmic tail containing two ITIMs. The activating NKG2C or D have a short cytoplasmic tail with a charged residue in the transmembrane domain, allowing the association with DAP10 [63], another ITAM containing adapter molecule.

Although no murine KIR with MHC class I specificity has been identified, two mouse genes related to human KIR, designated gp49A and gp49B, have been cloned and reported to harbor stimulatory or inhibiting activity, respectively [87-89].

**Human NK cell receptors with MHC class I specificity**

Human NK cells express inhibitory receptors from both structural families (Figure 2B). The receptors in the Ig superfamily of receptors are the killer cell immunoglobulin-like receptors (KIRs). These include the receptors initially termed p50.1 and p50.2 (activating) p58.1, p58.2, p70, p140. Their ligands are the classical HLA-A, -B and -C class I molecules. The KIR family is subdivided into two subfamilies based on structure, and a new nomenclature has been adopted. Some KIR members have two Ig domains (KIR2D) while others have three Ig domains (KIR3D). KIR members vary also in the length of the cytoplasmic tails. Typically, KIR with a long cytoplasmic tail (L) deliver an inhibitory signal, whereas KIR with a short cytoplasmic tail (S) can activate NK or T cell responses. This occurs through the interaction with DAP12 and a charged amino acid in the membrane spanning part, in analogy with activating Ly49 receptors.
The KIR are encoded by a gene family on chromosome 19. Genomic studies indicate that the KIR genes are evolutionarily recent. Their diversity is evolving at a fast rate, even by comparison to the MHC genes [90].

A related group of Ig-like receptors, some of which also contain ITIM, is termed “Ig-like transcripts (ILT)” or “leukocyte inhibitory receptors (LIR)”. These are expressed mainly on monocytes and dendritic cells, and one of them (LIR-1/ILT2/CD85) also on subsets of NK cells. Their ligands are not known, except for ILT-2/LIR-1 and ILT-4/LIR-2. These receptors have a very broad specificity, binding to both classical and non-classical MHC class I molecules. Interestingly, the affinity of LIR-1/ILT2 for the viral MHC class I homologue UL18 of human cytomegalovirus is 1000 fold higher than for human class I molecules.

As in the mouse, humans express also heterodimers of CD94/NKG2A, C or D. The ligand for the CD94/ NKG2A (inhibiting) and CD94/NKG2C (activating) is the non-classical class I molecule HLA-E, with analogous function to Qa-1 (see above)[91-93]. The inhibitory CD94/ NKG2 heterodimer is expressed by the majority of NK cells.

Humans have an NK complex located on chromosome 12, syntenic with mouse chromosome 6. An Ly49-like gene has been found in the human NKC but no functional transcript has been detected [94].

**Rat NK cell receptors**

Rat NK cell receptors, can like human and mouse receptors deliver inhibitory signals. As in the mouse, rat NK cell mediated alloreactivity is controlled not only by the MHC, but also by the NKC [95]. The rat NKC is located on chromosome 4, syntenic with mouse chromosome 6. Ly49 gene-homologues have been identified within this regions as well as rat homologues of the NKR-P1 genes, CD94 and NKG2 [96-98]. Two recently described antibodies, STOK1 and STOK2, identify an NKC-encoded inhibitory MHC receptor expressed on rat NK cells [99].
The missing self model

In the seventies and early eighties, investigators sought an explanation for the peculiar patterns observed in relation to MHC controlled rejection of parental BM in F₁ hybrid mice (A/A graft rejected by A/B host). This was not predicted by the laws of transplantation; there were only syngenic MHC molecules in the parental BM graft, so why was it rejected? It became clear that NK cells are responsible for the rejection in this situation [100], and also the rejection of MHC mismatched lymphocytes studied mainly in the rat [101], but the specificity remained unknown. The dominating paradigm suggested MHC encoded recessive Hh determinants as targets for NK cells [102]. Such determinants would be expressed by homozygous, parental BM cells, but not by heterozygous F₁ hybrid hosts; hence they would not be tolerant to them.

Models to study how natural resistance to tumors was influenced by MHC class I expression in the host were developed in parallel. Determinants for hybrid resistance against tumors as well as against BM grafts were mapped to the MHC class I D-locus of the H-2 region. On the other hand, the cytotoxic action of NK cells clearly did not require MHC class I expression of the target cells – in fact many of the most NK sensitive tumor targets expressed no or only low levels of MHC class I molecules.

In the missing self hypothesis, Klas Kärre stated that one function of NK cells is to detect and eliminate cells that lack or express reduced levels of self class I molecules. As opposed to T cells, NK cells would thus not require expression of novel, foreign antigens. They would instead react when normal self molecules are absent or down-regulated [103-105]. This could explain the NK mediated rejection of allogeneic and parental BM or tumor grafts (which lack at least one set of MHC alleles expressed by the recipient), as well as the efficient killing of tumors with poor MHC class I expression. It predicted that a tumor of e.g. H-2ᵇ type that was rejected in a (H-2ᵇ x H-2ᵇ) F₁ hybrid but not by the host of origin (H-2ᵇ), would be rejected in the latter if it lost its MHC class I expression. The rejection should be due to the increased sensitivity to NK cells from that host. These predictions were tested and verified in 1985 and 86, by work in mouse models [105, 106].
The missing self models and further developments of it became the platform for identification of the receptors reviewed in the previous chapter. For example, the inhibitory MHC class I specific receptors were predicted by one of the two mechanistic models proposed to account for missing self recognition by NK cells, the effector inhibition model [107]. According to that model, the activation program of NK cells would be initially triggered by less selective receptors recognizing ligands also on many normal cells; the ultimate decision would however be taken by inhibitory receptors, cancelling the activation program upon recognition of sufficient amounts of self MHC class I molecules on the target.

During the years from the postulation of the "missing self" model until I started my thesis study, many investigators contributed to the development of the model in understanding how MHC class I molecules control NK cells. It is not possible to give a complete review here, but some additional key findings that had been reported before I started are listed below.

**Findings related to MHC class I expression of target cells**

The first evidence that interferon mediated protection from NK recognition can occur through increased expression of MHC class I molecules; demonstration that IFN-γ can increase metastatic potential of melanoma cells, in parallel with enhanced MHC class I expression and reduced NK sensitivity [108, 109].

The first evidence for missing self recognition in a human system using naturally occurring HLA variants. NK susceptibility was linked to the HLA region on chromosome 6 [110].

Direct, transfection based evidence for MHC class I-mediated inhibition of (human) tumor cell killing by NK cells [111].

Direct, transgene based evidence for host MHC class I control of NK cells in transgenic mouse models [112].
Direct, transgene based evidence that normal (BM) cells can be protected from NK cells through expression of a self MHC class I allele [113].

Direct evidence that deleted MHC class I expression can render normal cells sensitive in vivo: rejection of BM cells with disrupted β2-microglobulin genes in some of the first strains of knock-out mice ever made [114, 115].

Evidence for interdomain MHC class I allele-specific recognition sites in the mouse [74].

Site-directed mutagenesis in the peptide binding groove renders the non-protective HLA-A2 allele protective [116].

The first mapping of inhibitory NK cell recognition sites, to and within HLA-C molecules [117, 118].

**Findings related to NK cell receptors**

The first NK clones (human) with allospecificity linked to the MHC [119].

Identification of the first murine (Ly49) and human (p58, NKB1) MHC class I-specific receptors in functional studies [74, 120, 121].

