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INTERCELLULAR PROTEIN TRANSFER
AND REGULATION OF INHIBITORY
NK CELL RECEPTOR ACCESSIBILITY

Katja Andersson

Stockholm 2007
To me, myself and I
ABSTRACT

NK cells are important players of innate immunity and capable of promoting specific responses of the adaptive immune system. NK cells possess the ability to recognise and eliminate virus-infected cells, tumour cells and allogeneic bone marrow grafts. The effector functions of NK cells are regulated by a fine-tuned balance of signals from activating and MHC class I–binding inhibitory receptors. In this thesis I investigated the interactions between inhibitory Ly49 receptors and their MHC ligands. In particular, effects of these interactions, like intercellular protein transfer and reduced cell surface expression of receptors, as well as the functional consequences thereof were studied. In addition a tumour therapy approach based on blockade of these interactions was explored.

I: Bidirectional intercellular transfer of proteins across the inhibitory NK cell immunological synapse (IS). Here we show that for both murine and human cells, target cells expressing MHC class I ligands could acquire cognate inhibitory NK receptors. Along with these, other cell surface proteins could co-transfer. The extent of KIR acquired from NK cells correlated with the level of expression of cognate MHC class I protein on the target cells. Transfer of MHC molecules to the NK cell also occurred and the target cell cytoskeleton influenced intercellular transfer of proteins in both directions. Constitutively expressed KIR could not be removed via mild acid wash treatment while a fraction of acquired KIR could. However, an accumulation of phosphotyrosines at the location of the transferred KIR suggests a signalling capacity for NK cell proteins transferred to target cells. Recent data from our and other groups, regarding intercellular protein transfer, suggest that this kind of cellular communication might play an important role in immune surveillance.

II: NK cells, expressing inhibitory Ly49A receptors, specifically acquire their cognate MHC class I ligands, H-2D\(^d\), from surrounding cells in vivo. Here we introduce three different in vitro systems, supporting Ly49A\(^-\) dependent acquisition of H-2D\(^d\) by splenic NK cells. Kinetics experiments revealed that transfer of H-2D\(^d\) was observed already after 1 minute, while downmodulation of the Ly49A receptor occurred later, suggesting that MHC class I transfer precedes receptor downmodulation. Furthermore, the acquired H-2D\(^d\) molecules interfered with the capacity of Ly49A to receive inhibitory signals delivered by ligands on target cells. Interestingly, when Ly49C was co-expressed with Ly49A on NK cells, the ability to acquire H-2D\(^d\) increased, but only in the presence of the Ly49C ligand H-2K\(^b\) on the target cell. The transferred H-2D\(^d\) molecules may fine-tune, through cis interactions with Ly49A expressed on the same cell, the accessibility of inhibitory Ly49A receptors and thereby regulate the NK cell immune functions.

III: The issue of accessibility of inhibitory receptors at the NK cell surface is an important question as the sensitivity of individual NK cells to inhibitory interactions is a critical determinant for NK cell function, not only at the effector stage, but also during NK cell development. The cis-interaction is formed between Ly49A and H-2D\(^d\) both expressed on the same NK cell surface. We quantified accessibility of the Ly49A receptors by using an established protein transfer assay, measuring the amount of H-2D\(^d\)-GFP molecules transferred to Ly49A expressing NK cells. Constitutive expression of H-2D\(^d\) molecules on B6.D\(^d\) NK cells reduced the ability to acquire H-2D\(^d\)-GFP molecules and decreased the clustering of H-2D\(^d\)-GFP molecules at the NK-target-cell contact site. This correlated to a reduced sensitivity to H-2D\(^d\)-mediated inhibition in cytotoxicity assays. Ly49A\(^+\) NK cells from B6.D\(^d\) mice showed a 90 % reduction in Ly49A accessibility that was caused both by absolute lower expression of Ly49A and interactions in cis between Ly49A and H-2D\(^d\) at the NK cell surface. Thus, endogenously expressed H-2D\(^d\) ligands regulate Ly49A receptor accessibility through interactions both in cis and in trans, in this manner regulate central developmental processes or peripheral tolerance mechanisms.

IV: Therapeutic strategies for the treatment of cancer are being developed based on preventing NK cell inhibition or triggering NK cell receptors to activate NK cells. In this study we investigated, using a mouse model, whether it would be possible to identify a therapeutic interval for inhibitory receptor blockade, where NK cells would be induced to kill syngeneic tumours, but still leave normal cells untouched. Our approach was to block inhibitory Ly49C/I receptors that bind to MHC class I molecules (H-2K\(^b\)), with Ly49C/I specific F(ab’\(^\prime\)) fragments both in vitro and in vivo. In vitro, this resulted in blockade of up to 80% of the Ly49C/I receptors and induced killing of syngeneic tumour cells and lymphoblasts by activated NK cells in vitro. In vivo, a 80-85% blockade of Ly49C/I caused NK cell-mediated selective rejection of i.v. inoculated fluorescence labelled syngeneic tumour cells but not of syngeneic spleen cells, bone marrow cells or lymphoblasts administered in a similar manner. The anti-tumor effect was maintained after 2 weeks of continuous receptor blockade without induction of autoreactivity or NK cell anergy. Our data demonstrate that inhibitory receptor blockade results in increased rejection of syngeneic tumour cells, but no killing of ‘normal’ syngeneic cells in vivo.
LIST OF PUBLICATIONS

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


* These authors contributed equally to the work in this manuscript
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<th>Full Form</th>
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<tbody>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
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<tr>
<td>β2m</td>
<td>β2-microglobulin</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>Con A</td>
<td>Concavalin A</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorter</td>
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<tr>
<td>FSC</td>
<td>Forward Scatter</td>
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<tr>
<td>H-2</td>
<td>Histocompatibility-2</td>
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<tr>
<td>HA</td>
<td>Haemagglutinin</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HLA</td>
<td>Human Leucocyte Antigen</td>
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<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IS</td>
<td>Immunological synapse</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
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<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>ITSM</td>
<td>Immunoreceptor tyrosine-based switch motif</td>
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<tr>
<td>KIR</td>
<td>Killer cell immunoglobulin-like receptor</td>
</tr>
<tr>
<td>LAK</td>
<td>Lymphokine activated killer cells</td>
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<tr>
<td>LFA</td>
<td>Lymphocyte function-associated antigen</td>
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<tr>
<td>LIR</td>
<td>Leucocyte Immunoglobulin-like receptor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MΦ</td>
<td>Macrophage</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NKC</td>
<td>NK gene complex</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>SMAC</td>
<td>Supramolecular activation cluster</td>
</tr>
<tr>
<td>SLAM</td>
<td>Signalling Lymphocytic Activation Molecule</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporters associated with antigen processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>ULBP</td>
<td>UL16-binding protein</td>
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INTRODUCTION

A GENERAL OVERVIEW OF IMMUNOLOGY

Historically, in the ancient Rome, immunity (from the Latin immunitas; protected) described the exemption from various duties and legal prosecution offered to Roman senators, which were immune during the tenures in office. In fact, still today this term is used in the political arena. In time, the term immunity meant protection from disease, i.e. resistance to reinfection. The cells and molecules responsible for immunity constitute the immune system. In general, the immune system is divided into two major branches: innate and adaptive immunity.

The innate immune system is the first line of defence, including physiological barriers to pathogen invasion like skin, mucosal membranes, pH and temperature. Additionally, soluble blood-borne mediators (such as cytokines and enzymes) circulating complement molecules, spontaneous “unspecifie” phagocytic (macrophages, neutrophils) and cytotoxic (natural killer) cells belong to the components of the innate immunity. The innate immune system deals successfully with many of the infections. However, infection that can not be handled by the innate immunity trigger the adaptive immune response characterised by clonal selection and expansion of antigen-specific lymphocytes, B- and T-cells, which together generate remarkable diversity, specificity and memory. The adaptive immune responses are dependent on these lymphocytes, providing life long immunity that follows exposure to disease and vaccination.

The efficient collaboration that exists between these two branches of the immune systems, in form of cell-cell interactions and cytokines, provides an effective defence system that is crucial for the survival of the individual. Remarkably, even though we spend our lives exposing ourselves to potentially pathogenic microbes in our environment, immunity ensures that we become ill relatively rarely.

MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

In 1996, Rolf Zinkernagel and Peter Doherty were awarded the Nobel prize in Medicine and Physiology for their discovery that cytotoxic T lymphocytes (CTL) have the ability to recognise combinations of viral antigens and Major Histocompatibility Complex (MHC) class I molecules on the surface of infected cells (2), i.e they proposed that antigen recognition was MHC-restricted. This finding profoundly changed the view on how immune responses were initiated and regulated.

The proteins coded by the MHC gene complex play a central role in the innate and particularly in the adaptive immune response (3). The main function of MHC molecules is to present peptide fragments from potential antigens to different functional subsets of T cells (4). The MHC molecules are divided in two groups; termed MHC class I and II. MHC class I
molecules are expressed by the majority of cells in the body and deal mainly with presenting intracellular antigen (e.g. virus-derived protein fragments) to CTLs. On the other hand, MHC class II molecules, expressed mainly on specialised antigen presenting cells (APC), such as dendritic cells (DCs) and macrophages (MΦs), are responsible for presenting extracellular antigens to helper T cells (T_{H1} and T_{H2}) (5). In the last decades, it has become evident that MHC class I molecules play a key function also in NK cell development, education and recognition. However, different requirements are needed to influence T and NK cells. T cells are stimulated while NK cells, in general, are inhibited by interactions with MHC class I molecules.

The human MHC class I molecules are termed Human Leukocyte Antigens (HLA)-A, -B, and –C and the genes are located on chromosome 6. In mice, the MHC is composed of a large group of genes and is located on chromosome 17. In the murine system, there exists two types of MHC class I genes, composed of class Ia and Ib loci. The Ia genes are called H-2K, H-2D and H-2L. The MHC region is highly polymorphic, i.e. there are multiple allelic variants at each locus. Beside the Ia gene, there are a number of class Ib (non-classical) MHC I genes, e.g. Qa-1. In humans, examples of MHC class Ib molecules are HLA-E, HLA-F and HLA-G (6). Unlike the MHC class Ia molecules, the class Ib display a rather limited polymorphism. Two separate polypeptide chains, a membrane bound heavy chain and a smaller non-covalently linked β2-microglobulin (β2m) subunit, form the MHC class I. The heavy chain consists of three extracellular domains (α1, α2 and α3), a transmembrane region and a cytoplasmic tail. The three domains fold in a certain structure to generate a narrow cleft, where a short peptide, derived from a degraded intracellular protein, is able to bind and be presented. The interaction between the β2m subunit and the heavy chain stabilises the binding of peptide. β2m is a single immunoglobulin-like domain that non-covalently associates with the heavy chain of the MHC class I molecule. In the absence of β2m, MHC class I molecules are unstable and are therefore found at very low levels of the cell surface (7).
NATURAL KILLER (NK) CELLS

A historical perspective of NK cells

Originally NK cell activity was demonstrated in a study showing that lethally irradiated mice were still capable of rejecting allogeneic or parental strain bone marrow cell (BMC) allografts (8, 9). The latter refers to a phenomenon termed “hybrid resistance” that argued against the current classical transplantation law (10). Irradiated F₁ hybrid H-2 heterozygous (a/b) mice, derived from crossing of two different inbred mouse strain (a/a x b/b) were able to reject parental homozygous BMC (a/a or b/b). Already in 1976, George Snell suggested that “hybrid resistance” could be explained by a mismatch between host (a/b) and donor (a/a) MHC class I molecules that could trigger rejection of the graft (3). (He would be proven right). Other groups reported on mouse spleen cells that mediated spontaneous killing of allogeneic tumour cell lines in vitro and in vivo. These peculiar radioresistant lymphoid cells, mediating bone marrow (BM) rejection and cytotoxicity against tumours in vitro and in vivo in a MHC-regulated manner, became known as ‘Natural Killer’ cells for their ability to lyse target cells without need of priming (11-16).

“The Missing self hypothesis”

Further investigations into the functions and specificity of natural killer (NK) cells revealed that NK cells were able to distinguish between different target cells based on their MHC class I expression. According to “the missing self hypothesis” proposed by Klas Kärre (17), NK cells possessed the exquisite ability to distinguish and eliminate a cell that displayed reduced self MHC class I expression occurring after, e.g. transformation or virus infection, or even failed to display self-MHC class I molecules completely on the cell surface. Consistent with the hypothesis, induction of suitable MHC class ligand on MHC-deficient target cell was sufficient to abrogate rejection mediated by NK cells. NK cells thus represent a significant complementary and alternative effector mechanism in the immune system, since they demonstrated the capacity to recognise alterations that were not detected by the peptide/MHC specific T cells. An important feature of “the missing self hypothesis” was the requirement of an initial activating contact between the target cell and the NK cell, presuming that the NK cell had already triggered its lytic program, which will proceed by default unless the inhibitory signals are transduced (18, 19). NK cells probably utilise several parallel recognition mechanisms to distinguish and eliminate aberrant cells (20, 21). Thus “the missing self hypothesis” did not exclude the possibility that other target cell properties, like activating ligands, costimulatory receptors and adhesion molecules, influenced the sensitivity to NK cells.
Figure 2. A simplistic view of “The missing self hypothesis” (17). Reduced or absence of MHC class I expression as well as high expression of activating ligands renders the target cells susceptible for NK cell killing. Only the target cell to the left survives NK cell scrutiny.

A brief description of NK cells

NK cells are large granular lymphocytes (LGLs) derived from the bone marrow (BM), sharing a common progenitor with T cells. NK cells, the third major population of lymphocytes, constituting 5-15% of the peripheral blood lymphocytes in human and 3-5% of the lymphocyte population in a mouse spleen. Unlike B and T cells, the first and second major population of lymphocytes, NK cells develop normally in severe combined immune deficiency (scid) mice or in mice with defected RAG-1 or RAG-2 genes (rearrangement genes), indicating that gene rearrangement is not required for their development, differentiation and triggering of activation. An NK cell-mediated response is characterised by thymus independence, rapid onset and no requirement of priming or pre-immunisation, features different than those required for clonal expansion and effector responses of CTLs. Contrasting to this view, recent evidence suggest that some NK cells in fact require the thymus to develop (22). These NK cells are identified by their expression of the IL-7R and have reduced killing capacity. After leaving the thymus, these NK cells localise preferentially...
to lymph nodes, suggesting a unique functional property. Until recently, NK cells in both mouse and human currently had to be identified by both positive and negative criteria due to the absence of a truly NK-specific marker. Cell surface markers, which were considered to be specific for NK cells, e.g. NK1.1, DX5 (mouse) and CD56 (human), are also present on certain T cell subsets, but the lack T cell specific marker, such as CD3 and T cell receptor (TCR) allowed for identification of NK cells. Lately, a activating NK cell receptor, termed NKp46 (23-25) (also called MAR-1 in mice), has become a suggested marker of NK cells since it is expressed, presumably by all NK cells and not by T cells.

NK CELL BIOLOGY

NK cells are active in rejection of bone marrow grafts and in resistance to tumour growth and metastasis of tumours. In NK-depleted mice, growth of some tumours and metastases are augmented (26). Additionally, NK cells are involved in the innate immune response against certain viruses (e.g. cytomegalovirus CMV), intracellular bacteria (e.g. Mycobacterium tuberculosis) and parasites (e.g. Plasmodium falciparum) (27). NK cells play an essential role in the interplay between the innate and adaptive immunity. Loss of MHC class I from cells owing to transformation or infection may lead to NK cell activation, according to the “missing-self” hypothesis, provided that an activating receptor is engaged. NK cells can directly lyse of target cells by exocytosis of granules, containing perforin and granzymes, or by producing various cytokines and chemokines. A number of cytokines are secreted by NK cells, promoting haematopoiesis, such as granulocyte-monocyte colony stimulatory factors (GM-CSF) and granulocyte-colony stimulatory factor (G-CSF). Upon activation, NK cells secrete cytokines, such as interferon (IFN)-γ, and tumour necrosis factor (TNF)-α, promoting specific immune responses of the adaptive immune system to the infection. In addition, NK cells also secrete chemokines, such as CCL3, macrophage inflammatory protein (MIP1-α), CCL4 (MIP1-β) and CCL5 (28). Recently, it has been reported that NK cells also secrete T_h2 cytokines, such as IL-15, IL-10 and tumour growth factor (TGF)-β. The activity of NK cells is likely dependent on surrounding cytokines in the current milieu. During infections by viruses and other intracellular pathogens, NK cells respond rapidly to interferon (IFN)-α/β, IL-12 and IL-18 which are mainly secreted by activated dendritic cell (DC) and macrophages (29). An additional effector mechanism of NK cells is mediated through interaction of Fas-ligand (Fas-L) with the extracytoplasmic domain of the Fas receptor, inducing Fas trimerisation and activation of the apoptotic cell death process. Fas-related membrane receptors contain death domains in their cytoplasmic part. The Fas/Fas-L system plays a major role in the cytotoxic activity of immune cells and the regulation of immune response. In vivo, Fas-L expression induces tumour cell rejection (30). Additionally, TNF-related apoptosis inducing ligand (TRAIL) binds to death domain-containing receptors on target cell leading to their apoptosis in a perforin-independent way (31-33).