The first cloning of genes encoding human NK receptors [122, 123].
Aims of this thesis

When I started my studies, the concept of "missing self" recognition by NK cells started to become established. Several studies by different groups had demonstrated that cells with no or markedly reduced expression of all MHC class I molecules were more susceptible than their wild type control cells to NK lysis in vitro and NK mediated rejection in syngeneic mice in vivo. In studies based on transfection of tumor target cells or transgenic mice, it had also been demonstrated that a single MHC class I gene/molecule could dramatically influence NK cell mediated killing. However, molecular aspects on mechanisms and specificity were lacking.

The focus for my studies developed as follows.

1) Are syngeneic MHC class I molecules required to protect a target cell against killing by NK cells? If so, is a complete set of syngeneic molecules needed? Can allogeneic MHC class I molecules confer protection? These questions were addressed in paper I.

Our results indicated that D^d had a particularly strong influence on protection against killing in mice of the H-2^d haplotype. An important role for D^d was also evident from previous studies of transgenic mice [112, 113, 124]. Furthermore, Yokoyama et al. identified a receptor (termed Ly49, later renamed Ly 49A) that inhibited NK cell killing upon recognition by D^d [74]. This led us to our next study, asking

2) Can the allelic specificity of the Ly49A/D^d interaction be mapped to one particular domain of the MHC class I molecule? This was studied in paper I (the general role of the α1/α2 versus α3 domains) and paper II (the role of α1 versus α2 domains in Ly49A recognition).

Our results indicated that the α2 domain was essential for allele specific recognition by Ly49A. By comparison of sequences between D^d and other, non-protective MHC
class I molecules such as D\textsuperscript{b}, as well as studies of the crystal structures of the latter [125], it became possible to model and predict motifs within the \( \alpha_2 \) domains of D\textsuperscript{d} that might be responsible for Ly49A recognition of this molecule. We thus asked:

3) **What is the role of motifs in the antigen binding groove for Ly49A recognition, in particular a hydrophobic ridge across the cleft in D\textsuperscript{b}, absent in D\textsuperscript{d} (paper III)?**

The results in paper III showed a partial impairment of Ly49A recognition by introduction of the hydrophobic ridge in the D\textsuperscript{d} molecule. We concluded that there must be additional motifs that differ between D\textsuperscript{d} and D\textsuperscript{b}, and that are critical for Ly49A recognition. One candidate motif was revealed as the structure of the D\textsuperscript{d} molecule was solved [29]: a loop in the N-terminal part of the \( \alpha_2 \) domain (formed by amino acids 102-109), beneath the \( \alpha \)-helix, but exposed to the solvent. This loop was localized within a larger region of D\textsuperscript{d} to which the binding of the antibody 34-5-8S had been mapped [126]. This is the only antibody which can inhibit the Ly49A/D\textsuperscript{d} interaction efficiently [127]. We thus set out to test the following hypothesis:

4) **Is the 102-109 loop in the D\textsuperscript{d} molecule an epitope for this antibody as well as for the Ly49A receptor (paper IV)?**

During the course of the last study, a few other studies based on mutagenesis of D\textsuperscript{d} and Ly49A recognition were published. In addition, Tormo et al. published the structure of a cocrystal of Ly49A and D\textsuperscript{d} [72]. A final aim of this thesis has been to discuss our results in relation to these findings.
Results and discussion

Allelic specificity in MHC class I mediated protection from NK cell recognition

The purpose of the first study in this thesis, in collaboration with the first author Rickard Glas, was to investigate the allelic specificity in MHC class I mediated protection from NK cells in vivo. It was already clear that restoration of the complete set of syngeneic MHC class I molecules could inhibit NK cell lysis and rejection in the mouse [112, 113]. It was of interest to elucidate the role single of syngeneic and also allogeneic MHC class I molecules. The latter could be relevant for e.g. transplantation. In theory, the allogeneic molecules could be inert, stimulatory or inhibitory for reactivity mediated by NK cells, thereby influencing graft survival.

We thus asked: are syngeneic MHC class I molecules required to protect against killing by NK cells? Are all syngeneic allelic products needed, or are some of them more important? Can allogeneic MHC class I molecules confer protection? In paper I we studied this in vivo in H-2D<sup>d</sup> SCID mice (lacking B and T cells), by inoculation of tumor cells expressing different MHC class I alleles due to mutation and/or transfection, followed by monitoring of graft rejection or growth. SCID mice have a defect in DNA rearrangement of the BCR and TCR genes. In the recombination process DNA breaks occur, but joining of fragments is defective. Only rare joints are seen and most of these have abnormal features. There is however, some "leakiness" of functional T and B cells due to occasionally productive joining. The SCID mice have normal NK cells.

The H-2<sup>b</sup> lymphomas RBL-5, RMA and EL-4 (B6 background) are all moderately tumorigeneic in C.B-17 SCID mice (H-2<sup>d</sup>, BALB/c background). At a tumor dose of 10<sup>3</sup>, these allogeneic tumors grew progressively in between 22% and 43% of the mice. Depletion of NK cells by treatment of the mice with anti-asialo GM1 [128] resulted in 100% acceptance of all the tumor cells. This suggested that NK cells were able to reject all three allogeneic lymphomas in C.B-17 SCID mice. This model to study graft survival in SCID mice is of course dependent on the dose of tumors inoculated. All tumors will grow progressively if enough cells are inoculated, and none of the tumors will grow at a too low dose of inoculation. In order to assess
potential positive as well as negative effects of transfected MHC class I molecules, it is important to be within the correct dose range of rejection/survival of control cells.

Furthermore, it is important to rule out that differences in tumor takes in vivo are due to differences in intrinsic growth of the different mutant and transfectant lines. This was assessed by comparing the in vitro growth of the different tumor cells (paper I, data not shown).

Can allogeneic MHC class I molecules confer protection? When TAP-2 defective RMA-S cells (mutant subline of RMA), [105] were inoculated into SCID mice, none of the mice developed tumors. Treatment of mice with anti-asialo GM1 antiserum restored tumor formation outgrowth in 78% of the mice. Transfection of the TAP-1 and TAP-2 to the RMA-S cells restored expression of H-2K^b and D^b, both of which are allogeneic visavi SCID mice (H-2^d). Such transfectant cells grew in 44% of the mice at the dose 10^3. This was comparable to grafting RMA cells (tumor growth in 43% of the mice). We thus concluded that allogeneic MHC class I molecules have at least a partial protective effect against NK mediated rejection in this combination. In light of more recent findings, it must now be noted that the restored expression of the TAP dependent non-classical MHC molecule Qa-1^b may have contributed to the protective effect against NK cells in vivo. Qa-1^b is nonpolymorphic, and functions as a ligand for the inhibitory NK cell receptor CD94/NKG2A. The result nevertheless showed that the MHC class I expression of tumor cells could mediate a partial protection against NK cells in SCID mice, even in the absence of classical class I molecules matching the MHC of the host. Furthermore, observations on the effect of transfecting the D^p gene to RBL-5 in the same paper (further discussed below), clearly showed that an allogeneic product could confer protection from NK mediated rejection.

Can syngeneic MHC class I molecules give complete protection against in vivo rejection by NK cells? Are all syngeneic allelic products needed, or are some of them more important? To address this question each of the syngeneic products D^d, K^d and L^d was transfected into H-2^b tumor cells and inoculated into SCID mice. Untransfected RBL-5 cells grew in only 22% of the mice, whereas depletion of NK cells allowed growth in 100%. Transfection of D^d resulted in almost complete
protection: 82% of the mice developed tumors. Similarly, RMA cells grew in 43% whereas RMAD\textsuperscript{d} grew in 92% of the mice. A third cell line, EL-4, showed a similar result; 25% of mice developed tumors, compared to 80% when the corresponding D\textsuperscript{d} transfectant was inoculated. In conclusion, there was a strong protection conferred by the syngeneic D\textsuperscript{d} allele. We further concluded that this pattern of protection occurred in tumors of different etiology (RBL-5/RMA being virally induced and EL-4 chemically induced). This conclusion cannot be maintained on the basis of those findings, as two recent reports have demonstrated that the RBL-5 line, from which RMA and RMA-S were derived, is most probably a subline of EL-4. This was based on the finding that EL-4 and RBL-5 have exactly the same TCR rearrangement. [129].

D\textsuperscript{d} transfection to RMA-S cells did not result in protection against rejection. This indicated that the presence of the D\textsuperscript{d} gene \textit{per se}, with only a limited number of D\textsuperscript{d} molecules at the cell surface, either empty or devoid of TAP-dependent peptides [130], was not sufficient to confer protection from NK cell-mediated rejection. A moderate effect (from 43% to 75% tumor takes) of K\textsuperscript{d}-transfected RMA cells was observed. L\textsuperscript{d} conferred no additional protection when transfected into RBL-5 or RMA cells. In summary, while one of the self class I molecule (D\textsuperscript{d}) could efficiently protect by itself from NK cell-mediated rejection \textit{in vivo}, other equally self class I molecules (L\textsuperscript{d}, K\textsuperscript{d}) had no or only partial influence.

This unequal influence of different self alleles, as well as the inhibitory effect of allogeneic class I products in certain situations, can easily be explained in light of today’s knowledge about the specificity of Ly49 receptors (Figure 2A, in the introductory part). These usually recognize a group of MHC class I alleles, often from different haplotypes; some MHC class I molecules are recognized by several Ly49 receptors, and some may not be efficiently recognized by any receptor. This can explain why an allogeneic allele can have a protective effect. It can also explain why a syngeneic allele does not always have an additional protective effect when introduced in an allogeneic tumor – there may be no Ly49 receptor for the syngeneic molecule, or there may be one which crossreacts with one of the already present allogeneic molecules.
This is well illustrated by another set of results in paper I. To address the question whether the NK-mediated rejection was linked to the D locus, another D locus product, D^p, was transfected into RBL-5 and inoculated into SCID mice. Indeed, the expressed D^p molecule conferred as efficient protection from rejection as the D^d molecule when compared with untransfected RBL-5 (73% versus 22%) cells. This was in contrast to a report by Höglund et al. who found no protective effect of D^p when the in vivo survival of transfected cells was monitored within 24 hours of intravenous inoculation [124]. In studies performed later by Sentman et al. and Alheim et al. [131, 132] evidence was presented that D^p and D^d confer crossreactive protection because they are both recognized by the receptor Ly49A. However, D^p did not appear to protect as efficiently as D^d, suggesting a lower affinity in the Ly49A/D^p interaction.

Finally, it has now become clear that introduction of an allogeneic class I molecule into a target cell or a transplant may also lead to rejection by positive recognition, i.e. mediated by MHC class I specific triggering receptors of NK cells. Öhlén et al. reported that D^d transgenic BM was efficiently rejected by NK cells of B6 mice, but concluded that this was not necessarily due to positive recognition of the D^d product; possible indirect effects of the transgene, e.g. downregulation of other H-2^b MHC class I products (i.e. self to the transplant recipient) were considered. However, later studies by George et al., have unequivocally demonstrated that Ly49D is a triggering NK receptor specific for H-2D^d [133]. A small subset of B6 NK cells that expressed Ly49D, but lacked expression of inhibitory Ly49 receptors specific for D^d and K^b class I molecules expressed on the target cells (Ly49A, C, G2, I), was responsible for rejection of D^d expressing B6 cells [134]. This demonstrates that NK cells expressing stimulatory but not inhibitory receptors specific for target cell class I ligands can be responsible for the rejection of BM allografts. A similar phenomenon may explain also the results of Vaage et al, who provided evidence for a NK cell triggering ligand mapping to the RT-1C region [28].
Mapping of specificity at the level of intramolecular domains; studies with chimeric MHC class I molecules

By using tumor cells transfected with chimeric class I genes as targets for NK cells in vivo and in vitro, it was possible to investigate which part of the MHC class I molecule that is required for an allele specific protective effect. A first step in this direction was taken in paper I. We took advantage of the differential effect of D\textsuperscript{d} and L\textsuperscript{d} on tumorigenicity in SCID mice of H-2\textsuperscript{d} genotype. RBL-5 cells transfected with chimeric D\textsuperscript{d}/L\textsuperscript{d} class I genes were inoculated into C.B-17 SCID mice (paper I). RBL-5 cells expressing the $\alpha_1/\alpha_2$ domains of D\textsuperscript{d} and the $\alpha_3$ domain of L\textsuperscript{d} showed a marked increase in tumorigenicity, comparable to RBL-5D\textsuperscript{d}. In contrast, RBL-5 expressing the $\alpha_1/\alpha_2$ L\textsuperscript{d} domains and the $\alpha_3$ domain of D\textsuperscript{d} had no effect. This mapped the protective effect of D\textsuperscript{d} to the $\alpha_1/\alpha_2$ domains of the molecule. The same pattern was seen when these transfectants were inoculated into D\textsuperscript{d} transgenic B6 mice [135, 136] or tested for in vitro sensitivity to NK cells from B6 and Balb/c mice [137]. Furthermore, Karlhofer et al. demonstrated in the first report on an inhibitory NK receptor (Ly49, today termed Ly49A) that its recognition of the D\textsuperscript{d} molecule could be blocked only by an antibody known to recognize the $\alpha_1$ and $\alpha_2$ domains, and not by an antibody binding to the $\alpha_3$ domain [74]. The more precise specificity of this antibody was studied further in paper IV. Finally, these results in the murine system were well in line with a previous study demonstrating that an allele specific protective effect of HLA-B27 against human NK cells could be mapped to the $\alpha_1/\alpha_2$ domains of this class I molecule [138].

As a next step we asked whether the allele specific effect of protection could be mapped further either to the $\alpha_1$ or the $\alpha_2$ domain. Our expectation, based on emerging mapping studies of the allelic specificity of human NK cells, was that the $\alpha_1$ domain would be critical for inhibitory recognition [139]. Our starting point was the notion that there must be some important feature for inhibitory recognition by NK cells within the D\textsuperscript{d} molecule that is not present in D\textsuperscript{b} or K\textsuperscript{b} molecules. This was clear from our own studies in paper I, as well as from studies by Höglund et al, and Öhlén et al. on D\textsuperscript{d} transgenic B6 mice as recipients for B6 derived tumor or BM grafts [112, 113]. Moreover, the first results of Yokoyama and colleagues on the receptor Ly49A suggested that this molecule was a good candidate for a mechanism that could
distinguish D\textsuperscript{d} from MHC class I molecules of H-2\textsuperscript{b} haplotype [73, 73].

We thus decided to 1) base the further construction of chimeric molecules as well as mutant molecules on the comparison between D\textsuperscript{d} and D\textsuperscript{b} 2) test the the effect of chimeric molecules not only on NK cell recognition in general, but also more specifically in relation to NK cells expressing the receptor Ly49A. In retrospect, one may note that Ly49A may actually bind at least weakly to D\textsuperscript{b} (Jakob Michaelsson, submitted for publication). Moreover, this does not change our interpretation since there seems to be a much more efficient interaction with D\textsuperscript{d}.