The cytolytic activity and the cytokine production of NK cells are under tight regulation. The susceptibility of a target cell to an NK cell is dependent on the expressed repertoire of activating and inhibitory receptors on the NK cells, as well as their ligands on the target cell, reflecting a complex system for NK cell recognition. The crucial process that regulates or
selects the composition of receptors to ensure effector function and self-tolerance is still a controversial topic of discussion (34).

**NK cell receptors**

*Activating receptors*

The absence of self-MHC molecules is not sufficient in itself for triggering of NK cells. NK cell activation requires engagement of specific stimulatory receptors by ligands expressed on infected and transformed cell, but also by normal cells. The positive stimulation may be initiated through a combination of signals, received from a multitude of receptor/ligand pairs accumulated at the point of cell-cell contact. The stimulation initiates several signalling cascades that eventually influence the rearrangement of the Golgi apparatus (GA) and microtubule-organising centre (MTOC), which orients the cytolytic machinery of the NK cell toward the target cell. The triggered signal transduction results in secretion of cytokines and release of cytoplasmic granules, containing perforin and granzymes that are responsible for delivering the ‘lethal hit’ of the target cell, thus triggering apoptosis and target cell death (35).

Lymphocyte function-associated antigen-1 (LFA-1, \(\alpha\beta\)) is a \(\beta\)-integrin that binds intercellular adhesion molecule-1 (ICAM-1) and is important for adhesion to the target cells (36, 37) Its binding to ICAM-1 stabilises the intercellular adhesion and conjugation between the NK cell and its target cell. Interestingly, LFA-1-mediated adhesion by itself has been shown to trigger an early activating signal, promoting the accumulation of NK cell activating receptors in lipid rafts and delivery of cytotoxic granule contents towards the susceptible targets (38, 39).

DNAx accessory molecule-1 (DNAM-1, CD226) (40), is able to enhance cytolytic activity and cytokine production in both T and NK cells. Recently, the polio virus receptor (PVR, CD155) and nectin-2 (CD112), members of the nectin family, were identified as the ligands for DNAM-1 (41). These two ligands are highly expressed in certain tumour cell lines, including melanomas, carcinomas and neuroblastomas (42). Moreover, nectins are by no means tumour-specific antigens as they are also widely expressed on normal cells, e.g. in epithelial and endothelial cells. Notably, these normal cells are not killed by NK cells because of their high MHC class I expression (43).

**KIR2DS and KIR3DS** are two activating killer cell Ig-like receptors (KIRs), on human NK cells. Most KIR receptors recognise MHC class I molecules and are inhibitory. They will be more discussed in the section concerning inhibitory receptors. Activating KIRs that contains two extracellular immunoglobulin domains and a short cytoplasmic tail are designated KIR2/3DS. Most ligands for activating KIRs are not known, but it has been proposed that they may also recognise MHC class I molecules (44). They may also recognise MHC class I molecules (44). Genetic studies have implicated that some KIRs are in involved in control of viral infections and malignancy, susceptibility to autoimmunity, and reproductive success (45, 46). For instance, KIR2DS2 has been shown to be associated with vasculitis in patients with rheumatoid arthritis (RA) (47) and with susceptibility to psoriasis vulgaris (48). In HIV-1 positive individuals, who co-express KIR3DS1 and HLA-Bw4, show delayed progression of
AIDS, indicating that the KIR subset somehow limits the viral spreading (49). Activating KIR and Ly49 molecules contain a positively charged amino acid, such as arbinine or lysine, in their transmembrane domain. These positively charged residues permit association with DAP12, an immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor protein allowing transduction of an activating signal (50). In the mouse, activating Ly49 receptor are able to activate NK cytotoxicity when interacting with MHC class I-expressing cells by an analogous mechanism, i.e., through adaptor proteins DAP12 (51). For example, Ly49H (52, 53) triggers a strong cytolytic response through DAP12 when binding specifically to m157 (54, 55), an ‘MHC I-like’ molecule, expressed upon infection by murine cytomegalovirus (MCMV) (56, 57).

The CD94 and NKG2 receptors recognise nonconventional MHC class I-like ligands (human HLA-E and mouse Qa1b). CD94 and NKG2 are type II transmembrane protein, belonging to the C-type lectin family. NKG2C and NKG2E are expressed on the cell surface as heterodimers, covalently associated to CD94 and coupled the ITAM-containing adaptor molecule DAP12 (described later) (58).

The Fc receptor CD16 (FcγRIIIA) is present on both murine and human NK cells. It has an activating function after binding the Fc part of IgG. CD16 mediates antibody-dependent cellular cytotoxicity (ADCC) (59, 60). Activation initiates signalling through the FcεRIγ and CD3ζ adaptor proteins in humans. In mice, CD16 couples only with FcεRIγ (61).

In humans the “natural cytotoxicity receptors” (NCRs) include NKp46 (62, 63), NKp30 and NKp44 (24). The murine homologue to NKp46 is termed murine activating receptor-1 (MAR-1 or NCR-1) (23, 24). NKp46 and NKp30 are present on both resting and activated NK cells, while NKp44 (64) is expressed upon activation (65). Human NKp44 and NKp46 bind to haemagglutinins (HA) of influenza virus (23, 66–68). The NCRs associate with different adaptor proteins containing ITAMs, including DAP12, CD3ζ and FcεRIγ. The cellular ligands recognised by the NCRs are still unknown. However, NCRs represent NK cell markers that allow for NK cell-mediated tumour cell lysis (69). Cells of different histotypes express the ligands, at least after tumour transformation or viral infection.

**NK cell receptor protein 1 (NKR-P1),** is also known as killer cell lectin-like receptor B1 (KLRB1) in mice. NKR-P1-family members are homodimeric C-type-lectin-like molecules. **NKR-P1A, NKR-P1C and NKR-P1F** are three activating members of the NKR-P1 family (70). NKR-P1C was the first to be identified and is also known as NK1.1 (71). It is commonly used as a marker in combination with CD3 to identify NK cells (NK1.1+CD3+) in certain mice strains, such as B6. Furthermore, NKR-P1C has a charged transmembrane residue that associates with the γ-chain of FceRI and triggers mouse NK cells (72). Its ligand is still undefined. NKR-P1F binds CLR-G (also known as OCILrP2) expressed on DCs and macrophages (73, 74). In human, only NKR-P1A has been identified and its function is still unclarified (75).

**CD2 family members** regulate NK cell lytic activity and inflammatory cytokine production when engaged by ligands on tumour cells (76). The most widely studied member of the CD2 subfamily, the **2B4 (CD244)** receptor, is capable of transducing both activating and inhibitory
signals in NK cells (77). The cytoplasmic part of 2B4, containing a so-called immunotyrosine switch motif (ITSM), recruits and binds to SLAM-associated protein (SAP) crucial for 2B4-mediated activation. However, if 2B4 engages with its ligand CD48 in the absence of the SAP protein, resulting in binding of phosphotyrosine phosphatases (PTPs), e.g. SHP-1, and an inhibitory signal will be transduced (78, 79). In contrast to mouse 2B4, human 2B4 seems to be mainly an activating receptor (80, 81). In humans, 2B4 can also be inhibitory but only in the absence of functional SAP. This situation is seen during NK cell development and in patients with X-linked lymphoproliferative syndrome (XLP) (78, 82).

The NKG2D receptor is expressed by a fraction of T lymphocytes and by a majority of NK cells and is involved in mediating both cytotoxicity and cytokine release (83-85). In the mouse, there are two isoforms of NKG2D generated by alternative splicing. NKG2D-L, the long form, interacts with the adaptor protein DAP-10 whereas the short form, NKG2D-S, associates with either DAP-10 or DAP-12 (referring to next section) (86, 87). In humans, only NKG2D-L has been identified (40, 88). NKG2D recognises a family of related ‘stress’ inducible ligands, including unique long (UL) 16-binding proteins (ULBPs) (89), MHC class I related chains A and B (MICA and MICB) (83) in human. In mouse, there are multiple ligands, including the minor histocompatibility antigen (H60), retinoic acid-early inducible (Rae)-1 and murine ULBP-like transcript (MULT)-1 (90-95). DNA damage has been demonstrated to mediate induction of NKG2D ligands in response to genotoxic stress (96). The expression of NKG2D ligands can be induced in mature cells by stress, infection or transformation. NKG2D emerges to be a key player in immunity against tumours and infections (97, 98).

Figure 3. Some human and murine activating NK cell receptors and their ligands.
**Inhibitory receptors**

A balance between positive and negative signals delivered by activating and inhibitory receptors normally controls immune responses. The result of the negative regulation could be apoptosis, anergy and growth inhibition, as well as termination of activating signals. Its main purpose would be to prevent undesired effector functions, such as tissue damaging cytokine production and cytolysis of autologous cells. In NK cells, such negative signals are mediated by specialised inhibitory receptors, preventing signalling cascades initiated by activating receptors (figure 4 and 5). In the beginning of the 1990’s, the first MHC class I-specific inhibitory receptor Ly49 (now known as Ly49A) was discovered in the mouse (99-102). The Ly49A receptor binds various MHC class I ligands, of which H-2D\(^d\) is the strongest and most well studied, not at least in the work presented in this thesis.

**MHC-binding inhibitory receptors**

In mice, the major inhibitory receptor family is the Ly49 receptors, which recognise the polymorphic H-2 class-I molecules on mouse target cells and subsequently inhibit NK cell-mediated cytotoxicity. (Ly49D and Ly49H are two activating members, described in the previous section). The Ly49 receptor family is located on mouse chromosome 6 in the “NK gene complex”, a cluster of genes mainly expressed by NK cells (70, 103). Ly49 receptors belong to the C-type lectin superfamily and are type II membrane glycoproteins (intracellular N-terminal) expressed on the cell surface as disulphide-linked homodimers. Expression of Ly49 receptors is a late event in the development of the NK cells. Ly49\(^+\) NK cells appear gradually during ontogeny and adult levels are reached after 6-8 weeks of life (104). Interestingly, the distinct repertoire of Ly49 inhibitory receptor expression on the cell membrane of NK cells is dependent upon the genetic background mouse strain and is also shaped by influences of the host MHC class I haplotype (105). In humans, chimpanzee and gorilla, a single Ly49 nonfunctional pseudogene has been found, which is poorly transcribed (106-108). However, rat and horse NK cells also express Ly49 receptors with the equivalent function as in mice (109). Furthermore, these receptors are found on NKT cells and memory CD8\(^+\) T cells. Lately, it has been highlighted that Ly49A receptors on T cells can mediate inhibition (110, 111).

In humans, the equivalent to Ly49 receptors are the KIRs that belong to the the immunoglobulin superfamily and are type I transmembrane glycoproteins with 2 or 3 extra cellular domains, recognising class Ia HLA-C, B or A molecules (62). The number of KIR genes in the genome of any given individual varies within the population. The KIR2DL2 and/or KIR2DL3 receptors for HLA-C group 1 are present in all individuals. The KIR2DL1 receptor for HLA-C group 2 is found in 97% and the KIR3DL1 receptor for HLA-Bw4 alleles is found in approximately 90 % in the Caucasian population (112-116).

Another NK cell inhibitory receptor of the immunoglobulin superfamily, leukocyte immunoglobulin-like receptor-1 (LIR-1) /immunoglobulin-like transcript (ILT)-2, binds HLA-G molecules. HLA-G is a non-classical MHC molecule characterised by limited polymorphism and a restricted expression to immuno-privileged sites, such as at the fetal-maternal interface (117). HLA-G expressed by tumours may inhibit NK cell cytotoxicity by
interacting with NK cell LIR-1 and/or KIR2DL4 receptors, implicating that HLA-G maybe is involved in immune escape of tumour cells (118, 119).

In addition to inhibitory Ly49 or KIR receptors, some NK cells express another MHC class I-specific receptor, the molecular complex formed by CD94/NKG2A/B. The CD94/NKG2 receptor has been detected on both murine and human NK cells. The CD94/NKG2A and B receptors have been shown to execute inhibitory signals upon binding to Qa-1b (mouse) (120-122) or HLA-E (human) (123), non-classical MHC class I molecules on target cells. An interesting feature concerning Qa-1b and HLA-E is that the most abundant peptides bound to these molecules are derived from the leader segments of different classical MHC class I proteins (124, 125).

**Figure 4.** Some inhibitory NK cell receptors binding to MHC class I

**Non-MHC-binding inhibitory receptors**

Recent work has revealed other systems of NK cell inhibition that are independent of MHC class I molecules (77). The most studied receptor is the 2B4 receptor, expressed by all human and mouse NK cells (126). 2B4 belongs to the SLAM family, a subfamily of the CD2 family of immunoglobulin receptors, implying that 2B4 contains 2 immunoreceptor tyrosine based switch motifs, ITSMs, in the intracellular part. Most studies indicate that mouse 2B4 functions as an inhibitory receptor. 2B4 inhibits both NK cytotoxicity and IFN-γ production when it is engaged with targets that express its ligand CD48 (127-129). Nevertheless, as mentioned above in the section about activating receptors, 2B4 in mouse has also an activating function, according to some studies (130).

Ligation of NKR-P1B and NKR-P1D receptors cause inhibition of NK cell effector functions in mice (131, 132). NKR-P1B and NKR-P1D recognise and bind to CLR-B (also know as OCIL) (74). NKR-P1D and CLR-B interaction inhibits NK killing of syngenic cells or tumour cells, expressing low levels of MHC class I molecules (73), which could be one mechanism that explains how NK cells could maintain self-tolerance. CLR-B and CLR-G (also known as OCIL and OCILrP2 respectively) molecules are expressed on DCs and macrophages (73, 74).
CLR-NKR-P1 interactions appear to enable the NK cells to distinguish between normal and transformed cells (133).

Carcinoembryonic antigen-related cell adhesion molecule 1, **CEACAM1**, belongs to a multifunctional immunoglobulin superfamily and is the first identified member, CEACAM5 (also known as CEA), is used as a marker of colon cancer (134). CEACAM1, containing two ITIMs in the cytoplasmic tail, is the only member expressed by human NK cells and the ligand is CEACAM1 itself (135). Recent studies have revealed that CEACAM1 has the capacity to mediate NK cell inhibition (136). Furthermore, CEACAM1 is able to prevent NK-cell autoagression in absence of self-MHC-class I molecules (137).

Killer cell lectin-like receptor G1, **KLRG1**, (also known as MAFA) (138, 139) is an inhibitory ITIM-carrying NK cell receptor. Its crosslinking mediates reduced cytokine production and lytic NK cell activity (140). The broadly expressed classical cadherin molecules have lately been identified as the ligands of KLRG1 receptors. Upon crosslinking and following phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITIM) tyrosine, KLRG1 preferentially recruits SHIP-1 and SHP-2, but not SHP-1 (141-143).

Sialic-acid-binding immunoglobulin-like lectins, SIGLECs. Humans have 11 SIGLECs and mice 8 SIGLECs. **SIGLEC7**, in humans, was originally identified as an inhibitory receptor, containing two ITIMs and is expressed by all NK cells (144). One ligand that has been characterised for SIGLEC7 is GD3, a glycosphingolipid, and NK cells do not lyse GD3-expressing cells.

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<tr>
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<tr>
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<td>KIR family</td>
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<td>CD94/NKG2A</td>
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**Figure 5.** Some human and murine inhibitory NK cell receptors and their ligands
Accessibility of Ly49 receptors

Since NK cell are regulated by a balance between activating receptors and MHC class I-specific inhibitory receptors, the accessibility of the receptors is crucial for the final outcome of the NK cell activity and especially for some of my projects. I will therefore address the issue of Ly49 receptor accessibility and how it may be regulated in more detail.