Together with first author Jonas Sundbäck, exon shuffling of genomic DNA clones was used to produce the domain swapped molecules D\textsuperscript{b}\textsubscript{α1}D\textsuperscript{d}\textsubscript{α2}D\textsubscript{d}\textsubscript{α3}, D\textsuperscript{d}\textsubscript{α1}D\textsuperscript{b}\textsubscript{α2}D\textsuperscript{d}\textsubscript{α3}, D\textsuperscript{d}\textsubscript{α1}\textsubscript{α2}D\textsuperscript{b}\textsubscript{α3} and D\textsuperscript{b}\textsubscript{α1}\textsubscript{α2}D\textsuperscript{d}\textsubscript{α3}. The latter two were important to confirm that the allelic specificity mapped to the α\textsubscript{1}/α\textsubscript{2} domains in the first place (compare the results on L\textsuperscript{d}/D\textsuperscript{d} chimeric molecules discussed above). We decided to construct the chimeric molecules in combination with the D\textsuperscript{d}\textsubscript{α3} domain whenever possible, in order to identify positive clones after transfection into an H-2\textsuperscript{b} cell line. To study the role of these molecules in vivo, we inoculated RBL-5 tumors separately transfected with the different chimeric genes into D8 mice. In order to assess the role of NK cells, some groups of mice were treated with anti-NK1.1. mAb (PK136) one day before inoculation. This treatment has been shown to abrogate NK cell activity in vivo [140].

In this type of assay with mice that have not been preimmunized, it is our experience that NK cells strongly influence early events, i.e. whether a palpable tumor will form or not, while an adaptive T cell response, once it is initiated, can lead to rejection of established tumors. A T cell response had to be considered since the chimeric molecules were potentially allogeneic; even if each MHC class I domain was present in the recipient mice, new interactions between domains could result in structures sufficiently different from the complete D\textsuperscript{b} and D\textsuperscript{d} self-molecules to be perceived as "altered self". It was thus important to monitor the growth for an extended time period in order to catch early as well as late events, and we did this by weekly palpations for up to 6 weeks. Challenging doses of tumor cells were chosen on the basis of previous experiments so that tumor outgrowth of D\textsuperscript{d} negative cells would be prevented by NK cells in the majority of mice. D\textsuperscript{b}\textsubscript{α1}\textsubscript{α2}D\textsuperscript{d}\textsubscript{α3} transfectants were not able
to form tumors in more than 25-30% of mice, comparable to untransfected RBL-5 (10-25%). In contrast Ddα1α2Dbα3 transfectants grew out in 100% of the mice. This confirmed that the allelic specificity of protection resided in the α1,α2 domains. When we compared the transfectants expressing the same Dd derived α3 domain, but swapped α1 and α2 domains, RBL-5-Dbα1Ddα2α3 grew out in the majority of the mice (60-75%), whereas RBL-5-Ddα1Dbα2Ddα3 was markedly less tumorigenic (20-27%). These results indicated that motif(s) in the α2 domain of Dd were sufficient for protection, even in the context of the α1 domain from the non-protective Db allele. It should be noted that the expression levels in the transfectants of the non-protective chimeric molecule Ddα1Dbα2Ddα3 were lower compared to the transfectants of the protective Dbα1Ddα2α3, making it difficult to completely exclude a role for the α1 domain of Dd in protection against natural resistance. It was thus possible that the α2 domain was sufficient, but not necessary; protection would then be dependent on the presence of either the α1 or the α2 domain of the Dd molecule. Both RBL-5-Dd and RBL-5-Dbα1Ddα2α3 tumors eventually regressed in many mice. We interpret these late rejections as a T cell response, since the RBL-5-Dbα1Ddα2α3 cells grew progressively in BALB/c nu/nu mice (data not shown).

Our interpretation of these results was further supported in the in vitro studies, in collaboration with Mary Nakamura and colleagues at UCSF. In order to focus on the Ly49A receptor, the in vitro experiments were performed with the Ly49A transfected rat NK-like cell line RNK-16, a spontaneous NK cell leukemia from F344 rats with phenotypic and functional characteristics of rat NK cells. As targets, we used the rat myeloma YB2/0. Neither Ly49 molecules, nor H-2 molecules are expressed endogenously in this rat model system, which is an advantage when studying murine receptor/ligand interations. The chimeric molecules were transfected into the YB2/0 cells, and the resulting cell lines were then examined in cytotoxicity assays. The data were consistent with the in vivo findings. Protection against killing by Ly49A transfected RNK-16 cells was seen with the YB2/0-Ddα1α2Dbα3 and YB2/0-Dbα1Ddα2α3 but not the YB2/0-Dbα1α2Ddα3 and the YB2/0-Dbα1Ddα2Ddα3 transfectants. Blocking by anti-Ly49A monoclonal antibody restored lysis of the protected targets. No protection was seen when wild type RNK 16 cells, without Ly49A expression, were used. Since YB/20 cells turned out to give reproducibly high levels of all transfected murine MHC class I genes, the in vitro results allowed the conclusion that the α2
domain is both necessary and sufficient to induce protection mediated by the Ly49A receptor.

Taken together, the data demonstrated 1) an essential role for the $\alpha_2$ domain of $D^d$ in the inhibition of natural killing by NK cells in vivo and that 2) this allele specificity mapping to the $\alpha_2$ domain can be explained by the specificity of the Ly49A receptor. One interpretation of these results was that Ly49A binds only to the $\alpha_1$ domain of the MHC class I molecules. Secondly, it was possible that the Ly49A receptor requires binding to motifs in both domains: some shared by $D^d$ and $D^b$ in the $\alpha_1$ domain, and some unique to $D^d$ in the $\alpha_2$ domain. A third, more remote possibility was that the Ly49A contact is solely within the $\alpha_1$ domain, although the site is critically dependent on a conformational state of the class I molecule that is imposed by the $\alpha_2$ domain of $D^d$.

Our conclusions may be considered in the perspective of later findings. There are two studies in the literature that have employed chimeric MHC class I molecules to map the specificity of murine NK cells. Milisaukas et al., using natural chimeras between $D^d$ and $L^d$ molecules in mutant mice, mapped protection (against rejection by NK cells in mice of H-2$^{d/b}$ haplotype) to be the $\alpha_1$ domain and the N-terminal part of the $\alpha_2$ domain [141]. Matsumoto et al. reached a similar conclusion using in vitro recombinants of $D^d$ and $K^d$ genes [142]. Our studies is well in line with those results. Furthermore, our study predicted that it should be possible to abrogate the protective effect of the $D^d$ molecule (visavi the Ly49A receptor) by mutations at specific motifs in the $\alpha_2$ domain. This has been confirmed, as discussed further below in the context of papers III and IV. Here, one can simply note that at the time of our study, the $D^d$ structure was not known, and it was thus difficult to pinpoint crucial differences between $D^d$ and $D^b$. We could only tell that these two molecules differ by 13 amino acids in the $\alpha_2$ domain, and we had to limit our further analysis to these residues.

Finally, one may note that our expectation that the $\alpha_1$ domain would be critical for inhibitory recognition, as shown for human NK cells, was not fulfilled. This predicted that at least for the studied human and mouse receptors, the interaction with the class I molecule would be totally different, one being oriented towards the $\alpha_1$ domain and the other towards the $\alpha_2$ domain. Today, structures of cocrystals of one human
and one mouse NK (Ly49A) receptor-ligand pair have been solved. All our results will be discussed in relation to these structural studies below. However, it is interesting to note already here that the predictions were partly right, partly wrong: the human and mouse receptor in question indeed differ widely in their contact areas, although both contact the $\alpha_1$ as well as the $\alpha_2$ domain.

**Mapping of specificity at the level of structural motifs; studies of mutated MHC class I molecules**

**Influence of the antigen binding groove**

The results with chimeric molecules described above led to the obvious next question. Is it possible to predict critical motif(s) within the $\alpha_2$ or $\alpha_1/\alpha_2$ domains that are responsible for Ly49A recognition? We decided to first focus on the antigen binding groove, given the importance of this part of the molecule for T cell recognition.