Downmodulation of receptor expression at the cell surface

Shortly after the discovery of the first MHC class I-specific inhibitory NK receptor, Ly49A (99), it became clear that the cell surface levels of inhibitory Ly49 receptors were modulated by the MHC class I molecules of the host. In a H-2D\textsuperscript{d} transgenic mice (D\textsubscript{8}) the expression level of Ly49A were reduced 30-50 % in comparison to control non-transgenic B6 mice (145-147). A hypothesis related to the receptor repertoire or expression levels of MHC class I-specific inhibitory receptors was postulated. “The receptor calibration model” suggested that NK cells interact with self and non-self MHC in the current environment and subsequently adapt their receptor repertoire in order to detect alterations of self-MHC expression. According to “the receptor calibration model”, it was beneficial for the host to downregulate the expression of the inhibitory receptor on the NK cells if the corresponding MHC class I ligand is present. Host NK cells would enhance their ability to discriminate and detect slight changes in expression levels of the MHC class I ligands and by this means kill infected or transformed cells, expressing reduced levels of MHC class I molecules. Lower levels of receptors would require an increase in the number of class I alleles on the target cell in order to achieve an inhibitory signal. Thus, “The receptor calibration model” proposed that down-regulation of Ly49 receptors on NK cells may be “useful” for the NK cells to discriminate between normal and reduced levels of MHC class I molecules. NK cells, expressing either Ly49\textsuperscript{high} or Ly49\textsuperscript{low}, require different amount of MHC class I molecules on the target cell to receive an inhibitory signal. Even lower levels of MHC ligands turn off the Ly49\textsuperscript{high} NK cells, whereas Ly49\textsuperscript{low} NK cells require increased expression of the ligand to be turned off properly. Speculations about mechanisms behind the regulation of NK receptor expression suggested that the NK cell repertoire could be determined during the development of immature NK cells or NK cells might continuously adapt to the self-MHC class I milieu (145, 146, 148-152). Mechanistically, the reduced accessibility of Ly49A receptors in D\textsuperscript{d}-expressing mice was thought to result from physical removal of Ly49A receptors from the cell surface in the form of shedding or from a reduction caused by receptor internalisation.

Cis interaction

Hints towards an alternative explanation for modulation of Ly49A receptor accessibility came from two directions. The first was the crystal structure of the complex between Ly49 and H-2D\textsuperscript{d} (121), which pointed towards the possibility of an interaction between these two molecules in cis, i.e. when sitting next to each other in the cell membrane. In addition, work using MHC class I mosaic mice from our laboratory, by Kåse et al., demonstrated that H-2D\textsuperscript{d} expression on the NK cells was necessary to maintain low Ly49A receptor levels after IL-2-activation in vitro, suggesting that endogenous H-2D\textsuperscript{d} molecules on the NK cells play an active and modulatory role in the regulation of Ly49A receptor expression. Also here, it was possible that the low Ly49A expression could be retained, through a physical cis interaction, between H-2D\textsuperscript{d} molecules and Ly49A receptors at the cell surface (152). Doucey and colleagues presented
direct evidence of a *cis* interaction between Ly49A and H-2D<sup>d</sup> at cell surfaces. They further suggested that low cell surface expression of Ly49A was caused by the inability of most Ly49A-monoclonal antibodies (mabs) to bind to Ly49A receptors since the receptors were bound to the endogenously expressed H-2D<sup>d</sup> molecules on the NK cells in *cis* (153). My work in paper III expands further into this issue. It was further suggested that Ly49A receptors could exist in a backfolded conformation, allowing and interaction with H-2D<sup>d</sup> ligands on the NK cell (*cis*) and also in an extended conformation allowing functional binding to H-2D<sup>d</sup> molecules on target cells (*trans*), resulting in NK cell inhibition (153, 154).

The ‘co-crystal’ structure of Ly49A-H-2D<sup>d</sup> indeed showed 2 possible interaction sites: site 1 and site 2 (figure 6). It has later been shown that both the *cis* and the *trans* interaction occurs through binding at site 2, the functional site for inhibition (155, 156), excluding simultaneous *cis* and *trans* interaction. Consequently, the *cis* interaction restricts the accessibility of Ly49A receptors for target cell MHC class I (interaction in *trans*), which then could provide one explanation for why H-2D<sup>d</sup>-positive effector cells have reduced expression level of Ly49A and demonstrating inefficient inhibition by H-2D<sup>d</sup>-expressing targets. The *cis* interaction therefore seems to provide the NK cell with a regulatory system that could modulate the threshold at which NK cells exceeds NK cell inhibition, allowing NK cells distinguish abnormal from normal cells sufficiently to maintain self-tolerance (153).

**Figure 6.** Co-crystal of of the H-2D<sup>d</sup> Ly49A and the potential interactions; site 1 and site 2 (157)
NK cell signalling

Activating Signalling

Engagement of NK cell activating receptors and adhesion molecules initiates intracellular signalling that involves several steps. Initially, there is rearrangement of actin cytoskeleton and formation of an NK immune synapse (NKIS). This follows by reorientation of the Golgi complex and the MTOC to polarise the lytic granules toward the target cell, resulting in the release of lytic substances as well as cytokines. The map of signalling pathways is still not complete and further deep-diving investigation in this complex field is required. The complexity gets even more complicated since the signalling network changes constantly depending on which and how many receptors are involved.

The β2-integrin LFA-1 participates in adhesion and conjugate formation and is also involved in triggering the earliest signal transduction event, inducing the recruitment of NK cell activating receptors to lipid rafts. VAV-1 is found downstream LFA-1, organising the cytoskeleton by triggering RAS-related C3 botulinum substrates-1 (RAC-1) (158), proline-rich tyrosine kinase-2 (PYK-2) (159) and extra-cellular signal-regulated kinase (ERK) pathways that polarise the granules towards the target (160).

A common feature of haematopoietic activating immunoreceptors resides in their association at the cell surface with transmembrane signalling adaptors, such as the DAP-12 (DNAX-activating proteins of 12kD), also called KARAP (killer cell-activating receptor-associated protein) (161, 162), CD3ζ or FceRIγ (163). These adaptors harbour intracytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs), containing YxxL/Ix6-8YxxL/I residues, where x is any amino acid. Upon crosslinking, the ITAMs are phosphorylated by Src family protein tyrosine kinases (PTKs) such as Lck and Fyn, which subsequently recruit the Syk family PTK members, such as ζ-associate protein ZAP-70 or Syk (164-167). ZAP-70 or Syk signalling pathways stimulate downstream events, involving phospholipase (PL)-Cγ and MAP kinases, which eventually cause Ca^{2+} influx, degranulation and transcription of cytokine and chemokine genes (58, 168, 169). DAP-12 has been shown to both activate and inhibit activation. It has been suggested that the quality of the cellular responses is modulated by the avidity of the interaction between the DAP-12-associated receptor and its ligand. Available data for DAP12 indicate that the main role of DAP12 is to modulate the threshold for cellular activation in response to stimuli (170).

Recently, it has become clear that ITAM-containing adaptors may also mediate inhibitory signals, propagated through tyrosine residues within ITAMs, providing to set the activation threshold of the cell. The negative regulation depends on the particular receptor, number of ligands, and affinity of the ligand or cell type involved. Inhibition might be a result of recruitment and activation of phosphatases or other dampening signalling components. Alternatively, recruitment and sequestration of kinases in that way deprivating other receptors of the kinases needed for their activation. Otherwise, it might be caused by induction of immune-suppressive cytokines that have the capacity to diminish cellular responses initiated by other activating receptors (168).
In contrast, the signalling pathway of another transmembrane adaptor **DAP-10** (DNAX-activating proteins of 10kD), containing a YxxM, includes PI3K, Grb-2, PLC-γ2, SLP-76, and is independent of Syk family PTKs (88, 171). Once activated, DAP-10 couples to two adaptors that propagate separate branches of the signalling pathways. Grb2 couples with Vav-1, responsible for PLCγ2 and SLP-76 phosphorylation (172), whereas PI3K activation leads to ERK phosphorylation (173). Both of the pathways are required for calcium flux and DAP10-mediated NK cell cytotoxicity (171, 174). DAP10 signalling seems to be enough to trigger mouse cell cytotoxicity but insufficient for inducing cytokine (e.g. IFN-γ) production (173). On the contrary, ITAM-dependent signalling has the ability to trigger both cytotoxicity and cytokine secretion (86, 175, 176).

**Inhibitory signalling**

Inhibitory receptors are able to block the activation of NK cells and therefore prevent attack on normal cell and tissues. The engagement of inhibitory Ly49 (177, 178) or KIR receptors (179, 180) with their ligands results in interruption of early activating signals in NK cells in *trans* by ITIM phosphorylation by Src PTKs and recruitment of phosphotyrosine phosphatases (PTPs), e.g. Src homology 2 (SH2) domain-bearing tyrosine phosphatase-1 (SHP-1) (181, 182), SHP-2 and/or SHIP proteins. PTP recruitment may result in decreased tyrosine phosphorylation of effector molecules, belonging to the activating signal pathways, such as Syk, Vav-1 (183) and PLCγ. SHIP dephosphorylates PLCγ substrate, PIP2, inhibiting Ca^{2+}-dependent signalling and activation of PKC (184). Thus, the PTPs dephosphorylate important stimulating intermediates in the intracellular tyrosine-based signalling that subsequently suppress NK cell effector functions, i.e. cytotoxicity and cytokine secretion.

**NK cell regulation by cytokines**

Cytokines are able to affect the effector functions of the NK cell (185). Interleukin (IL)-2, IL-15 and IL-21 (186) are capable of inducing proliferation and activation of NK cells, but only IL-15 (187) has shown to be critical for the development and maintenance (185, 188). IL-2 and IL-7 protect NK cell from death by apoptosis and up-regulate bcl-2 expression (189). In addition, IL-2 has been reported to induce IFN-γ production by human (190) and murine (191) NK cells. Stem cell factor, SCF, andflt3 ligand have been reported to be important for early NK cell differentiation (192). IL-12 and IFN-α/β exert potent stimulatory effects on NK cells. Endogenous IL-12 plays an important role in the normal host defence against infection by a variety of intracellular pathogens. Additionally, IL-18 in combination with IL-12 is particularly effective in augmenting the NK cell function (193, 194). Both IL-12 and IL-18 are also important modulator of Th1 responses and predominately produced by DCs and macrophages (195). They are able to stimulate NK cell production of IFN-γ as well as augment NK cell proliferation and cytotoxicity (196, 197). It is possible that cytokines such as TNF-α and IFN-γ may cause suppression of NK cell responses and further undergo an activation-induced
cell death (AICD) (72).

**NK cell interactions with DCs and T cells**

NK interact with DC in inflamed peripheral tissues and secondary organs, where both cell types are recruited by chemokines, leading to modulation or amplification of different innate and adaptive mechanisms. This cross-talk between NK cells and myeloid dendritic cells (DCs) results in NK cell activation and in DC maturation. Activated NK cells have the ability to kill DCs that have failed to undergo proper maturation (“DC editing”). During NK-CD interactions NK cells are induced to secrete TNF-α, triggering DC maturation. During acute inflammation, DCs induce, by secreting IL-12, proliferation of NK cells that enhance the NK cell cytotoxicity and production of IFN-γ, which is involved in regulation of DC-mediated priming of T cells. Therefore, NK cells play a central role during DC-induced T-cell priming and subsequent polarisation both indirectly and directly, according to some reports (198, 199).

NK cells have the ability to promote or inhibit the activation of autoreactive T cells during the initiation of autoimmunity. Several mechanisms, by which NK cells could modulate autoreactive T cell by promoting or inhibiting them, have been addressed. Through production of IFN-γ, activation of APCs, costimulation of T cells and/or direct antigen presentation by the NK cells to the T cells, NK cells can promote the development of autoreactive T cells. Alternatively, the NK cells might inhibit autoreactive T cells through the lysis of DC or T cells, production of regulatory cytokines (such IL-10 and TGF-β) or regulation of cell cycle progression (200). A recent interesting role for NK cells in relation to T cell priming has been illustrated in lymph nodes. CXCR3-dependent recruitment of NK cells seems to correlate with the induction of T helper cell type 1 (T\(_{\text{H}1}\)) responses. NK cell depletion experiments show that NK cells provide an early source of interferon-γ (IFN-γ) that is necessary for T\(_{\text{H}1}\) polarization, implying an essential role of NK cells in secondary lymphoid organ (201).

Recently, it has been demonstrated that regulatory T cells (Treg) directly inhibit NK cell function in both tumour (202) and BMT situations (188). It has been shown that Tregs have the capability to suppress NK cell effector functions, i.e. proliferation, cytotoxicity and IL-12 mediated IFN-γ production in vitro and in vivo. The mechanisms behind the inhibition of NK cells in mice are still under considerations but soluble, surface-bound TGF-β and IL-10, produced by Tregs, are strong candidates (203-205).

**THE IMMUNOLOGICAL SYNAPSE**

The immunological synapse (IS) is defined as an intercellular contact, involving at least one cell of the immune system, at which encounter causes proteins to segregate into micrometer-scale supramolecular organisation of surface molecules. Potential molecular mechanisms involved in the formation of IS include a role for the cytoskeleton, segregation of proteins according to size of the extracellular domains and association of proteins with lipid raft (206-209). The major function of the IS is still under investigation, but there are several speculations that concern signalling, triggering of activation, secretion or internalisation of receptors. The first IS intensively studied in three-dimensional analysis was the interface between T cells and APCs, which showed that receptors and intracellular proteins were
organised into supramolecular activation cluster, (SMACs), presumably regulating the fate of the T cell activation (210). The mature IS has been defined by the bull’s eye arrangement of SMACs. The central region, called cSMAC, is enriched in TCR and MHC-peptide complexes. The peripheral ring, pSMAC, encloses LFA-1 and the counter receptor ICAM-1 expressed on the effector and target cell, respectively (211-213). Heterogeneity in the SMAC of IS arises from the involvement of different cells, external stimuli from the surrounding and levels of surface protein expression, proposing that formation of the IS influences the outcome of the intercellular communication and therefore of importance in various immunological situations (214).

Activating NK cell synapse

The contact site between the NK cells and target cells has been termed the NK cell immune synapse (NKIS). Together with a susceptible target an NK cell forms the cytolytic NKIS (cNKIS). Similar to the IS, the NK cell activating receptor-ligand pairs and effector signalling molecules accumulate at the cNKIS, forming a cSMAC, surrounded by adhesion molecules, including LFA-1 and Mac-1, pSMAC, which both bind ICAM-1 on the target cell (215). Beneath the adhesions molecules gather the actin-binding protein talin, linking to the cytoskeleton (216). Delivering of the “kiss of death” (217), e.i. the process of NK cell degranulation, includes rearrangement of the actin cytoskeleton, reorientation of the Golgi apparatus and the MTOC to polarise and release lytic granules at the cNKIS (218, 219). Additionally, the cNKIS lipid rafts become polarised to the site of interaction upon crosslinking of activating receptors that follows by stimulation of signalling molecules, including Src and Syk family PTKs (220). Thus, the balance between activating and inhibitory signals at the cell surface of the NK cells affects the distribution of cytoskeletal proteins, assembly of the NKIS, and hence NK cytotoxicity (206).

Inhibitory NK cell synapse

An NK cell in contact with a resistant target forms an inhibitory NK cell immune synapse (iNKIS). Inhibitory receptors, like activating receptors, assemble and interact with their cognate ligands at the contact site, referred to as the supramolecular inhibition cluster (SMIC), leading to gathering of phosphatases that are responsible for dampening the initiated activating signalling cascade. Daniel Davis et al. was first with the visualisation of the NKIS, demonstrating that in the human iNKIS a peripheral ring of KIR/HLA interactions formed around the central cluster LFA-1/ICAM-1 cluster, the opposite of the mature T cell IS (221). Immediately after conjugation, the position of SHP-1 discriminates the cNKIS from the iNKIS. SHP-1 is located peripherally in the late cNKIS, whereas SHP-1 accumulation is central in the early iNKIS (222, 223). In addition, ezrin, CD43 and CD45 are excluded from the iNKIS (224). The rate of KIR clustering is regulated by actin cytoskeleton that also plays a role in stabilising the conjugate formation (39). Surprisingly, clustering of receptor-ligand interactions occurs independently of inhibitory signal transduction. Nevertheless, KIR signalling is crucial for preventing lipid raft polarisation, which is an important step in NK cell cytotoxicity (225). The inhibitory signalling cascade mediated by CD94/NKG2A receptors ligated to their HLA-E ligands, prevents actin-dependent recruitment of raft-associated activating receptors, such as NKG2D, to the SMIC (226).
INTERCELLULAR PROTEIN TRANSFER

Intercellular transfer of plasma membrane fragments, protein and surface molecules between cells seems to be a common feature among the cells of the immune system that may be of great importance in the induction and regulation of immune responses (227, 228). This phenomenon is a central theme in this thesis, both in direct studies and as a tool to determine Ly49 receptor accessibility. Imaging of intercellular communication between immune cells and their targets has revealed that there exist a strong correlation between accumulation of receptor/ligand cluster at the IS, intercellular protein transfer and functionally changes of the effector cells (229).

Numerous reports have documented intercellular transfer between players in the innate and adaptive immune system. The original studies about intercellular protein transfer were published between 1970-80, reporting about transfer of B cell Ig, MHC class I and II molecules to T cells (230-235). Not until two decades later, these observations were studied in depth and analysed thoroughly. T cells can acquire MHC class I and II molecules (236), co-stimulatory proteins (41Hwang, 2000 #192) and membrane fragments (237) from APC and endothelial cells. Acquisition of antigen by B cell from targets leads to enhanced processing and presentation to T cells (238). NK cells rapidly acquire MHC class I ligands from surrounding cells, which is followed by down regulation of cell surface expression of corresponding inhibitory receptor (239-242). Moreover, bidirectional protein transfer of MHC class I molecules and inhibitory receptors occurs across the cell-cell contact in inhibitory murine and human NK-target-cell interaction (243). Bidirectional exchange of MICB and NKG2D occurs between NK cells and MICB-expressing target cells (244). Furthermore, MICA is transferred to NK2D-positive NK cells (245).

Mechanisms for intracellular protein transfer

The mechanisms behind intercellular protein transfer are still obscure, but several have been proposed including uprooting, proteolytic cleavage, membrane bridges, trogocytosis, exosomes, spontaneous cellular dissociation or membrane nanotubes (228, 246). Uprooting of protein from the surface membrane, while being ligated to a receptor that pulls away, could be one possible explanation (247). Alternatively, the T and NK cell-activating ligand MIC is enzymatically cleaved from ‘stressed cells’ and blocks its receptor, NKG2D, and hence dampening the NK
and T cell immune response (248). Secretion of exosomes has been demonstrated to deliver proteins between APC and T cells (249, 250). HLA-G molecules are found on melanoma-derived exosomes, which may provide a novel way for tumours to modulate the immune response of the host (251). Trogocytosis (252, 253), also called absorption (254) is a process that allows spontaneous rapid transfer of cell surface proteins and fragments of cell membrane at the synapse (237, 255-257). Membrane bridges are intercellular membrane fusions, seen between interacting CTLs and target cells, facilitating protein transfer (258). More commonly observed are membrane nanotubes, which connect a variety of immune cells (259-263) and provide another possible way of trafficking exchange of proteins. Further investigations and more evidence are necessary to confirm the mechanisms mentioned.

**Functions of intracellular protein transfer**

The biological significance of the intercellular protein transfer between immunological cells is a matter of debate, but different possibilities might be considered, each implementing functional consequences for immune cells. The acquired molecules might be presented to nearby cell to balance immune responses (264, 265). CD4\(^+\) T cells that have acquired peptide-MHC class II complexes can present these to nearest CD4\(^+\) T cells may trigger proliferation or result in anergy and apoptosis, depending on the activation status of the responding CD4\(^+\) T cells (266, 267). CTLs acquire and internalise specific peptide/MHC class I complexes that are degraded and presented at the cell surface, resulting in fratricidal killing (236, 268). Ligand internalisation might interfere in intracellular signalling; causing inhibitory effects on the immune response such as tolerance induction via deletion or anergy (269). Bidirectional exchange of the activating receptor NKG2D and its ligand MICB at the cytotoxic NKIS appears to cause reduction of NKG2D-dependent NK cytotoxicity (244). Additionally, NKG2D-positive NK cells acquire MICA proteins from target cells and present the acquired ligand to neighbouring NKG2D\(^+\) NK cells, which triggers degranulation, indicating a possible occurrence of NK-NK fratricide (245). NK cells that acquire HLA-G1 molecules stop proliferating, are no longer cytotoxic, and behave as suppressor cells capable of inhibiting cytotoxic functions of other NK cells (270). Protein transfer may also provide an instructive role in the recipient cell, establish affinity maturation or allow cellular dissociation (254, 271). Intercellular contacts permits transfer of CD21 onto NK cells, the B cell marker and receptor for the Epstein-Barr virus (EBV), facilitating EBV infection of NK cells (272). Data also support that enveloped particles of HTLV-1 and HIV-1 spread very efficiently through cell-to-cell contact (273-275).

**NK CELL TOLERANCE**

Unresponsiveness to self-antigen, “self-tolerance”, is achieved by educating the lymphocytes of the immune system to actively prevent an immune attack on autologous cells and tissues, but still retain their activity against foreign organisms, substances and transformed cells. To date, the mechanisms behind the development of NK cell tolerance are still under investigation and various models have been introduced where the missing self-hypothesis has been a guiding principle for understanding target cell recognition. A cellular adaptation of the NK cell repertoire to the MHC molecules expressed on the host seems to occur and host MHC class I molecules are the determinants that regulate development of NK cell reactivity and tolerance (276).
Initially, there were at least two possible models that tried to explain the mechanism of MHC class I mediated protection against NK cell killing. “The target interference model” postulated that the MHC molecules prevent a putative NK cell receptor from binding to its real ligand by shielding or masking. Alternatively, “the effector inhibition model” suggested that NK cells that are triggered by activating receptors, adhesion or costimulatory molecules distributed on the target cells, simultaneously receiving negative signals from inhibitory receptors that are able to cancel the activated lytic program after recognition of self-specific MHC class I alleles expressed on the surface of the targets. The “effector inhibition” model, which was eventually proven correct, as several inhibitory receptors were identified both in the murine and human system (19).

Transgenic and MHC class-deficient mice

In order to reveal the mystery of NK tolerance and its relation to MHC class I molecules both transgenic and knockout mice have been used. For example, studies of a B6 (H-2b) mouse transgenic for H-2Dd (the D8 mouse) demonstrated that when the transgene was present, the NK cell rejected the H-2b grafts, a reaction not observed in non-transgenic B6 mice (277, 278). In addition, introduction of H-2Dd in H-2b lymphoma or in bone marrow graft resulted in acceptance in D8 mice (278, 279). From these results it was concluded that upon introduction of H-2Dd transgene NK cell specificity was altered toward missing self-recognition of cells lacking H-2Dd. This suggests that NK cells in D8 mice are taught to request H-2Dd expression in order to save the target from being killed (277, 278, 280).

The β2m-deficient mouse, expressing impaired levels of MHC class I molecules, was another important tool for studies of the influence of MHC class I on NK cell development. In normal β2m+ mice, NK cells rejected cells derived from β2m- mice, emphasising the fact that normal haematopoietic cells express activating ligands for NK cells. Thus, mechanisms responsible for maintaining self-tolerance are crucial to prevent killing of endogenous cells in vivo. However, NK cells in β2m- mice were unable to recognise and kill normal β2m+ cells, which indicated that they had developed self-tolerance independently of MHC class I (279, 281, 282). NK cell tolerance can thus be broken either by interference with class I expression in the graft or by addition of novel MHC class I allele in the host, illustrating the impact of host MHC expression on the NK cell education. On the other hand, NK cells in the β2m- mice showed defects in cytotoxicity and rejection of allogeneic bone marrow grafts (282) However, MHC class I deficient mice contained normal or even elevated numbers of NK cells, indicating that self tolerance in these mice were not a result from NK cell depletion nor failure of NK cell development. It was thus hypothesised that NK cells in the β2m- mice were hyporesponsive or anergic. Studies with the MHC class I-deficient mice showed that self-tolerance of NK cells is not only dependent on inhibitory interactions of NK cells with MHC class I molecules (19, 283, 284) (figure 8).
It was proposed that autoreactivity caused by NK cells could be avoided if all NK cells lacking MHC class I-inhibitory receptors were deleted. This *negative selection* would predict that MHC class I-deficient mice would lack all NK cells or have remarkable alterations in their NK cell receptor repertoire, which is not the case. MHC class I deficient mice carry regular numbers of NK cells and display an intriguingly balanced repertoire of activating and inhibitory receptors, arguing against this idea (279, 281, 282, 285).

"The receptor calibration model"

Our group and others found that MHC class I molecules of the host modulate the expression of Ly49 receptors. A model, regarding NK cell self-tolerance, related to the receptor repertoire or expression levels of MHC class I-specific inhibitory receptors was postulated. The model, previously mentioned, called "The receptor calibration model" proposed that NK cells interact with self and non-self MHC and subsequently adjust their receptor repertoire expressed on the NK cell surface in order to detect modifications of self-MHC. According to this model, MHC class I self downregulation of inhibitory NK cell receptors made the NK cells more sensitive to normal and reduced levels of MHC class I molecules (149, 150). This model is discussed more in the section on Ly49 receptor accessibility earlier in this chapter.

"The at least one model"

Until fairly recently, one of the ideas was that NK cell tolerance arises by ensuring that each NK cell expressed at least one inhibitory receptor specific for self MHC molecules, termed the "at least one model" (21). This model implies a selection against NK cells that fail to
express self-MHC inhibitory receptors or a sequential cumulative expression of additional
receptors until the correct host MHC class I-specific inhibitory receptor is expressed (104, 105, 113, 148, 286).

“The disarming model” vs “The licensing model”

The “at least one model” was refuted when self-tolerant NK cells, lacking self-specific
inhibitory receptors were found in ‘normal’ mice (287, 288), confirming the earlier studies in
the MHC class I deficient mice that demonstrated that the expression of “at least one” self-
MHC class I specific inhibitory receptor, in conjunction with its ligands, is not necessary for
self-tolerance (280, 283). In parallel, two groups performed comparable studies on NK cells
lacking MHC class I-specific inhibitory receptors, although using different experimental
approaches and made similar findings i.e. NK cells lacking self-MHC class I specific inhibitory
receptors are functionally impaired (287, 288). Nevertheless, the interpretations of the
underlying mechanisms, leading to self-tolerance of NK cells, were completely different and
lead to two distinct hypotheses. In the study by Fernandez et al. these self-tolerant NK cell
exhibited a hyporesponsive functional phenotype, including impaired ability to reject target
cells from MHC class I-deficient mice and produce IFN-γ (288), a similar functional phenotype
as seen in MHC class I-deficient NK cells. A very recent published paper shows that
hyporesponsive NK cells are also present in human peripheral blood, lacking self-MHC class I
specific inhibitory receptors (289). However, hyporesponsive NK cells are not necessarily
functional incompetent. Under certain conditions, e.g. in response to MCMV and Listeria
monocytogenes infection, the NK cells become activated and secrete IFN-γ most likely due to
upregulated activating ligands och triggering cytokines (288, 290). Furthermore, it should be
noted that these NK cells display all markers thought to identify fully mature NK cells,
indicating that these cells have reached complete functional maturity. Additionally, normal
expression of activating receptors is found on these NK cells, including NK1.1, NKG2D,
Ly49D and CD16.

The hypothesis presented in Fernandez et al. proposes that developing NK cells, lacking
inhibitory receptors for self MHC, which are exposed to persistent triggering that are not
counterbalanced by inhibitory signals, adopt a hyporesponsive state comparable to anergy of T
or B cells (34, 276, 288). In this current model, termed “The disarming model”, it is proposed
that hyporesponsiveness is a result of alterations in signalling pathways that lead to dampening
of stimulatory signals in the NK cells. The model is based on the assumption that NK cells are
constantly exposed to stimulating ligands expressed on normal autologous cells in the body
(105). The model is supported by previous reported data (291-294).

In contrast, Kim et al. propose that NK cells acquire functional competence through specific
interactions with host MHC class Ia molecules, a process termed “licensing”. The final
outcome from the maturation process, according to “the Licensing model”, would results in an
NK cell repertoire consisting of functionally competent (“Licensed”) and incompetent
(‘unlicensed’) NK cells (287, 295). Yokoyama and colleagues have been presented two
potential models for how self-MHC specific inhibitory receptors, through interaction with host
MHC class I molecules, could “license” NK cells. In “The Stimulatory model” MHC class I-
specific inhibitory receptors trigger the “licensing” process to develop mature functional NK
cells. In contrast, “The Inhibitory model” implies that the MHC class I-specific inhibitory receptors prevent activating signals, which could lead to overstimulation and eventually NK anergy (296). These models were once demonstrated and named as ‘the positive model’ and “the negative model”, respectively, by Wu et al. (293). (The last model is very alike ‘the disarming model’, mentioned and suggested above).

To summarise; in “the licensing model” recognition of MHC class I-positive cells by NK cells is necessary to induce functional NK cell maturation. Without a MHC class I-mediated signal the NK cells remain hyporesponsive i.e. “unlicensed”. In “the disarming model”, NK cells are “armed” unless they interact with cells lacking cognate class I ligands, in which case constant stimulation, lack of inhibitory input from MHC class I molecules, render the NK cells hyporesponsive, i.e. “disarmed” (figure 9). The hyporesonsive state might be reversible through the action of cytokines or by certain stimulatory molecules.

![Figure 9. The disarming model and the licensing model](image)

As a matter of fact, studies performed a couple of years ago attempted to test just these two predictions by examining NK cell development in chimeric (293), mosaic (292) or H-2D\(^d\) transgenic mice wherein the H-2D\(^d\) expression could be extinguished by Cre recombinase (294), in which host cells consisted of a mixture of cells that did or did not express relevant MHC class I ligand. All three groups demonstrated that hematopoietic and non-hematopoietic MHC class I-negative cells could dominantly induce tolerance to this phenotype, suggesting that tolerance is ensured to all present MHC class I phenotypes by continuous NK cell interactions with multiple cells in the environment and appears to be dominantly controlled by the presence of cells lacking a specific MHC class I ligand (291). For example, if H-2D\(^d\)-positive and H-2D\(^d\)-negative spleen cell from the mosaic mice, DL6, were separated and cultured in IL-2, tolerance to H-2D\(^d\)-negative cells was broken in the H-2D\(^d\)-positive population, suggesting reversible adaptation (292). The tolerance process seems to be active during the entire life span of the NK cell and most likely a reversible adaptation to the local
environment and available cytokines in normal, infected and cancer tissues are other factors of impact.

Independently of which self-tolerance models one believes in, the strength of signalling pathways from one or several inhibitory receptors might be relevant for the fate of the developing NK cells. In addition, NK cell tolerance induction may be a quantitative process where frequency of interactions may play an important role as well as receptor-ligand avidity. As indicated in a recent publication from our group NK cells seem to be more or less potent, depending on which MHC allele and the number of alleles present, emphasising ‘the educating impact’ of different MHC class I alleles (297). Moreover, cis interaction may contribute to the signal strength of the inhibitory receptors, the frequency of ligand-receptor interactions and expression level of self MHC class I specific inhibitory receptors on the NK cells (153, 242, 296, 298, 299). The tolerance process seems to be active during the entire life span of the NK cell and may be a reversible continuous adaptation to the local environment. Available cytokines in normal, infected and cancer tissues are other factors of impact.

**NK CELL CANCER THERAPY**

NK cells play an important role in tumour immunosurveillance in mice and humans. The role of NK cells in cancer has been extensively examined and NK cells have been demonstrated to help control tumours and reduce spread of metastases. Since the balance of activating and inhibitory signals determines whether or not a NK cell responds upon encounter with other cells, NK cell-based cancer therapies of today regard both activating stimuli and removal of inhibitory elements. NK strategies have been considered for cancer therapy, which are based on shifting the responses in favour of NK cell activation by increasing activating receptor signals or by blocking inhibitory receptors (300).

NK cells are mainly used in adoptive cell-based therapies due to their migration to tumour sites and persistence in vivo. NK cells may be of particular benefit in blood-borne cancers, such as leukemias/lymphomas, due to the predominance of NK cells in the peripheral blood and spleen. As hematopoietic stem cell transplantation (HSCT) is an important treatment of leukemias and lymphomas, there have been some interesting studies regarding immunotherapy involving NK cells. There is always a risk that the immune system of the recipient rejects the transplant, immunological reactions termed host-versus-graft, HvG. Allogeneic transplants are also associated with a beneficial graft-versus-tumour, GvT, (e.g. graft-versus-leukemia, GvL) effects, but also graft-versus-host-disease, GvHD, caused by donor T cell attacking solid organs. Allogeneic NK cells are able to recognise and kill lympho-hematopoietic cells in HvG and GvH directions. However, NK cell do not harm solid tissues, meaning that NK cells, unlike T cells, can be transferred in an allogeneic setting without risk of initiating GvHD since they do not reject solid tissue allografts, making them attractive for use in HSCT (188, 301-303).
Preclinical mouse models and clinical studies

In pre-clinical murine studies, the administration of activated allogeneic NK cells following BMT has been shown to provide a GVL effect compared to syngeneic NK cell without mediating GvHD (304). NK cells mediate alloreactions when the mismatched allogeneic target cells lack expression of the cognate MHC class I alleles that inhibit their effector functions (‘missing-self’ recognition). Both in vitro and in vivo it has been observed that blockade of NK inhibitory receptors lead to an enhanced anti-tumour activity and survival, while using a murine leukemia model. F(ab')2 fragments anti-Ly49C/I (5E6) have been used to pretreat C57BL/6 (B6) mice injected with syngeneic leukemia cells intravenously (305). Ly49C/I bind to H-2Kb expressed in B6 mice (306). 5E6 F(ab’)2 fragments abrogate the resistance of the H-2Kb-positive targets to Ly49C/I+ B6 NK cells. Thus, blockade of MHC class I-specific inhibitory receptors of allogeneic NK cells promoted increased anti-tumour responses in vitro and in vivo. Blockade of Ly49C and Ly49I receptors on NK cells have been demonstrated to be of potential use in purging of tumour cells prior to autologous BMT (307). Inhibitory receptor blockade of MHC class I-specific syngeneic NK cells have proven to mediate more powerful anti-tumour effects than syngeneic NK cell against tumour cells in vitro and in vivo without abnormal effects.