During the progress of paper II, two papers adding valuable knowledge on MHC class I structures had been published. Young et al. crystallized the $D^b$ molecule and Corr et al. modeled the $D^d$ molecule on the basis of the HLA-B27 crystal structure [125, 143]. We compared the amino acid sequences and three-dimensional structures of these two MHC class I molecules, using also our own molecular models (paper III). The most prominent structural feature caused by the amino acid differences in the antigen binding groove appeared to be a hydrophobic ridge in the peptide binding cleft of the $D^b$ molecule, which was missing in $D^d$. This ridge is formed by the side chains of residues at positions 73, 147 and 156. It forces the peptide backbone to arch outwards at its C-terminal end. We introduced the ridge in the $D^d$ molecule by changing residue 73 (in the $\alpha_1$ domain) from serine to tryptophan (S73W) and residue 156 (in the $\alpha_2$ domain) from aspartate to tyrosine (D156Y). The corresponding nucleotide changes were introduced in the genomic clone of $D^d$. Residue 147 was conserved between the two MHC class I molecules. A mutant $D^d$ molecule with an altered amino acid outside the peptide binding cleft, in position 150 (alanine to serine), was also constructed. This relatively conservative change did not lead to any major structural differences in the molecular model.
We decided to mutate motifs in the D\textsuperscript{d} molecule into corresponding amino acids in D\textsuperscript{b} for two reasons. First, we believed that it would be easier to abrogate the effect of a protective motif by changing one or a few amino acids in the molecule, rather than building up a protective molecule by creating the corresponding motif in the opposite direction. Secondly, in order to transf ect and identify positive clones, we needed an antibody that would exclusively identify the product of the mutated gene after transfection to a cell line of the H-2\textsuperscript{b} type. This ruled out the alternative, i.e. to mutate the non-protective D\textsuperscript{b} into a protective molecule, as the antibodies would not distinguish such a mutated molecule from the endogenous D\textsuperscript{b} molecules. Although not shown in the figures in the paper, we always included the D\textsuperscript{d}\textsubscript{α3}-specific antibody to monitor transfectants, thereby enabling detection of molecules with mutated α\textsubscript{1} and α\textsubscript{2} domains of the D\textsuperscript{d} molecule.

The \textit{in vitro} model system to test the influence of transfected gene products in our lab at that time was based on the RBL-5 lymphoma as target and IL-2 activated B6 spleen cells as effectors in cytotoxicity assays. \textit{In vivo} natural resistance to these tumor transfectants was studied in D\textsuperscript{d} transgenic B6 mice. These rejected RBL-5 lymphoma cells, but not RBL-5 transfected with wild type D\textsuperscript{d} gene.

Wild type and mutated D\textsuperscript{d} genes were transfected into RBL-5 lymphoma cells and clones with comparable expression levels were chosen for further studies. When tested in a cytotoxicity assay with IL-2-activated B6 spleen cells, the RBL-5-D\textsuperscript{d}S73WD156Y cells were killed to a significantly higher degree than RBL-5-D\textsuperscript{d}A150S and RBL-5-D\textsuperscript{d} wild type cells, but still somewhat less efficiently than untransfected RBL-5. In this population of effectors there were several inhibitory receptors and the targets harbored at least four ligands; K\textsuperscript{b}, D\textsuperscript{b}, Qa-1\textsuperscript{b} and D\textsuperscript{d} mutant molecules. The possible interplay between these represented a complication. We therefore also tested sorted Ly49\textsuperscript{A+} or Ly49\textsuperscript{A–} B6 spleen cells as effectors. The same difference between D\textsuperscript{d} wild type and D\textsuperscript{d}S73WD156Y transfectants was seen when sorted Ly49\textsuperscript{A+/NK1.1–} cells were used as effector cells, but there was no clearcut protection by any of the D\textsuperscript{d} molecules with Ly49\textsuperscript{A+/NK1.1+} cells. Possible explanations for the somewhat reduced killing of RBL-5-D\textsuperscript{d} observed in occasional experiments with Ly49\textsuperscript{A+/NK1.1+} effectors include either contamination of Ly49\textsuperscript{A+} cells or the presence of Ly49G2\textsuperscript{+} cells in this population. The impaired protective capacity of the
D^{d}S73WD156Y molecule was confirmed by studies of clones derived from an independent transfection with the corresponding gene.

The \textit{in vitro} data thus showed that introduction of a hydrophobic ridge in the D^{d} molecule partially impairs the ability of this molecule to protect target cells against killing by Ly49A^+ NK cells. In analogy with the tumor outgrowth study in paper II, we were curious to investigate these D^{d} mutant cells lines in an assay for natural resistance \textit{in vivo}. In line with \textit{in vitro} data, the RBL-5-D^{d}S73WD156Y was much less tumorigenic compared to RBL-5-D^{d}A150S and RBL-5D^{d}. This difference was abrogated in NK1.1 treated animals. For the similar reasons as discussed in the previous section in relation to paper II, we monitored the early appearance of tumors (presumably reflecting the role of NK cells) as well as late regressions (presumably T-cell mediated). T cell mediated rejection was expected since the introduced mutations would potentially create D^{d} molecules perceived as "altered self".

Taken together, these studies showed that reconstituting the hydrophobic ridge present in the non-protective molecule D^{b}, results in impaired capacity to protect against NK cell-mediated killing \textit{in vitro} and \textit{in vivo}. It is well known that amino acid changes in the peptide binding cleft can change the pool of peptides presented \cite{144}. The ridge could mediate its effects through such altered peptide repertoire, in several different ways: a) Ly49A binding might involve the peptide directly b) the bound peptide might cause non-specific steric hindrance of Ly49A binding c) the peptide could cause secondary changes in other parts of the molecule. It is also possible that the mutations themselves have indirect effects on the structure of other parts of the D^{d} molecule, irrespective of the bound peptide.

At the time of publication of this paper, the D^{d} crystal structure was solved by Achour et al. \cite{29}. This made it possible to more thoroughly investigate the structural features important for MHC class I recognition by Ly49A. By comparing D^{d} and D^{b} (Young) crystal structures and focusing on amino acids in the \(\alpha_2\) domain, Achour et al. suggested two potential regions for Ly49A recognition: the N-terminal part of the peptide binding groove with adjacent areas and an area around one of the N-specific glycosylation site N176, including an external loop of the \(\alpha_2\) domain. The first region
is relevant to our results in paper III, while the second region will be discussed in relation to our result in paper IV.

Central residues in the first region involves amino acids W97, A99 and W114. These residues are buried in the antigen binding groove and can not be contacted by the receptor when a peptide is bound. They create sterical constraints for the double p2Gp3P peptide anchor requirements. The p2Gp3P motif assumes a very specific conformation and is buried in this narrow, deep part of the groove, wherafter the peptide climbs a midcleft wall (created by W97 and W114) leading to a broader and more shallow part of the groove. In D\textsuperscript{d} there is a plateau, whereas in D\textsuperscript{b}, residues W73 and Y156 form a hydrophobic ridge in the peptide binding cleft. This ridge is missing also in the other two Ly49A ligands; the D\textsuperscript{k} and D\textsuperscript{p} alleles, but it is present in the nonbinding ligand L\textsuperscript{d}.