Clinical studies in vitro on human primary lympho-hematopoietic lineage tumour cells show that alloreactive NK cell kill acute myeloid leukaemia (AML), chronic myeloid leukaemia, chronic lymphocytic leukaemia, non-Hodgkin’s lymphoma and multiple myeloma (308). Furthermore, it seems that in haploidentical (parent to child) HCST in combinations where NK cells from the donor have the potential for missing-self reactivity against the host, NK cells in the donor graft have the capacity to prevent leukemia relapse and the destructive complication of GvH and HvG in leukemia recipients (301). In some preclinical murine models and clinical transplantations, it has been demonstrated that alloreactive NK cells possess the potential capacity to eradicate acute leukaemia, favour engraftment by killing host T cells responsible for graft rejection and reduce GvHD by eliminating host-type DCs so that host antigens are not presented to graft T cells (309-313). Clinical findings show that adoptive transferred human haploidentical NK cells persist and expand in patients (314). Usage of inhibitory blockade or sorted NK cell subsets, lacking specific inhibitory receptors, seems to be a potential immunotherapy for treatment of cancer (303).
AIMS OF THE THESIS

Overall, my thesis is focused on accessibility of inhibitory NK cell receptor and its impact on NK cell effector functions after interactions with MHC class I-expressing target cells.

The specific aims during the studies were:

I. To illustrate and investigate bidirectional transfer of inhibitory receptors and cognate MHC class I ligands across the human and murine inhibitory NK cell synapse

II. To characterise phenotypical and functional consequences of MHC class I transfer from target cells to NK cells, occurring at the murine inhibitory NK cell synapse

III. To quantify the accessibility of Ly49A receptors expressed on NK cells by using an established intercellular protein transfer assay

IV. To study whether *in vivo* blocking of inhibitory NK cell receptors Ly49C/I could induce rejection of MHC class I expressing tumours without breaking tolerance towards normal syngeneic cells
RESULTS AND DISCUSSION

PAPER I

Bidirectional transfer

Background

Inhibitory NK receptor-dependent transfer of MHC class I molecules from target cells to murine NK cells had been reported during NK cell interactions with surrounding cells, both in vitro and in vivo (239, 241). In parallel experiments in humans, HLA-C molecules were demonstrated to transfer from target cells to human NK cell plasma membranes, and further into the cytoplasm, after accumulation of target cell HLA-C at the inhibitory NK cell immune synapse (240). As in the mouse, HLA-C transfer was dependent on MHC class I-specific NK cell receptor recognition, since HLA-Cw6 and -Cw4, but not -Cw3, transferred to KIR2DL1-expressing NK transfectants (240).

In paper I, we demonstrate bidirectional intercellular protein transfer of inhibitory NK cell receptors and their cognate MHC class I molecules across the human and murine inhibitory NK cell synapse (243).

Multiple proteins transfer from NK cells to target cells during inhibitory interactions

As a start of paper I, we took a general approach and simply asked if proteins in general could transfer from NK cells to target cells. For this, we developed a novel assay based on biotinylation of NK cell surface proteins before co-culture. After co-culture, target cells were probed using streptavidin to reveal all possible biotinylated proteins that had travelled across. Various proteins were transferred to target cells during inhibitory interactions with NK cells, but no sharing of cytoplasm between the cells was identified. Importantly, acquired biotinylated proteins remained detectable in the target cell up to 6 days after separation from NK cells, proposing that target cells could possibly remain influenced by these molecules for a period of time.

KIR2DL1 and Ly49A transfer onto target cells expressing cognate MHC I ligands

Among the proteins that transferred were inhibitory receptors KIR2DL1 (human) and Ly49A (mouse). A critical question that we asked was whether this transfer was specific. This was important to address since Tabiasco et al. and other groups had suggested that NK cells actively capture target cell membrane fragments (255), leaving open the possibility that protein transfer could be, at least to some extent, unspecific. In addition, prior investigation showed that activated CD8+ T cells could absorb unrelated molecules, such as MHC class II ligands, along with specific transfer of MHC class I molecules (315), and the synaptic transfers mediated by γδ T or CTLs cells involved target molecules unrelated to the cognate Ag and occurred independently of MHC class I expression by target cells (237, 316). One suggested transfer mechanism that could explain unspecific transfer is trogocytosis, a process in which membrane patches containing many proteins transfer between cells following immune synapse formation.
Consequently, during trogocytosis all molecules enclosed within a certain membrane area are transferred from one cell to another, including some that do not participate in the cell-to-cell crosstalk (317).

In our system, the KIR2DL1 receptor transferred only onto target cells expressing HLA-Cw6, a cognate MHC class I ligand for KIR2DL1, but not to target cells expressing HLA-C alleles to which KIR2DL1 did not bind. Similarly, the murine inhibitory Ly49A receptor transferred only to target cell expressing the cognate MHC class I ligand, H-2D\(^d\), but not to H-2D\(^d\)-negative cells (figure 10). Thus, there is clearly a specific component in KIR/Ly49A transfer that was dependent on MHC class I interactions. However, it was also clear from our data that multiple other proteins in addition to KIR were transferred from human NK cells to target cells. The effort to clarify their identity remains as does the question of whether or not transfer of those proteins were dependent on other specific receptors or occurred in a nonspecific manner during the KIR/MHC class I interaction. Most likely, protein transfer between cells includes both specific and non-specific events. As to the mechanisms responsible for NK receptor transfer, they are unclear to us at present. Apart from trogocytosis, other possible intercellular mechanisms such as uprooting, proteolytic cleavage, membrane bridges, exosomes, spontaneous cellular dissociation or membrane nanotubes have been proposed. (I have summarised them in the introduction). To date, none of these mechanisms can be excluded, except perhaps proteolytic cleavage since the entire KIR2DL1 protein, including the cytoplasmic part, was transferred. It is also unclear whether this bidirectional transfer takes place within in a single area of contact or via the formation of separate structure where membrane traffic follows in parallel in opposite directions.

![Bright field Ly49A GFP Overlay](Bright field Ly49A GFP Overlay)

**Figure 10.** Transfer of Ly49A receptors from the NK cell (red) to the H-2D\(^d\)-GFP expressing target cell (green) at the inhibitory NK IS. The red dots, on the target cell, are transferred clusters of Ly49A receptors. Coexpression of Ly49A and H-2D\(^d\) at the synapse (yellow). (Made by Bruno Vanherbergen)

The amount of acquired KIR2DL1 correlates with the level of MHC class I expression on the target cells.

The quantity of KIR2DL1 receptor that transferred correlated positively with the MHC class I expression level on the target cells, but not with expression of MHC class II molecules, CD53 or intercellular adhesion molecule (ICAM)-1, indicating the KIR2DL1 transfer is MHC class I specific and that its extent is quantitatively controlled by the target cell. The strength of receptor-ligand interaction could be a feasible explanation. Among all interacting pairs, the KIR2DL1/HLA-Cw6 interaction could presumably be the strongest. Previously, it has been
revealed that the amount of acquisition of CD80 by T cells was shown to be directly related to both the strength of peptide-MHC (pMHC) interaction and the amount of CD80 on the APC (318). Furthermore, Hudbrisier et al. claims in a recent published paper that different final functions of CTLs vary in proportion to the quantity of captured target cell components, such pMHC complexes and costimulatory molecules (227).

The cytoskeleton of the target cell influences the intercellular transfer of proteins

Treatment with cytoskeleton inhibitors demonstrated that the cytoskeleton of the target cell influenced transfer of proteins in both directions. KIR2DL1 transfer was also reduced by inhibiting active actin polymerisation in NK cells. In contrast, intercellular transfer of HLA-Cw6 from target cells to NK cells was not dependent on polymerisation of the actin cytoskeleton in NK cells, which confirms a preceding study (240). Interestingly, treating the target cell actually increased transfer of HLA-Cw6 to NK cells. This surprising finding suggests a more active role for the target cell in the NK/target cross-talk than simply as a victim of the NK cell attack. Our results on the role of the cytoskeleton are supported by published data suggesting that the actin cytoskeleton plays a key role in the stability of conjugates with either sensitive or resistant target cell and regulates the rate at which KIR clusters at the inhibitory NK cell synapse (39). In fact, instability of the conjugate with a HLA-Cw6-expressing target cell and slow accumulation of KIR2DL1 receptors at the synapse, a critical step, would in all probability reduce KIR2DL1 transfer.

Orientation of the transferred KIR molecules and phosphotyrosine colocalisation

The acquired KIR molecules were easily removed by a slight acid wash, whereas endogenous KIRs were not, indicating that the orientation of the transferred KIR molecules on the cell surface of the target cell is distinctive from the endogenously expressed KIRs. Acid wash has been demonstrated to disrupt cell surface proteins complexes resulting from associations of individual polypeptides, such as monoclonal antibodies bound to antigen (319), peptide and β2m complexes with the MHC class I heavy chain (320) and inhibitory Ly49 receptors bound to MHC class I in cis at the NK cell membrane (153, 242, 299, 321). Thus, one interesting possibility would be that acquired KIR receptors would exist in complex with another protein in cis, e.g. MHC class I. Disruption of this complex would then result in shedding of the acquired KIR receptors from the target cell surface and loss of fluorescence. However, another explanation could be quenching of extracellular GFP by exposure to low pH, resulting in decreased KIR2DL1 fluorescence (322), which seems to be the case since the following antibody stainings revealed that the extracellular portion of the transferred receptors was still accessible. Together, whether the reduced GFP signal is caused by acid treatment, different membrane anchoring, a cis interaction with MHC class I or a changed conformation of the acquired KIR molecules is not clarified. Intriguingly, the acquired KIR receptor colocalises with cotransferred phosphotyrosine, implying that the transferred receptors might be able to signal after transfer. The interesting questions remain to be answered whether the signalling molecules coassociated with transferred KIR receptors retain their capacity after transfer, which signalling molecules they would sequester in the target and what possible functional affects they would have.
Potential functional consequences and biological significances of bidirectional transfer

Transfer of NK cell receptor

It is not obvious to see the logical explanation for transfer of inhibitory NK cell receptors to target cells. In an inhibitory NK cell encounter, both the target and NK cell dissociate alive from the interaction. One speculative role of inhibitory NK cell receptor transfer would be “tagging” of target cells, i.e. the NK cell leaves an “NK trace” in the target cell membrane. A possible reason for such “tagging” could be to reduce the need for repeated scanning of same normal target cell by several NK cells and in this manner optimise immune surveillance by NK cells. How subsequent NK cells encountering a “tagged” target cell would interpret the “tagging” in molecular terms remains and interesting issue to work out.

Recent evidence has demonstrated bidirectional intercellular transfer of NKG2D and MICB proteins at the eNKIS (244). The intercellular exchange of MICB molecules to NK cells and NKG2D receptors to target cells resulted in reduced NKG2D-dependent cytotoxicity. The entire MICB protein was acquired and colocalised with NKG2D at the cell surface of the NK cell. The fate of the colocalised proteins was not clarified, but a number of suggestions have been put forward regarding how transferred MICB to NK cells may affect NKG2D function. One possibility is that MICB acquisition could lead to internalisation and degradation of NKG2D. However, cluster of these molecules have not been detected intracellularly. Another option could be that acquired MICA molecules would physically mask the extracellular part of NKG2D, e.g. forming a potential cis interaction, on the NK cells and thereby reduce the accessibility of the NKG2D receptors for ligands displayed on other surrounding cells, which could subsequently lead to decreased cytotoxicity capacity (229, 244).

Figure 11. Acquisition of H-2D\(^{d}\) ligands (green dots) by Ly49A\(^{-}\) NK cells from H-2D\(^{d}\)-GFP – expressing target cells (green).

Transfer of MHC class I molecules

In contrast to transfer of inhibitory receptors to target cells, transfer of MHC class I molecules in the opposite direction is more easily understood in terms of potential functions (figure 11). In paper II, we present evidence to suggest that acquired MHC class I molecules are able to mask the inhibitory NK receptor Ly49A by forming an interaction with this receptor in cis. This consequence of transfer would results in decreased accessibility of the Ly49A receptor to MHC class I ligands on surrounding cells and consequently enhanced probability to kill target cells. A speculative notion is that such a mechanism is part of an NK cell’s adaptation to the MHC class I environment, leading to an NK cell that is better tuned and optimised to changes in the status.
of inhibitory or activating ligands on potential target cells. The functional and biological consequences of MHC class I transfer was dependent on the activation status of the cell and is also most likely influenced by which ligand-receptor pair that is involved, which cytokines that are present, the surrounding environment and the activity of the adhesion molecules expressed on the cells participating in the interaction.

Another potentially interesting option is that NK cells might act as antigen-presenting cells (APC), presenting the peptides that are associated with their acquired MHC class I molecules to T cells. Recent published data indicate that CD4⁺ T cells can acquire peptide/MHC complexes and costimulatory molecules, such as CD54 and CD80 (B7-1) from syngeneic APCs, and by this acquire the potential to act as potent APCs for unprimed T cells (323, 324). Upon CD80 acquisition, the T cells sustain their proliferative response in the absence of APCs and the acquired costimulatory molecule CD80 seems to be functionally involved in subsequent T to T cell interactions and might play a role in the immunoregulation of T cell responses (325). By capturing the "antigen presentasome" (APS; eg, antigen-presenting complex) from APCs, these naive T cells thereby release the constraint of APC requirement for further activation (266, 318). Conceivably, the APS, including constimulatory molecules, together with the MHC class I molecules might be transferred to NK cells, which would give them APC properties. In fact, direct interactions between NK and T cells have been observed, suggesting that NK cells possess APC-like properties and have the ability to directly regulate T cell activation (326).

Finally, Rajagopalan and colleagues recently demonstrated that stimulation of resting NK cells by soluble HLA-G molecules, which get endocytosed by KIR2DL4, inducing NK activity by this means secretion of cytokines and chemokines, indicating that HLA-G transfer might be a likely mechanism of activation (327). In contradiction, it has been observed that HLA-G molecules transfer from tumour cells to activated NK cells, which results in termination of proliferation and cytotoxicity. On top, the NK cells start behaving as suppressor cells capable of inhibiting cytotoxic functions of other cells, implying that HLA-G transfer could be a potential mechanism of immune escape (270). These contradictory data suggests that transfer is both activating and inhibitory, depending on the present status of the NK cells and circumstances, such as transformation and infection.

PAPER II

Intercellular MHC class I transfer

Background

As described in paper I and paper II and previously in (239-241), murine and human NK cells have the ability to acquire cognate MHC class I molecules from target cells across the inhibitory NKIS and subsequently express the transferred molecules on the cell membrane. In an in vivo study, using an adoptive transfer model, it was shown that H-2D<sup>d</sup>-negative NK cells, with high expression of Ly49A, acquired H-2D<sup>d</sup> from surrounding cells when they were transferred to H-2D<sup>d</sup>-positive mice. In addition, when the rat NK cell line RNK-16 transfected with Ly49A genes were mixed with H-2D<sup>d</sup>-expressing cells in vitro H-2D<sup>d</sup> molecules were acquired by the
NK cells, confirming the in vivo data. Further analysis demonstrated that Ly49C-positive NK cells also acquired their cognate MHC class I ligands, H-2Kb (239).

In paper II we investigated the MHC class I transfer onto Ly49A-positive NK cells and assessed the functional and phenotypical consequences of the transfer.

In vitro transfer assays

To facilitate investigations concerning MHC class I transfer between target cells and NK cells and its consequences and mechanisms, we established three different in vitro systems. First, EL4 tumour cells expressing GFP-tagged H-2Dd molecules were generated (Figure 12). The cells were called EL4Dd-GFP cells and were also used in paper I and III. To confirm the biological integrity of the fusion molecule, the ability of the H-2Dd-GFP molecules to inhibit NK cell lysis upon interaction with Ly49A+ NK cells was verified. We also demonstrated that the staining of extracellular H-2Dd molecules correlated with the intensity of GFP fluorescence. Mixing naïve Ly49A+ NK cells and EL4Dd-GFP cells resulted in successful transfer of H-2Dd-GFP molecules to the NK cells, which was abrogated when an anti-Ly49A antibody was added to the coculture. The entire H-2Dd-GFP fusion protein was incorporated into the cell membrane of the NK cell, including the intracellular part where the GFP gene was tagged. Importantly, all GFP+ NK cells also stained positive for H-2Dd, excluding the possibility that only the GFP molecule transferred. Thus, EL4Dd-GFP cells provided a reliable measurement of the H-2Dd expression and were qualified to be suitable for further MHC class I transfer studies. Furthermore, two additional in vitro systems were established, including one based on bone marrow stroma cells, supporting maturation of NK cells from immature precursors (286). After coinubcation with bone marrow stroma, acquired MHC class I ligands were detected on the NK cells. The MHC class I acquisition was blocked by adding an anti-H-2Dd antibody (HB102) to the coculture. In the third system, Con A-activated lymphoblasts were cocultured with naïve NK cells, resulting in efficient acquisition of H-2Dd molecules. (146, 150, 152). Furthermore, Ly49A-positive NK cells could also acquire their weaker cognate H-2Dk ligands (147, 328, 329), but to less extent and with reduced efficiency than they acquired H-2Dd molecules. Both H-2Dd and H-2Dk are cognate ligands to Ly49A, of which the interaction with H-2Dd is the strongest (330). Furthermore, the strength of binding between Ly49A and the two different alleles correlates with the degree of functional inhibition mediated by the receptor (331). The decreased amount of H-2Dk transfer could be explained by the weaker binding between Ly49A and H-2Dk compared to the strong Ly49A-H-2Dd interaction. This finding supports the results in paper III, which demonstrate that MHC class I transfer reflects the strength of interaction between NK cells and target cells.
Kinetics of H-2D\textsuperscript{d} transfer and Ly49A downmodulation

A large body of evidence has been generated that suggest that host MHC class I molecules have been suggested to be responsible for modulating the expression of MHC class I-specific NK cell receptor (146, 147, 150, 152). “The receptor calibration model” proposed that NK cells interact with self and non-self MHC in the present milieu and adapt the receptor repertoire to sense changes of self-MHC class I expression. The modulation of receptor expression has been described in more detail in the introduction. Briefly, down-regulation of Ly49A receptors expression level has previously been reported to occur as a rapid adaptation process post interaction with surrounding H-2D\textsuperscript{d} ligands both in vitro and in vivo (239:Kase, 1998 #5). The downmodulation of the level of receptors have been explained by two possible biological mechanisms. One is absolute lower levels of the receptors on the NK cells and the second one implies that the accessibility of receptors is modulated by a cell-autonomous \textit{cis} interaction formed between the receptors and the endogenously expressed MHC class I ligands on the NK cells (153, 242, 299). (The \textit{cis} interaction is more thoroughly described the introduction). To find out the potential relation between ligand transfer and downmodulation we performed kinetic transfer experiments. Using either the assay based on EL4D\textsuperscript{d}-GFP or H-2D\textsuperscript{d}-positive lymphoblasts, we observed rapid transfer already after a couple of minutes in parallel with Ly49A downregulation. After 30 minutes the maximum value of H-2D\textsuperscript{d} acquisition was reached and the reduced level of Ly49A expression was stabilised. To elucidate whether \textit{cis} interactions were involved in the downmodulation the cells were exposed to an acid wash treatment, pH 3.3, in order to disrupt the \textit{cis} interaction and alter the conformation of the MHC class I molecules (320, 332). Interestingly, the level of Ly49A receptor expression was restored, suggesting the downregulation was due to a \textit{cis}-interaction formed between the acquired MHC class I molecules and the Ly49A receptors (figure 13). This finding might implicate that Ly49A-positive NK cells, while circulating in the body, acquire and bind cognate H-2D\textsuperscript{d} ligands in \textit{cis}. The \textit{cis} interaction modulates the Ly49A expression by reducing the accessibility to \textit{trans} interactions, which decreases the sensitive to inhibition of the NK cells. This renders the NK cells more sensitive activating ligands, induced by transformation or infection, on H-2D\textsuperscript{d} –expressing target cells and by this means distinguish abnormal host cells from normal.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure13.png}
\caption{Cocultured NK cells showed reduced levels of Ly49A receptors, which were restored completely after low pH exposure.}
\end{figure}
How is it possible for Ly49A to interact in both cis and trans with H-2D^d?

Dam et al. recently presented evidence to suggest that Ly49A exist in a dynamic equilibrium between a ‘closed’ form, allowing engagement with only one MHC class I molecule, and an ‘open’ form, which permits bivalent binding (figure 14). They proposed that the ‘closed’ state mediates a cis interaction with MHC class I, whereas the predominantly ‘open’ state would mediate a trans interactions. The presence of along stalk in the Ly49A molecule would make this doable by allowing a back-folded conformation to bind MHC class I in cis (closed) and extended conformation to bind in trans (open). Proposed factors that determine the state of the Ly49A receptors could be local conditions, such as cytokines milieu and pH, conformation of the stalk and interactions with other proteins on the NK cell membrane (333). In contrast, data from a recent paper provide evidence that Ly49A/H-2D^d cis interactions remain stable upon target cell recognition and trans H-2D^d ligands can not easily compete with cis H-2D^d ligands. This data is supported by binding of soluble multimers to H-2D^d expressing Ly49A-positive cells and limited recruit of Ly49A receptors and H-2D^d to the iNKIS. This finding indicates that the backfolded Ly49A conformation is relatively more stable and in favour of rebinding in cis rather than in trans (321).

![Figure 14. Model for cis and trans interaction between Ly49A with H-2D^d molecules. In the cis interaction (left), the “closed” Ly49A dimer binds one H-2D^d molecule. In the trans interaction (right), the “open” Ly49A dimer binds two H-2D^d molecules.](image)

**H-2D^d transfer causes less sensitivity to H-2D^d-mediated inhibition**

To assess if the killing capacity of the NK was affected by the H-2D^d transfer, NK cells were coincubated on H-2D^d-positive (D8) or H-2D^d-negative (B6) bone marrow stroma cells and following challenged against H-2D^d- or H-2D^d-expressing tumour cells. NK cells cocultured on D8, and by this had acquired H-2D^d molecules, killed YB2/0D^d (YB2/0 cells transfected with H-2D^d), and YAC-1 cells (displaying low levels of H-2D^d) whereas NK cells coincubated on B6 did not (figure 15). These results imply that the created cis interactions with the acquired H-2D^d ligands reduced the threshold at which NK cell activation exceeds inhibition by modulating accessibility of the Ly49A receptor. This finding was substantiated by a reduction of Ly49A expression by 50 % after H-2D^d acquisition, consistent with a blocking effect on antibody epitopes by the cis interaction with H-2D^d. Thus, these results demonstrate a clear and logical effect of acquired H-2D^d molecules that also imply a possible
regulatory role for MHC class I transfer to NK cells. Moreover, when tumour cells lacking H-2D\textsuperscript{d} expression were used no difference in killing was observed between NK cells cocultured on B6 or D8 stroma cells.

According to previous data reported by Sjöström et al., in contrast to our present results, H-2D\textsuperscript{d} transfer did not abrogate the ability of rat RNK16 cells transfected with Ly49A\textsuperscript{T} to receive inhibitory signals from YB2/0-D\textsuperscript{d} target cells (239). Furthermore, acquisition of H-2D\textsuperscript{d} by these NK cells was accompanied by a partial inactivation of cytotoxic activity against H-2D\textsuperscript{d}-negative target cells. One plausible reason for the discrepancy between our present study and our previous work might be that H-2D\textsuperscript{d} molecules fail to associate in cis with Ly49A at the cell surface of the rat RNK16 transfectants. Possibly, various molecules, including chaperons and glycosylations, might be involved in forming the critical cis interaction at the cell surface. A possibility is that some such factors are missing on the cell membrane of the rat RNK16 transfectants, precluding the cis interaction to occur. Alternatively, the mouse Ly49A receptor itself may not be processed and expressed properly in RNK-16 cells, since it is in fact a rat cell line, which could also affect the capacity of Ly49A to bind H-2D\textsuperscript{d} in cis. Finally, it is also possible that the H-2D\textsuperscript{d} molecule would behave differently at the surface of rat cells, perhaps due to the potential association with rat β\textsubscript{2}m, which could potentially affect the cis interactions with Ly49A. A role for β\textsubscript{2}m has been demonstrated in Ly49/MHC class I binding (334, 335). The testing of these interesting possibilities require further work and is important since it can provide important insights into the regulation of the cis interaction and indirectly of Ly49 receptor accessibility.

**Figure 15.** Reduced sensitivity to inhibition. NK cells that had acquired endogenous H-2D\textsuperscript{d} molecules killed H-2D\textsuperscript{d} targets more efficiently suggesting that acquired H-2D\textsuperscript{d} molecules masked Ly49A receptor epitopes by interacting in cis and prevented them from interacting with target cell H-2D\textsuperscript{d}.

**IL-2-activated NK cells acquire less H-2D\textsuperscript{d} molecules than naive NK cells**

In order to figure out whether the amount of transfer changed upon NK cell activation, we compared the efficiency of H-2D\textsuperscript{d} transfer to naive or IL-2-activated NK cells. The amount of H-2D\textsuperscript{d} molecules transferred was indeed influenced by the activation status of the NK cells. The degree of H-2D\textsuperscript{d} transfer was reduced after NK cell activation in comparison to the naive NK cells, rendering the activated NK cells more sensitive to inhibition. Though, the most obvious would be augmented extent of H-2D\textsuperscript{d} transfer since the acquired H-2D\textsuperscript{d} molecules probably bind Ly49A receptors in cis, not allowing binding in trans, which means keeping the Ly49A receptor in “closed” conformation, resulting in less sensitivity to inhibitory signals. Nevertheless, the activated NK cells may be require more inhibitory input to retain their tolerance towards normal cells, which also display activating ligands but to less extent than
the tumour cells. Furthermore, the IL-2 activated NK cells may have another composition of extracellular membrane proteins or be regulated differently than naïve cells.

**Coexpression of several Ly49 receptors and MHC Class I Ligands**

Hanke and colleagues revealed that multiple Ly49 receptor interactions have a cumulative impact on the NK cell development and effector activity (336). Receptor combinations and the MHC class I milieu seem to have significant effects on NK cell tolerance and functional inhibition. We wondered what would occur when a NK cell equipped with both Ly49A and Ly49C receptors encounter a target cell expressing both H-2D\textsuperscript{d} and H-2K\textsuperscript{b} ligands. To address this question we decided to utilise MHC class I deficient NK cells from Kb-/-Db-/- that have been shown to display high expression levels of both Ly49A and Ly49C receptors (337, 338), providing a system to study the behaviour of coexpression of Ly49A and Ly49C receptors on the same cell. Bone marrow stroma cells of MHC class I-deficient mice, B6 (H-2\textsuperscript{b}+), H-2D\textsuperscript{d}-single mice or D8 mice, expressing both H-2D\textsuperscript{d} and H-2K\textsuperscript{b}, were used as donor cells.

It has been proven earlier that Ly49C\textsuperscript{+} NK cells are able to acquire their high affinity MHC class I ligands, H-2K\textsuperscript{b} molecules. The H-2K\textsuperscript{b} transfer is followed by rapid Ly49C receptor downregulation, in similar manner to what has previously been described for the Ly49A/H-2D\textsuperscript{d} receptor-ligand pair (239). Recent evidence established that the Ly49C receptors are able to bind and form \textit{cis} interactions with endogenously expressed H-2K\textsuperscript{b} on the NK cell membrane (299). The question whether or not Ly49C binds in \textit{cis} to H-2K\textsuperscript{b} ligand after acquisition from target cells remains unsolved.

As expected, Ly49A\textsuperscript{+}C\textsuperscript{-} cells were able to acquire H-2D\textsuperscript{d} molecules from both H-2D\textsuperscript{d}-single positive and D8 stroma cells. Ly49A\textsuperscript{+}C\textsuperscript{+} cells acquired H-2D\textsuperscript{d} molecules from H-2D\textsuperscript{d}-single positive but unpredictably acquired increased amounts of H-2D\textsuperscript{d} after coincubation on D8 stroma, where both the H-2D\textsuperscript{d} and H-2K\textsuperscript{b} ligands were present. Noteworthy, it is known that Ly49C can bind weakly to H-2D\textsuperscript{d} (331). The presence of Ly49C could be one explanation for augmented H-2D\textsuperscript{d} transfer since both receptors might bind to H-2D\textsuperscript{d} ligands and mediate transfer. Nonetheless, if this was the only reason, enhanced H-2D\textsuperscript{d} transfer would have also been detected by Ly49A\textsuperscript{+}C\textsuperscript{-} cells after culture on H-2D\textsuperscript{d}-single positive stroma cells. Occurrence of another Ly49/MHC class I receptor-ligand pair, promoting a cumulative effect on present transfer procedure, is an alternative explanation. Moreover, an additional receptor-ligand couple present possibly leads to a prolonged conjugate or synapse formation with higher avidity. This model would explain why Ly49A\textsuperscript{+}C\textsuperscript{+} cells were able to acquire H-2D\textsuperscript{d} molecules, but only in the presence of H-2K\textsuperscript{b} molecules. The weak affinity interaction between Ly49C and H-2D\textsuperscript{d} might be sufficient to attract H-2D\textsuperscript{d} molecules to an already existing synapse, formed by the Ly49C/H-2K\textsuperscript{b} interaction but insufficient by itself to initiate recruitment of molecules to form a synapse, indicated by the absence of H-2D\textsuperscript{d} acquisition by Ly49A\textsuperscript{+}C\textsuperscript{-} cells when H-2K\textsuperscript{b} is absent.

**NK-NK intercellular communication**

We found that Ly49A receptors and H-2D\textsuperscript{d} molecules had formed clusters at the contact site between the NK cells as well as transfer of H-2D\textsuperscript{d} molecules were detected on the NK cell that had not been in contact with the target cell. Direct cell-cell interaction and transfer
between two NK cells may well be an approach of the NK cells to adjust to the present environment they encounter. Another option might be that NK cells act as APC and re-present the cells for neighbouring cells. Nevertheless, to verify these speculations further experiments have to be executed. NK-NK cellular communication has recently been demonstrated when the NKG2D ligand MICA was transferred, after accumulation of MICA at the cNKIS, from target cells to NK cells and could later be seen to be exchanged between the NK cells. The acquired MICA molecules were observed to engage NKG2D receptors on neighbouring NK cells, triggering NK cell degranulation (245). Thus, NK cells can possibly influence other NK cells with proteins captured from target cells suggesting that NK cells could lyse other NK cells, NK-NK fratricide, upon recognition of activating ligands acquired from target cells. This mechanism could constitute an important function for immunoregulation of NK cell activity (245).

Intercellular ligand transfer and cell-cell interactions between same types of immune cells might have feedback inhibitory effects on the immune response, such as suppression via fratricide killing or anergy (339). Fratricide has previously been described as an immunoregulation mechanism of T cell responses induced by high viral load. At high antigen concentrations, T cell mediated adsorption of peptide-MHC complexes from APCs. Reappearance of the APC-derived peptide-MHC complexes rendered T cells susceptible to fratricidal lysis by neighbouring CTLs (236). Furthermore, it was demonstrated that regulatory T cells were able to acquire alloantigen from APC, present the alloantigen to activated syngeneic CD8\(^+\) T cells and at that moment send death signals to CD8\(^+\) T cells (269).

Furthermore, in a recent publication it was demonstrated that NK cells, acquiring HLA-G1 from tumour cells immediately stopped proliferating, lost their cytotoxic capacity and behaved as suppressor cells capable of inhibiting cytotoxic function of others NK cells (270). In addition, similar observations were seen among CD4\(^+\) and CD8\(^+\) T cells. The T cells could acquire HLA-G1 from APCs and instantly reverse function from effectors to new type of regulatory cells (340). Transfer of HLA-G from tumour cells to T cells or NK cells might be a mechanism of immune escape for tumour cells. A related immune escape mechanism could be the reason for the decreased NK cell cytotoxicity after H-2D\(^d\) transfer to Ly49A\(^-\) RNK-16 cells previously reported (239). Thus, acquired H-2D\(^d\) ligands from the tumour cells may cause a kind of NK cell anergy, as described above, impeding the proliferation and hampering the cytotoxic capacity.