Apart from the study of the 73/156 motif in this paper, both the 73/156 and the 97/99/114 motifs have been studied by Nakamura et al. [145]. These authors did not observe any effects on Ly49A-mediated inhibition when they introduced the hydrophobic ridge (S73WD156Y) in the D\textsuperscript{d} molecule. The discrepancy between their results and ours might reflect differences in the effector cell systems used (Ly49A transfected RNK-16 cells in their case, Ly49A expressing NK cells in our case), target cells used (rat YB2/0 versus mouse RBL-5) or expression levels of receptor and ligand genes. However, the study of Nakamura et al. agrees with ours in the sense that alterations in the floor of antigen binding groove could influence Ly49A recognition. In their hands, a combination of mutations of the three residues W97, A99 and W114 completely abrogated the inhibitory recognition by Ly49A. The authors noted that the expression levels of these triple mutant molecules was slightly lower than for the control wild type D\textsuperscript{d}. Thus it remains possible that higher expression of the triple mutant could result in at least partly functional recognition by Ly49A.

Today, these results are relevant to discuss also in relation to the published cocrystal of Ly49A and H-2D\textsuperscript{d}. This will be done in connection with the discussion of paper IV below.
In the human system, transfection of class I genes into NK-sensitive targets can result in resistance to lysis by human NK cell clones. Storkus et al. were the first to show that there is critical involvement of residues within the peptide-binding cleft for control of NK cell susceptibility [116]. Mutation of position 74 (histidine) in the HLA-A2 allele, which is non-protective against NK cell killing, yielded a mutant molecule that could inhibit NK cell attack. The authors concluded by comparison with the solved structure of HLA-Aw68, that histidine 74 most likely blocks access to a side pocket in the HLA-A2 antigen binding cleft.

Role of the peptide
As to the role of the peptide, two papers published during the course of our study argued against direct Ly49A-peptide binding, and for an interaction of the inhibitory receptor solely with the MHC class I heavy chain. Five known Dd binding peptides tested [146], as well as peptides substituted with alanine at all but the anchor positions, could stabilize empty Dd molecules of TAP-deficient target cells and protect them against Ly49A+ effector cells [127]. This indicated that Ly49A recognition was peptide dependent but not peptide specific, i.e. inhibitory recognition required a bound peptide, but any Dd binding peptide would do. These results argue that effects on mutations in the Dd antigen binding groove on Ly49A recognition are more likely to be mediated by indirect effects of the peptide on other parts of the MHC class I molecule.

In contrast to Ly49A, there is good evidence that the Ly49C receptor is influenced by the contents of groove. Franksson et al. reported a peptide selectivity in protection of TAP deficient cells when loading with different Kb-restricted peptides and testing them as targets for Ly49C+ NK cells [147]. Using molecular modeling, Franksson et al. identified a conformational motif encompassing the C-terminal parts of the α1-helix (73-77) and the bound peptide that was common for the protective complexes. On the other hand, Su et al. reported that empty (peptide receptive) Kb molecules conferred better protection than Kb loaded with the protective peptide reported by Franksson et al. [148]. These studies clearly show that the interaction between Ly49C and Kb is influenced by the content of the MHC class I peptide binding groove.
Studies in the human system had already demonstrated a clear peptide selectivity for KIR. In particular, positions 7 and 8 of the peptide was implicated to affect inhibitory recognition [149-152]. This is easily reconciled with the crystal structure of the human KIR2DL2 complexed to HLA-Cw3 published this year [153]. The structure reveals that the KIRs binds in a nearly orthogonal orientation across the $\alpha_1$- and $\alpha_2$-helices of Cw3 and directly contacts positions 7 and 8 of the peptide. Most of the residues in the HLA-Cw3 making the contacts are conserved residues. A hydrogen bond between Lys 44 of the KIR and asparagine 80 of Cw3 confers the allospecificity. This is well in line with several studies based on mutagenesis; the allospecificity was defined to position 80 for both HLA-C alleles [154] and HLA-B alleles [155, 156].

Role of residues exposed to the solvent

By comparison of the crystal structures for D$^d$ and D$^b$, Achour et al. suggested two potential regions of interest for Ly49A recognition. The region residing in the antigen binding groove has been discussed in detail above. It will be touched upon again later in this section, in connection with the interpretation of results in the context of the Ly49/D$^d$ cocrystal published by Tormo et al. [72]. The other region is located around one of the N-specific glycosylation sites, N176, and includes an external loop of the $\alpha_2$ domain. This loop involves amino acids 102-109 with polymorphisms (differing between D$^d$ and D$^b$) at residues 103, 104 and 107. These residues are located beneath the $\alpha_2$ helix but are still well exposed to the solvent. We became interested in this loop for an additional reason. It is located within a larger region described to be recognized by the mAb 34-5-8S [126, 157-159]. This mAb is the only D$^d$-specific mAb able to completely block the Ly49A/D$^d$ interaction, as shown in both functional and binding studies [74, 76]. We therefore wanted to test whether the loop defined by positions 102-109 in the D$^d$ molecule is (i) an epitope for the mAb 34-5-8S and (ii) an epitope for the Ly49A receptor.

The loop defines the lower part of an open cavity in the D$^d$ molecule, which is closed in the D$^b$ molecule mainly through the positioning of W107. In addition, although R108 is conserved between the two molecules, the position of its side chain is altered in the D$^b$ molecule, probably as a consequence of the difference between E104 and G104. Due to salt bridges with E104, R108 is pulled downwards by this residue in the D$^d$ molecule, but not by G104 in the D$^b$ molecule. We speculated that
this altered position of R108 could contribute further to prevent protein-protein
docking in this region.

We introduced a combination of three mutations at residues 103 (valine to leucine),
104 (glutamic acid to glycine) and 107 (glycine to tryptophan) in the cDNA of the Dd
molecule. The mutant gene was first transfected into RMA cells for tests of antibody
binding, and into RMA-S cells for tests of peptide binding. For the purpose of testing
Ly49A binding, the mutant gene was cloned into a vector for tetramer construction,
i.e. in the form of a soluble molecule with a specific biotinylation site introduced in the
C-terminal end of the molecule. Such heavy chains were produced in E. Coli, and
after purification, refolded with β2-microglobulin and a Dd binding peptide. This
required that the mutant heavy chain retained the ability to bind the peptide, as we
indeed confirmed first by an assay based on peptide induced stabilization of empty
MHC class I molecules of RMA-S. The RMA-S cells had been transfected with the
mutant cDNA, and the Dd mutant molecules of these cells were equally well stabilized
by the peptide as Dd wild type molecules of control transfectants. This suggested that
the mutant heavy chain should be possible to refold with the peptide and β2-
microglobulin, as we indeed could confirm. Complete soluble Dd molecules were then
subject to size exclusion chromatography (FPLC), enzymatically biotinylated and
tetramerized.

Meanwhile, we found that binding of mAb 34-5-8S to Dd103/104/107 mutant
molecules expressed on transfected RMA and YB2/0 cells was completely abrogated
as compared to the Dd wild type transfected control cells. All clones transfected with
mutant DNA stained readily with the Dd α3-specific control mAb 34-2-12. Mutations
V103L, E104G and G107W in the Dd molecule were thus sufficient to completely
abrogate the binding of mAb 34-5-8S.

Binding of Dd mutant tetramer to RNK-16 cells transfected with the gene encoding
the Ly49A receptor was also markedly reduced, but not completely abolished, when
compared to wild type Dd tetramer. This pattern was evident also in studies of splenic
NK cells, indicating a severely impaired interaction with Ly49A receptors expressed
at physiological levels on freshly derived NK cells.
The tetramers with wild type and mutated D\textsuperscript{d} bound equally well to the mAb 34-2-12 in ELISA (data not shown), arguing that the quality and stability of the two tetramers were equal. However there was no binding of mAb 34-5-8S to the D\textsuperscript{d} mutant tetramer, confirming the loss of a critical epitope for this antibody (data not shown).