The communication through intracellular protein transfer between NK cells and other cells may play an essential role for a variety of reasons. The transferred H-2D\(^d\) molecules may fine-tune, through the cis interactions, the accessibility of inhibitory Ly49A receptor and in this manner regulate the central developmental processes or peripheral tolerance mechanisms. Transfer of molecules could render the activated mature immune cell from being a potent effector cell to a regulatory suppressor cell, suggesting that intercellular protein transfer might be an immune escape mechanism. Likewise, captured stimulatory ligands from target cells may render the immune cells susceptible to fratricidal lysis.
Reduction in accessibility of the Ly49A receptors

Background

NK cell education and specificity appears to be under constant adaptation, rather than a permanent feature established early through a developmental adaptation and selection step. Supporting this, the level of Ly49 receptor expression of mature NK cells can be modified, principally depending on the MHC class I environment the NK cells are exposed to. Thus, calibration of receptors aligned with surrounding host MHC class I ligands may be a continuous process that occurs throughout the life span of the NK cells. The NK cell activity is mainly regulated by the strength of the inhibitory signalling upon host MHC class I encountering, which depends on the number, affinity and accessibility of inhibitory receptor levels (292, 297).

Cell-autonomous cis interaction

Endogenous expression of MHC class I molecules displayed on the NK cells have according to previous studies an important function in the regulation of inhibitory receptor levels. Prior data demonstrated that H-2D^d+ NK cells, called B6.D^d in paper III, expressed lower Ly49A levels (145-147, 152, 341) and were less sensitive to inhibition than NK cells from H-2D^d-negative mice, called B6 in paper III (146, 149, 153, 292). One explanation for such reduced receptor accessibility could be the absolute levels of the Ly49A receptors at the NK cell surface. A second explanation might be the formations of cell-autonomous interactions in cis between the Ly49A receptors and the endogenous MHC class I ligands, H-2D^d, in the NK cell membrane itself. Such a potential cis interaction was initially predicted by Kåse et al., who showed that H-2D^d molecules on the NK cell itself led to sustained low Ly49A expression upon cellular activation while H-2D^d-negative NK cells that had downregulated Ly49A due to interactions with H-2D^d on surrounding cells rapidly upregulated Ly49A expression during similar culture conditions (152). Tormo and colleagues were the first to demonstrate, from the crystal structure, that the Ly49A dimer could interact with two H-2D^d molecules at distinct sites and suggested an interface where an interaction between Ly49A and MHC-I may occur on the NK cell itself, i.e. the cis-interaction (121). Doucey and colleagues were the first to deliver biochemical proof for the formation of a cis interaction with reduced sensitivity to inhibition as a consequence (153). Recent findings indicate that the accessibility of other MHC I-binding Ly49 receptors can also be modulated by the interaction with cognate MHC class I molecules in cis, e.g. Ly49C (299).

The two explanations (absolute lower cell surface levels and a masking cis interaction with MHC class I), would not be mutually exclusive but could work in parallel and thus cooperate to reduce the sensitivity of NK cells to target cell MHC class I.

Structural biology of the cis interaction

The x-ray crystal structure of the Ly49A.H-2D^d complex revealed that homodimeric Ly49A interacts at two distinct sites of H-2D^d: At site 1, Ly49A interacts with amino and carboxy terminal residues of the α_1 and α_2 chains of H-2D^d. Site 2, the binding site for CD8 and responsible for mediating NK cell inhibition (binding in trans), is located beneath the peptide-
binding groove, making contacts with residues of the $\alpha_2$, $\alpha_3$ and $\beta_2m$ domains (155, 156). Furthermore, it was shown that cis and trans interactions in fact use the same binding site, which is site 2, and that simultaneous binding in cis and trans is almost certainly excluded (153). More recently two states: a "closed state" and an "open state" for Ly49A have been proposed. Ly49A dimer can engage two MHC molecules, in cis and trans, showing that Ly49A exists predominantly in the ‘open’ state (333).

**Protein transfer assay as a tool**

The fact that an interaction in cis, in the NK cell membrane itself, between Ly49A and H-2D$^d$ could potentially regulate NK cell inhibition was very intriguing. To understand the impact of such a regulation, it was then important to clarify the magnitude of this cis interaction on Ly49A receptor function. For this, we needed a reliable and simple assay that could be used, quantitatively, to measure “accessibility” of Ly49 receptors at NK cell surfaces. Previous assays such as real killing experiments or binding of soluble MHC class I multimers to NK cells both have their limitations. For example, killing assays give a result that can not easily be interpreted in quantitative terms. Soluble MHC class I multimers represent a nice tool but one can not be sure that binding of such soluble MHC class I proteins to Ly49 receptors represents the binding characteristics of real NK cells to target cell expressing the same molecules in membrane-bound forms. We therefore sought to develop another type of assay that could be used to obtain quantitative measurements of Ly49A receptor accessibility and that was also dependent upon cell-cell contact. Intercellular protein transfer was found to fulfil those criteria.

As we demonstrated in **paper I**, Ly49A-positive NK cells are able to acquire H-2D$^d$ molecules from surrounding cells. In **paper II**, we demonstrated Ly49A-dependent intercellular transfer of GFP-tagged H-2D$^d$ molecules. Importantly, we found a linear relationship between the accessibility of Ly49A receptors and H-2D$^d$ transfer in this model. We thus used this assay to estimate the accessibility of the Ly49A receptors on NK cells, and hence to measure quantitatively the capacity of Ly49A receptors to interact with MHC class I molecules in trans.

**Reduced Ly49A inhibition in the presence of endogenous H-2D$^d$**

It was known that Ly49A cell surface expression levels were reduced in mice expressing a cognate MHC class I ligand of Ly49A (146, 147, 152, 341). Former data had also reported that H-2D$^d$ transfer was completely abrogated when Ly49A-positive NK cells also expressing H-2D$^d$, were tested, indicating that no Ly49A receptors were accessible (241). In contrast, it was shown by my group that Ly49A$^+$ NK cells, expressing endogenous H-2D$^d$ molecules, still acquired H-2D$^d$ ligands expressed on neighbouring cells (239). It was also clear that H-2D$^d$-NK cells could still be functionally inhibited by H-2D$^d$ on target cells, suggesting that accessibility could not be zero. We found, using our protein transfer assay, that the presence of H-2D$^d$ in the current milieu and on the NK cell itself (B6.D$^d$ mice) led to a 92% reduction in Ly49A accessibility showing that Ly49A receptors were still accessible to target cell H-2D$^d$. The reduction in accessibility could be explained both by lower Ly49A surface expression and by an association of Ly49A with endogenous H-2D$^d$ in cis (**figure 16**).
Less efficient recruitment of H-2D<sup>d</sup> molecules at the inhibitory NK cell synapse

Ly49A<sup>+</sup> NK cells from B6.D<sup>d</sup> mice were less efficient than Ly49A<sup>+</sup> NK cells from B6 mice to recruit H-2D<sup>d</sup>-GFP molecules expressed by the target cell at the inhibitory NKIS, which was a predicted outcome since B6.D<sup>d</sup> NK cells have a limited Ly49A accessibility, i.e. fewer free Ly49A receptors available for recruitment of target cell H-2D<sup>d</sup>-GFP molecules. Our results were confirmed in a recent publication, showing that endogenous H-2D<sup>d</sup> molecules were able to prevent Ly49A-dependent cell-cell adhesion and reduce the redistribution of Ly49A and the H-2D<sup>d</sup> of target cells at the contact site (321). Previous findings suggest that the degree of inhibitory ITIM-bearing NK cell receptor clustering correlates with the strength of the inhibitory signal (342). Nevertheless, a weaker, but sufficient, inhibitory signal transduction may occur without distinguishing clusters of either MHC class I molecules or inhibitory receptors. Reduced Ly49A accessibility and less clustering of H-2D<sup>d</sup> molecules at the iNKIS correspond to a decreased sensitivity to H-2D<sup>d</sup>-mediated inhibition. The latter has been proven by our group and others in cytotoxicity assays (146, 149, 153).

Mild pH exposure restores partially H-2D<sup>d</sup>-transfer

The reduced Ly49A accessibility on B6.D<sup>d</sup> NK cells could be due to diminished absolute levels of Ly49A receptors at the cell surface or by cis interactions between the Ly49A receptors and endogenous H-2D<sup>d</sup> molecules. To clarify if how much the potential cis interactions contribute to the constraint of Ly49A accessibility, we treated the NK cells with low pH. By acid treatment at pH 3.3 the MHC class I molecules are eliminated from the membrane of viable cells. At pH 3.3, MHC class I molecules instantly disrupt and alter conformation, which might be due to elution of the peptide or dissociation of the β<sub>2</sub>m subunit from the MHC class I heavy chain (320, 332, 343). A rapid incubation of NK cells from B6.D<sup>d</sup> mice at pH 3.3 before co-culture resulted in significantly enhanced H-2D<sup>d</sup> transfer from target cells. However, H-2D<sup>d</sup> transfer did not exceed more than 43 % of equivalent value for B6 NK cells; implicating that approximately 60 % of the reduced accessibility resulted from an acid-resistant mechanism, most likely lower absolute levels of Ly49A. This experiment also allowed us to estimate that 75 % of all Ly49A receptor expressed on B6.D<sup>d</sup> are engaged in cis with endogenously expressed H-2D<sup>d</sup> ligands. The remaining 25 % of Ly49A receptors on B6.D<sup>d</sup> NK cells are, “free” and accessible for trans interactions with H-2D<sup>d+</sup> target cells. Additionally, to find out whether a dynamic relationship exists between accessible Ly49A and Ly49A bound in cis, we pre-treated B6.D<sup>d</sup> NK cells with acid wash, replaced the cells in medium with neutral pH either with or without H-2D<sup>d+</sup> target cells. The analysis revealed that endogenous newly synthesised H-2D<sup>d</sup> molecules reappear at the cell surface accurately folded and bind accessible Ly49A, preventing the interactions in trans, thereby diminished capability to acquire H-2D<sup>d</sup> molecules expressed on surrounding cells (figure 17).
Binding site of JR9.318 and YE1/48 antibodies

Two different Ly49A antibodies were used, JR9.318 and YE1/48 to measure the Ly49A accessibility displayed on B6 and B6.Dd. Interestingly, YE1/48 staining of B6.Dd was only 28% in comparison to B6, whereas JR9.318 demonstrated a 40% reduction. We speculated that this inconsistency between the two antibodies was caused by the cis interaction. To assess if this was the reason, NK B6.Dd cells where exposed to pH 3.3 before Ly49A staining using the both antibodies. The results showed that the YE1/48 staining increased substantially, from 28% to 52%, implying that the epitopes of YE1/48 was blocked by the cis interaction. Interestingly, the JR9.318 staining did not change at all (figure 18). According to our estimation, YE1/48 should have detected about 10% if the antibody had recognised only the Ly49A receptors, which were not occupied in cis on B6.Dd. Since the staining of YE1/48 reached 28%, it seems like the YE1/48 antibody also can bind some Ly49A engaged in cis. To verify this idea we determine the transfer of H-2Dd-GFP to B6.Dd and B6 NK cells, pre-blocked with 125 ng/ml unlabelled YE1/48 exhibit equivalent intensity of Ly49A staining as B6.Dd NK cells. The experiment revealed that in spite of identical Ly49A staining, H-2Dd transfer was less efficient to B6.Dd NK cells in comparison to B6 NK cells, indicating that YE1/48 antibody recognise not merely free Ly49A. Probably, YE1/48 may bind free Ly49A epitopes as well as certain epitopes engaged in cis. Further investigation needs to be done to clarify how and where. Since binding of JR9.318 is not changed after acid treatment one could speculate about whether JR9.318 staining can be seen as a direct reflection of the absolute level of Ly49A receptors at the NK cell surface.

Figure 17. pH pre-treatment before coculture

![Graph showing relative endogenous Dd expression and relative transfer of Dd-GFP over time in neutral pH](image)

**Figure 17.** Relative endogenous Dd expression and relative transfer of Dd-GFP over time in neutral pH (min).

**Figure 18.** Increased accessibility of Ly49A receptor after pH exposure when staining with YE1/48 (black bars). The Ly49A expression was not altered when JR9.318 was used for staining (white bars).
Reduced absolute surface levels of Ly49A and cis interactions with endogenous H-2D<sup>d</sup>

We provide evidence in our study that an absolute reduced surface level of Ly49A in combination with a cis interaction between Ly49A and endogenous H-2D<sup>d</sup> contribute to decreased accessibility of Ly49A receptors on H-2D<sup>d</sup> positive NK cells (242). In our model, two effects of H-2D<sup>d</sup> molecules are suggested, explaining their major impact on the accessibility of Ly49A receptors in B6.D<sup>d</sup> mice. The first is claimed to be the importance of H-2D<sup>d</sup> molecules in trans. This interaction would result in an increased turnover of Ly49A molecules and an internalisation of cell surface Ly49A receptors. The internalisation may occur in a manner similarly to a peptide/MHC-dependent TCR internalisation, described elsewhere (268). Acquired H-2D<sup>d</sup> molecules might be involved in this process, which is implied in a previous report dealing with HLA-C transfer to human NK cells expressing the corresponding KIR receptor. In that paper, Daniel Davis’ group shows images of acquired HLA-C molecules both on the cell membrane but also intracellularly in the cytoplasm (240). Similar results were observed when H-2D<sup>d</sup>-GFP ligands transferred onto Ly49A-positive NK cells (unpublished data). Whether or not the corresponding receptors colocalise intracellularly with the acquired MHC class I molecules remains to be elucidated.

A second mechanism is the cis interaction (the engagement between the Ly49A and endogenous H-2D<sub>d</sub> ligand), restricting Ly49A accessibility on the NK cell surface. The cis interaction is principally responsible for masking Ly49A and thereby preventing further interaction in trans with H-2D<sub>d</sub>-positive target cells. Nevertheless, it cannot be excluded that a cis interaction also results in Ly49A internalisation. The cis interaction could probably be reversible and disrupted under certain physiological conditions, which are still not investigated. A pH-dependent regulation of protein conformations has been demonstrated in peptide loading on MHC class II molecules. The invariant chain is released inside lyzosomal vesicles, which affects the conformation of MHC class II molecules such way that they are available for peptide loading and subsequent transport to the cell surface (344). As a matter of fact, a subset of MHC class I molecules have been reported to be directed into comparable endosomal low pH compartment, indicating involvement in intracellular trafficking and presentation of antigen (345). Still it is unknown about how the accessibility of inhibitory Ly49 receptors is influenced by surrounding in vivo milieu, including cytokines, infection or inflammation. Yet, an inflammatory response could potentially elevate the degree of cis interactions, as a consequence of IFN-induced increase in number of MHC class I molecules on the NK cell surface. A suitable regulation during infection and inflammation since augmented NK cell activity would be needed (154).

Discrepancy between Andersson et al. and Doucey et al.

In the study by Doucey et al. it was suggested that a cis-interaction between endogenous H-2D<sup>d</sup> ligands and Ly49A represented the main reason why H-2D<sup>d</sup>-expressing Ly49A<sup>+</sup> NK cells were less sensitive to Ly49A-mediated inhibition compared to H-2D<sup>d</sup>-negative Ly49A<sup>+</sup> NK cells (153). Supporting this conclusion, the binding of soluble H-2D<sub>k</sub> multimers to Ly49A was almost completely impaired by the presence of endogenous H-2D<sup>d</sup> (153), something we confirmed in our study. In these two studies, various dissimilarities in the experimental setups could contribute to the discrepancy of results. Staining with multimers may show low
sensitivity depending on glycosylation, peptide-specificity, or to a non-linear responsiveness to the expression level of the receptor (346). A titration of free Ly49A epitopes illustrated that much higher expression level of the Ly49A was required to detect the receptors with the H-2D\textsuperscript{k} multimer compared with the YE1/48 antibody and H-2D\textsuperscript{d}-GFP transfer, implying that H-2D\textsuperscript{d} multimer staining fails to detect Ly49A receptors if they are expressed at low levels (Fig. 19). Furthermore, another potential important issue is usage of Ly49A-transgenic mice by Doucey et al. versus sorted Ly49A\textsuperscript{+} NK cells from Ly49A-non-transgenic mice, expressing Ly49A under physiological promotor, in our study. The intensity of Ly49A expression detected by JR9.318 is conflicting in the two models. In the study by Doucey et al. the staining of JR9.318 were similar for Ly49A-transgenic mice from B6 and B6.D\textsuperscript{d}, whereas we detected 60% reduction in Ly49A accessibility on Ly49A-non-transgenic B6.D\textsuperscript{d} NK cells compared to B6 NK cells by, using the same antibody. Thus, the question still remains how relevant is a potential cis-interaction in relation to a real reduction in absolute levels of free Ly49A receptors on the NK cell surface and in restricting H-2D\textsuperscript{d} inhibition (153, 242).