In conclusion, the changes introduced in D\textsuperscript{d} at positions 103, 104 and 107, located in an exposed loop in the N-terminal part of the \( \alpha_2 \) domain 1) had no profound effect on the binding of the antigenic peptide P18-I10 as compared to D\textsuperscript{d} wild type, 2) resulted in complete loss of the ability to bind the D\textsuperscript{d}-specific mAb 34-5-8S and 3) resulted in a marked impairment of the binding to the Ly49A receptor. The introduced mutations therefore directly or indirectly affect the conformational epitope(s) contacted by the mAb and the Ly49A receptor. It is thus possible that mAb 34-5-8S and the Ly49A receptor recognize at least partially overlapping epitopes. It is clear that they cannot dock to the D\textsuperscript{d} molecule in exactly the same manner. Nakamura et al. have reported mutations that influence one but not the other interaction, e.g. a mutation at residue 104 partially impaired the binding of mAb 34-5-8S, without affecting Ly49A [145].

It cannot be excluded however that there are two completely different binding sites for Ly49A and mAb 34-5-8S, one or both of which is distant from the exposed loop, but nevertheless influenced by it, via an indirect conformational effect transmitted through the molecule.

These and other results in mutagenesis studies can now be interpreted in the context of the solved structure of the Ly49A/D\textsuperscript{d} cocrystal [72]. The structure surprisingly revealed two contact sites between receptor and ligand in the crystal. On the MHC class I ligand, site 1 occupies an area of 994 Å\textsuperscript{2}, at the N-terminus of the \( \alpha_1 \) domain and the C-terminus of the \( \alpha_2 \) domain. Twelve (six in each of the \( \alpha_1 \) and \( \alpha_2 \) domains) residues on D\textsuperscript{d} are involved in hydrogen bond interactions with Ly49A. Binding site 1 contains polymorphic residues in the MHC class I molecule, which would be in line with an allelespecific Ly49A recognition motif. This site was suggested to be involved within Ly49A/D\textsuperscript{d} interactions in \textit{trans}, with the receptor and ligand on opposing cells, as expected in the usual effector-target interaction [72]. The more extensive site 2, occupying an area of 3342 Å\textsuperscript{2} in the D\textsuperscript{d} molecule, corresponds to a cavity formed by the \( \alpha_1/\alpha_2 \) (60%), \( \alpha_3 \) (15%) and \( \beta_2 \)-microglobulin (25%) domains, beneath the peptide
binding platform. Eighteen residues in D\textsuperscript{d} in this contact area are involved in hydrogen bonds with Ly49A (4 in \(\alpha_1\), 10 in \(\alpha_2\) and 4 in \(\beta_2\)-microglobulin). There are no polymorphic residues in the contact area of binding site 2. If this represents the functional contact area for transmission of negative signals, the allelic specificity must depend on distal polymorphic residues affecting the structure at the interface. Binding site 2 was suggested to mediate a \textit{cis} interaction [72], based on the idea that a Ly49A receptor can interact with D\textsuperscript{d} on the same cell as previously suggested by functional studies [44].

How can our present results be interpreted in relation to these two proposed binding sites? The 103/104/107 loop is not directly contacted at site 1, but is located very close to residues R169 and E166 on D\textsuperscript{d} (<7Å). The side chain of R169 projects out into the solvent in the structure of D\textsuperscript{d} [29], and is directly involved in the binding of Ly49A at site 1 in the cocrystal [72]. The mutations that we have created in the loop, and more specifically G107W, would most probably influence this segment of the binding site 1; when this mutation was introduced in the published structure of the cocrystal [72] through molecular modeling (paper IV, Figure 7C), the distance between W107 and R169 was less than 2Å. This is likely to influence/interfere with the interaction between R228 and D229 on Ly49A and E166 and R169 on D\textsuperscript{d}.

Although contact site 1 can be influenced by changes in positions 103/104/107, the loop they belong to is actually at least as close to contact site 2 [72]. The side chain of E104, one of the residues we mutated, is directly involved in a salt bridge (3.6Å) with residue R223 on Ly49A (paper IV, Figure 7D). Residue S2 of D\textsuperscript{d}, just adjacent to the loop, is directly involved in a hydrogen bond with R228 of Ly49A. Furthermore, residues R6 and R111, pointing down from the \(\beta\)-sheet of the peptide-binding cleft, are located very close to the loop (<6Å) and are both engaged in hydrogen bonds with residues D229, L240 and D241 on Ly49A.

Site 2 is interesting also in relation to two additional motifs that have been addressed above in the discussion of paper III: W97/A99/W114 and W73/Y156 [145, 160]. Both motifs differ between D\textsuperscript{d} and D\textsuperscript{b}; both are parts of the antigen binding groove; both have been shown to influence the Ly49A/D\textsuperscript{d} interaction in mutagenesis studies. W97/A99/W114 forms a steep wall in the middle of the cleft, and contributes to the
constraints for the particular tandem anchor motif p2Gp3P in D\textsuperscript{d} binding peptides [29, 143]. Positions R6 and R111, which are protruding out and down towards Ly49A in site 2, are located on or very close to residues W97, A99, and W114.

It is thus possible that mutations in the 103/104/107 loop as well as in the 97/99/114 pocket impose structural alterations that disturb the interaction with the Ly49A receptor at site 2. It should be noted that the 103/104/107 motif is part of a loop that connects the two β-sheets that carry residues W97, A99, and W114. In the same way, the motif created by position 73/156 (paper III), building a ridge across the midcleft of D\textsuperscript{b}, but absent in D\textsuperscript{d}, may have an impact on site 2. This ridge is adjacent to the W97/A99/W114 motif.

When considering our results here together with the effects of mutated residues in the antigen binding groove 97/99/114 and 73/156 respectively [145] (paper III), it may thus be premature to exclude contact site 2 in the crystal as functionally significant. These residues in the antigen binding groove may be important not only for interactions upwards, i.e. towards the peptide, but also for interaction downwards, i.e. in the contact zone at site 2, beneath the groove.

It may be appropriate at this point to critically discuss the tetramer based method to assess Ly49 receptor specificity. The advantage of the method is that it directly reflects the binding of Ly49 receptor to MHC ligand. The approach based on inhibition of cytotoxicity by transfectants, used in earlier studies in this thesis, is less direct. The outcome is influenced by a number of additional factors, e.g. activating receptors and ligands, expression levels of transfected genes for inhibitory ligands, general sensitivity to cytotoxicity. On the other hand, it reflects the function of the mutant molecules in a more physiological context, with receptors in relevant concentration, orientation and topology. The tetramer assay allows a free geometry of one of the components. It cannot be excluded that it overemphasizes a particular type of binding that is less important in the interaction between cells. This should be kept in mind when considering the results in relation to the two contact sites between Ly49A and D\textsuperscript{d} in the cocrystal. In addition, tetramer binding is highly dependent on the level of receptor expression, with sharp threshold effects (Jakob Michaelsson, personal communication). There is always a risk for false negative results due to too low
expression of the receptor on the cells used to assess binding.

In conclusion, the studies in paper IV demonstrated that mutations reconstituting the Db motif in the 103/104/107 loop abrogates binding of mAb 34-5-8S and markedly impairs Ly49A binding to Dd molecules. This is consistent with the finding by Nakamura et al. [145] that the determinants required for binding by 34-5-8S overlap with, but are not identical with the determinant for Ly49A. Structural analysis based on the cocrystal of Tormo et al. [72] indicates that the mutations that we have introduced lead to structural changes that can disturb each of the two contact sites between Ly49A and Dd [72].

The altered amino acids could affect the binding either directly (i.e. the loop interacts physically with the antibody) or indirectly, by introducing structural changes that are transmitted to an antibody binding epitope in another part of the molecule.

Role of the glycans
Since the Ly49 receptors contains a putative carbohydrate recognition domain, several mutagenesis studies have addressed the hypothesis that the Ly49 interacts with the glycan(s) of the MHC class I molecule. This issue had been approached in different ways as soon as it turned out that the first NK receptor for an MHC class I molecule was member of the C-type lectin like superfamily. Brennan et al. showed that fucoidan, an anionic sulfated polysaccharide, could inhibit cell adhesion of the 51Cr-labeled GM979 cell line to Ly49C transfected COS cells [160]. Moreover, the binding of the Ly49C-specific mAb 5E6 to COS cells expressing Ly49C was inhibited by fucoidan. Another study by Daniels et al. demonstrated a fucoidan-dependent inhibition on binding of fluorescent labeled C1498-Dd cells to CHO cells expressing Ly49A [161]. The authors also investigated the ability of fucoidan to restore lysis of Ly49A+NK cells towards Dd targets without success. However, glucose 6-SO4, another sulfated carbohydrate, significantly restored killing of the C1498-Dd targets by Ly49A+NK cells. They also showed that treatment of Dd target cells with tunicamycin prevents their binding to Ly49A+ cells and renders them susceptible to lysis by Ly49A+ NK cells.

Two studies on the role of the conserved glycosylation sites in Dd by using mutant
molecules have been published. Masumoto et al. demonstrated that D^d molecules with mutations at N86 and N176 still could inhibit of killing by Ly49A+ NK cells [142]. They also showed that fucoidan competed with the antibody A1 for binding to Ly49A, and blocked binding of C1498 cells transfected with the glycosylation mutant to CHO cells expressing Ly49A. They therefore opened for a reinterpretation of the study by Daniels et al. [161], since the tunicamycin treatment have consequences for other molecular interactions required for cell-cell recognition. Tunicamycin reduces the overall level of N-specific carbohydrates by inhibiting synthesis of the precursor lipid-linked oligosaccharide. Matsumoto et al. suggested that Ly49A has two distinct ligands, carbohydrate and Ly49A.

At the same time, Lian et al. reported contrasting results [162]. They found that the glycosylation at residue 176, but not residue 86, was important for the binding of D^d to Ly49A. As single mutations, only mutation of the glycosylation site at residue 176 reduced the binding to D^d but was still sufficient to induce partial inhibition of cytotoxicity of LY49A+ NK cells. The interaction was assessed by binding of D^d transfected GM979 cells to COS cells transfected with Ly49A. Two things should be noted. First, the mutations at the glycosylation positions 86 and 176 in D^d differ between the two studies. Matsumoto et al. changed asparagine 86 and 176 to glutamine, whereas Lian et al. changed serine 88 to glycine and threonine 178 to glycine. The impact of these different amino acids changes with respect other structural changes in the D^d molecule remains to be investigated. Second, in the Ly49A/D^d cocrystal, there is a space around position 176 in D^d where the glycan possibly could fit.

It should finally be noted that allele selective binding of Ly49 receptors to MHC class I molecules has been demonstrated also when the latter have been produced as nonglycosylated recombinant proteins in E. coli, and used in tetramer constructs or in surface plasmon resonance based assays [77-79] (paper IV). Altogether, the data argue that Ly49 receptors can bind to and distinguish different MHC class I molecules in a glycan independent manner. This does not exclude that the glycan has an additional positive or negative influence on binding in vivo. As already discussed above, the Ly49 receptors were initially classified as C-type lectins, but also their structure question their role as carbohydrate recognizing receptors.
Concluding remarks and future prospects

Compared to the starting point, the studies performed in this thesis as well as other reports in the literature have clarified a number of points regarding the allospecificity of NK cell receptors. We now understand that NK cells can recognize certain syngeneic as well as allogeneic class I molecules as protective, due to their overlapping sets of inhibitory receptors. We know that in certain combinations, one MHC class I molecule may be superior to others, and the structural basis for this may be mapped to the membrane distal, peptide presenting part formed by the $\alpha_1/\alpha_2$ domains. It is furthermore clear, at least for the Ly49A/Dd interaction, that certain changes in motifs created by a limited number of amino acids within these domains (or even within the $\alpha_2$ domain exclusively) may be sufficient to interfere with recognition.

Still, a lot remains to be explained. At the start of these studies, I and my colleagues would have expected that once it was possible to abrogate the NK receptor binding to a class I molecule by a set of mutations, and certainly once the structure of the receptor/ligand complex had been solved, it would be totally clear how the interaction works. This is not so; the cocrystal between Ly49A and Dd revealed two contact sites, and the mutated class I motifs that affect the interaction have limited direct interactions with these sites, although they are positioned so that they can influence either of them. The interaction therefore still carries many secrets. Are both contact sites important, and do they have different functions, i.e. in trans (between NK cell and target cell) and cis (between receptor and ligand on the NK cell surface itself)? Further studies of mutations in the direct contact residues involved in site 1 and site 2 will be required to elucidate the role of these two interacting sites, and the effect of the mutations must be evaluated in relation to different NK functions. Does the effect of mutations at multiple motifs within the Dd molecule, with limited direct interactions at the contact sites with Ly49A, reveal that the interaction is based on a delicate 3D structure where a variety of disturbances in the class I molecule may interfere with it indirectly? If so, the concept of missing self may have to be revised, and the NK cell surveillance extended to include different type of influences on class I molecules that may occur in aberrant cells.
Do all murine class I receptors interact with their ligands in a similar manner? If not, can the different interactions tell us more about the functions of different receptors, e.g. of those that are influenced by peptide versus those that are not, or of activating versus inhibitory receptors? It is interesting to compare the mutational and structural studies of Ly49A/D^d interactions with corresponding studies of the human KIR2DL2 binding to HLA-Cw3. In the latter case, mutagenesis experiments and the solved structure of the receptor/ligand crystal have provided a highly consistent picture; residues in both α-helices as well as in the peptide are engaged in contacts with the NK cell receptor, which docks across the cleft in a similar manner as TCR, although slightly tilted towards the N-terminal part of the peptide. Do these differences in general docking pattern and peptide specificity reflect a general difference between human and murine NK cell receptors? Perhaps not, since another murine receptor ligand interaction, between Ly49C and K^b, seems to be strongly influenced by the contents of the groove. Further mutational and structural studies of the receptors in the Ly49 and KIR families and their ligands are required to understand this.

The comparison leads to the more general question: why did the human and the mouse evolve distinct receptors for the same function? Human KIR diversify at a rapid rate, perhaps forced to do so by the rapid evolution of MHC class I molecules. It has been suggested that Ly49 receptors predominantly recognize polymorphic residues, while the KIRs appear to recognize "public specificities" i.e. residues that are conserved across multiple (but not all) HLA alleles. This type of specificity might have proven advantageous. Another possibility is that the glycans of the murine class molecules somehow modulate the Ly49 interaction, as discussed above, while the absence of this glycan on human HLA-molecules required a novel type of receptor.

Finally, one may ask: what is the point of understanding the nature of NK cell receptor/MHC class I ligand interactions? One reason relates to the allospecificity - why did NK cells evolve receptors that can distinguish between different MHC class I alleles? This was not necessary if detection of reduced or abrogated MHC class I expression was the main function required. If we can understand the molecular basis for allospecificity of NK cells, this may help us to understand the origins of the system. Another reason is practical: it may be possible to modulate NK cell function in different ways by probes that interfere only with certain receptors or receptor/ligand
contacts, and in a certain way. This could be useful in relation to cancer therapy, BM transplantation or donor lymphocyte infusions. It would require a complete understanding of NK receptor/ligand interactions.
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62


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