Figure 19. Limitation in using tetramers.

CD22, another cis-trans case

Recent data suggest that cis-bound Ly49A receptors are unable to changes to ‘free’ Ly49A conformation during target cell interaction (ref Back et al), similar to another cell surface receptor, CD22. The CD22 molecule, expressed on B cells, mediates intercellular adhesion and act as a coreceptor in antigen-induced B cell activation. CD22 redistributes to sites of cell-cell contact site by binding to trans high-avidity cis ligands on neighbouring cells despite of the presence of cis ligands, α2-6-linked sialic acids, implying that trans ligands efficiently compete with and replace cis CD22 ligands (347, 348). In contrast to the established cis interaction between Ly49A and H-2Dd that is stable and not reversible according to Back et al., suggesting that the interaction remains stable in the context of target cell recognition. However, the stability of cis interactions, i.e. the number of accessible Ly49A receptors, may vary under certain condition, as under NK cell development (321).

Whilst fewer inhibitory Ly49A receptors are accessible for functional trans binding the ensuing inhibitory signalling is relatively weak, rendering the NK cells extra sensitive to stress-induced activation ligands on H-2Dd\textsuperscript{+} target cells and as a result to detect unhealthy host cells. The degree of inhibition seems sufficient to maintain self tolerance and is adequate to optimally discriminate normal and abnormal host cells. Thus, NK cell function seems to be adjusted to the MHC class I- milieu via a cell-autonomous calibration and absolute cell surface reduction of Ly49A receptor expression, according to our study.
PAPER IV

Blockade of inhibitory NK receptors

In Paper IV we demonstrate that blockade of mouse inhibitory receptors induces rejection of MHC class I-expressing tumour cells without breaking the tolerance towards normal syngeneic cells.

Background

Reduced expression or absence of MHC class I molecules are common characteristics of tumour cells, leading to NK cell sensitivity, but not on all kinds of tumours. Whilst downmodulation or lack of MHC class I expression may protect tumours from T cell recognition, the tumours acquire sensitivity to NK cell activity instead. Besides, NK cells require interactions between activating receptors and their ligands expressed on the cell surface of the tumour cells. Thus, two features render the tumours susceptible to NK cell killing: 1) declined expression of MHC class I ligands and 2) upregulation of ligands recognised by NK cell activating receptors.

A number of studies have implicated that the activating NK cell receptor, NKG2D, and its ligands are deeply involved in tumour surveillance. The NKG2D receptor recognises defined antigens that are constitutively expressed on various different tumour cells (41) and plays a crucial role in immune surveillance against cancer (91). Data indicate that NKG2D ligand upregulation is associated with transformation, with a role for both oncogenes and tumour suppressors (349). Recently, Gasser et al. showed that the DNA damage response, which is activated early in tumourigenesis, induces expression of NKG2D ligands (350, 351). Engagement of NKG2D receptors often results in lysis of target cells although inhibitory receptors interact with MHC class I ligands. (More information, concerning NKG2D and its ligands, is available in the introduction).

The NCRs, (NKp46/NCR/MAR-1, NKp30, NKp44) have been suggested to be involved in recognition and killing of tumour cells. However, the cellular ‘tumour’ ligands of the NCRs are currently unknown. NKp46 blocking, by mAbs, revealed that NK-mediated killing of different types of tumours including melanoma, neuroblastoma and glioblastoma cell lines was inhibited (63, 352). Furthermore, deletion of a single NCR gene, the NKp46 mouse homologue (MAR-1, NCR1) reduced the ability of NK cells to clear RMA-S tumour cells in vivo at least on the 129 strain background (353).

NK cells in cancer therapy, as therapeutic tools

Strategies are emerging to apply NK cells as tools in cancer therapy. For instance, an adoptive transfer and in vivo expansion, induced by IL-2, of haploidentical NK cells have been successfully carried out in AML patients (314). Furthermore, Ruggeri and colleagues were first to demonstrate the benefits of using haploidentical mismatched transplants in recipients suffering from AML. The alloreactive NK cells mediated an enhanced GvL effect and declined
GvHD suggested being due to NK alloreactivity against host DC, thereby preventing presentation of host antigens to donor graft T cells. The long-term result was prevention in leukaemia relapse (354). These reports highlight the therapeutic potential in NK cell mediated “missing-self” reactivity. However, reports regarding other BMT studies, evaluating the potential benefit of haploidentical anti-host reactive NK cells, show mixed positive and negative results. Factors that have been suggested to be of relevance for the success is if donor and host are related or not and the use of T cell depleting reagents (ATG; anti thymocyte globulin) (311, 355, 356). A number of clinical analyses describe high risk for infections in consequence of DC depletion (357).

Methodology

Our study was based on the strategy to block the inhibitory Ly49C/I receptors displayed on the cell surface of 30-50 % of the NK cells. Ly49C/I (5E6) receptors bind H-2Kb (306, 358). This strategy has been described in earlier studies executed by Koh et al., showing that blocking of Ly49C/I receptors, using 5E6 F(ab')2 fragments, enhanced NK cell-mediated anti-tumour effects both in vitro and in vivo. The survival of mice, injected with tumours, was augmented after administration of 5E6 F(ab')2, blocking the Ly49I/C receptors, in comparison to untreated mice (305) (figure 20 and 21).

In addition, to confirm and extend the studies of Koh et al. we elucidated whether the blocking of Ly49C/I receptors break the tolerance and induce rejection of autologous cells. Could blockade of inhibitory receptors cause autoreactivity? Autoimmunity and inflammatory responses have been reported to be consequences of triggering activation of NK cells (Bellone, 1993). Importantly, the blocking of inhibitory receptors on IL-2 activated NK cells in vitro in co-cultures with BMC resulted in autoreactivity against syngeneic BMC during certain conditions, as demonstrated by a transient suppression of myeloid reconstitution in vivo (307).

**Figure 20.** 5E6 F(ab')2 fragments blocking inhibitory receptors Ly49C/I. Hypothesis: Increased levels of activating receptors on tumour cells compared to normal healthy cells may induce increased killing of syngeneic tumour cells after blocking, but not of normal syngeneic lymphocytes. *(Made by Gustaf Vahlne)*
**5E6 F(ab')₂ fragments block Ly49C/I receptors and kill syngeneic targets cells in vitro**

In vitro, 5E6 F(ab')₂ fragments are able to efficiently saturate up to 80% of Ly49C/I receptors on the NK cells, leading to induction of killing of syngeneic H-2b target cells, RMA and B6, confirming previous published results (305).

![In vitro blocking of Ly49I/C receptors after 5E6 F(ab')₂ injection i.v or i.p](image)

To obtain saturation of 80-85 % of the Ly49C/I receptors 83 – 250 µg 5E6 F(ab')₂ was injected i.p or i.v per mouse. Kinetics of Ly49C/I saturation after 5E6 F(ab')₂ treatment in vivo revealed that maximum saturation in spleen NK cells, approximately 80 %, was achieved 3-4 days post i.p injection and began to decline 5 days post injection. Regularly, blocking was measured by incubating lymphocytes from the treated mice with a fluorescence conjugated whole 5E6 mAb. We also used a conjugated mAb specific for Ly49I (clone YLI-90) in an attempt to measure saturation of Ly49I receptors. Intriguingly, YLI-90 staining of NK cells preincubated in vitro with 5E6 F(ab')₂ was unaffected or even increased, NK cells from mice treated with 5E6F(ab')₂ in vivo demonstrated a greatly reduced staining. In addition, direct detection of cell-bound 5E6 F(ab')₂ using a conjugated anti-Ig-kappa mAb could be done on in vitro pre-treated NK cells but not (or only weakly) on NK cells from in vivo treated mice (data not shown). Together these pieces of data suggest that Ly49I receptors may be downregulated (e.g. internalized) or induced to interact with Kb ligands in cis by in vivo treatment with 5E6 F(ab')₂. Still, it is not clarified whether the 5E6 F(ab')₂ saturation is due to ‘true’ blocking of Ly49C/I expression, downregulation by internalisation or masking of epitopes by cis-interactions between Ly49C/I receptors and endogenous H-2Kb molecules. Further experiments are required to illuminate this issue and if any of these mechanisms may be responsible for the functional effects in vivo.
5E6 F(ab’)2 treatment induces in vivo rejection of RMA, but not of syngeneic normal cells

Regardless of the mechanism of action treatment with 5E6 F(ab’)2 induced NK cell dependent-rejection of RMA cells in vivo. RMA-S cells and B6 β2m−/− spleen cells (susceptible MHC class I-deficient target cells), were rejected in untreated mice, exemplifying the strong NK cell-mediated effect against tumour and normal cells that fail to deliver inhibitory signals to MHC class I-specific receptors on NK cells (figure 22). Importantly, rejection of syngeneic B6; splenocytes, ConA blasts or BMC was not induced even though maximal saturation of Ly49C/I receptors was achieved, thus indicating self-tolerance. One plausible explanation for maintenance of robust tolerance is that normal cells may express less activating ligands in general. Moreover, another explanation might be that normal cells express additional inhibitory MHC class I ligands and/or non-MHC class I ligands. Normal cells also generally express less NKG2D and NCR ligands in comparison to transformed cells. However, it is not known which receptor-ligand pairs that may trigger RMA killing in this setting.

Contradictory, B6 con A-activated lymphoblasts were spared in 5E6 F(ab’)2 treated mice in vivo in spite of being killed by IL-15/IL-18 activated 5E6 F(ab’)2 treated NK cells in vitro. Our results point out that complete lack of MHC class I expression induce killing of normal cells in vivo and in vitro, but, under certain conditions, such as cytokine stimulation of NK cells, reduced inhibitory signal, in NK cells with partially blocked inhibitory receptors interacting with MHC expressing target cells, can be enough to change the balance in favour of activating signals and even induce killing of normal cells.

Figure 22. Selective rejection of syngeneic tumour cells vs. normal cells in vivo. Mice were injected with 200µg/mouse i.p. of 5E6 F(ab’)2, followed by inoculation of CFSE labelled RMA and B6 spleen cells. Treatment and target cells analysed are indicated beneath the graphs. NK1.1 indicated NK cell depleted recipient mice.

Intriguingly, blocking of Ly49C/I did not induce rejection cells H-2Kb-single spleen cells, expressing H-2Kb as their only inhibitory MHC class I ligand. H-2Kb-single cells do not express H-2Db and Qa-1b molecules, which are normally expressed on B6 cells, but may express other MHC or non-MHC class I ligands recognised by inhibitory NK cell receptors, e.g. 2B4 or NKR-P1B/D, which may be possible to investigate by expanding the blockade to other inhibitory receptors. An alternative explanation for absence of rejection is that 80-85% blocking of Ly49C/I is not enough to abrogate the inhibitory signal from Kb. Nevertheless, this finding
argues against the likelihood that a compensatory effect by other MHC class I molecules was critical for protection of host syngeneic cells.

**Ly49C/I blocking reduces rejection of β2m-/− spleen cells but not of RMA-S cells**

Unexpectedly, 5E6 F(ab’)2 treatment led to a small, but consistent NK cell-dependent reduction in the capacity to reject ‘normal’ β2m-deficient spleen cells in vivo. This outcome supports our observation in some, but not all, in vitro experiments that blockade of Ly49C/I caused significantly reduced killing of β2m-/− Con A blasts and RMA-S (data not shown). One credible reason, based on “weak inhibitory signalling”, is that 5E6 F(ab’)2 fragments may trigger a weak dampening signal, which is much weaker than the one received from interactions with cognate MHC class ligands, to the NK cell that influences rejection of β2m-/− target cells. Consequently, blockade of MHC class I-specific inhibitory receptors would upon encounter with MHC class I expressing tumour cells still lead to increased killing, while a decreased net killing would be observed for the β2m-/− spleen cells. On the other hand, 5E6 F(ab’)2 treatment may give rise to longer conjugation time between NK cells and normal autologous cells in the host, without induction of killing, due to a prolonged decision process in the NK/target synapse when the inhibitory input is quenched. A competition situation generated where NK cells become occupied dealing with autologous cells, would lead to declined rejection of β2m-/− spleen cells. Since a diminished killing was sometimes observed when inhibitory receptor blockade was applied in *in vitro* assays with RMA-S cells as targets it is worth taking into consideration potential consequences while applying this type of treatment to a clinical situation. If killing of MHC class I deficient tumour cells is actually reduced by inhibitory receptor blockade in vivo, such treatment might act to promote malignancy in cases where the tumour downmodulate or turn off its MHC class I expression. However, in our in vivo experimental series we never observed this reduced NK mediated elimination for RMA-S cells – in vivo it was only seen with β2m-/− spleen cells. One possible explanation for the difference between in vivo and in vitro results may be that our in vivo assay favours NK cell mediated rejection of RMA-S cells in a way that a small reduction cannot be detected. Potentially modifications in inoculated cell numbers and/or in the time span between cell inoculation and analysis of retrieved cells should be tested in order to definitely rule out that inhibitory blockade reduces rejection of MHC class I deficient tumour cells.

**Long term treatment of inhibitory receptor blockade**

**Functionally**

Anti-tumour effect and tolerance to normal cells was as effective after 15 days of continuous blockade as after one single treatment with 5E6 F(ab’)2 in vivo. No obvious signs of either autoreactivity or hyporesponsiveness were perceived. One could have expected that constitutive blocking of inhibitory input may cause mature NK cells to tune their activation threshold and eventually react against normal autologous tissue and cells. Alternatively, the NK cells could have evolved into hyporesponsive, “disarmed” or “non-licensed” NK cells due to overstimulation in absence of “normal” inhibitory signals, leading to downmodulation of stimulating signalling cascades. A prolonged blockade might theoretically lead to a hyporesponsive NK cell condition or even NK cell death. Although these obtained data do not exclude that blockade for an even longer period of time or under other conditions may affect the
NK cell education, they indicate that it is possible to retain an increased killing against syngeneic tumour cells for extended periods of time.

Phenotypically

No ‘side effects’, unpleasant treatment-related effects, were revealed after 13 weeks of twice weekly treatment with 5E6 F(ab’)2. A histopathological examination of the mice treated with inhibitory receptor blockade up to 13 weeks was executed. No macroscopic or microscopic abnormalities were detected, confirming absence of destructive NK attack on normal cells, in an examination of more than 40 separate organs or tissues in 24 individual treated and untreated mice respectively. This histopathological analysis is of great importance in preclinical models, if inhibitory receptor blockade would be applied in cancer therapy.

Tolerance perspective

In a tolerance perspective it would be exciting to find out what occurs if mice were treated with 5E6 F(ab’)2 throughout the estimated period of NK cell development. Would we detect any phenotypical and/or functional alterations of the NK cells? In a previous publication, it was reported that in vitro blocking of the interaction between Ly49C receptors and MHC class I molecules of H-2b haplotype inhibited the development of mature cytotoxic NK cells in a bone marrow culture setting. This indicates that specific interaction between inhibitory self-reactive Ly49 molecules and MHC I molecules may be crucial for NK cell functional development (319). As covered in the introduction, a large number of studies of MHC class transgenic and knockout mice have shown the importance of interactions between NK cells and MHC class I for development of NK cells function and specificity (reviewed by Johansson et al. 2006, Rautel et al. 2006 and Yokoyama et al. 2006). The mechanisms behind functional development and tolerance induction could be further studied using this tool to block MHC – Ly49 interactions during NK cells development.

Future prospects

In the near future, we will try blocking of additional inhibitory NK cell receptors, such as NKG2A. Whether or not blockade of other inhibitory pathways, such as NKG2A or 2B4, contributes to even greater anti-tumour effects will be interesting to investigate further. Additionally, it would be fascinating to explore further if the NK cell reactivity induced by inhibitory receptor blockade is possible to combine with other kind of stimulus and still maintain tumour selectivity. Cytostatic drugs, cytokines and stimulating expression of activating NK cell ligands would be suitable candidate to start with.

NKG2D-based cancer therapy

Therapeutic strategies aimed at upregulation of NKG2D ligands and NKG2D could further trigger the anti-tumour effects. However, one has to bear in mind several studies have revealed that sustained exposure to NKG2D-ligands expression may cause NKG2D downregulation and impairment of NK cell cytotoxicity, e.g. through dysfunctional DAP10- and DAP12- signalling (359, 360). Moreover, soluble NKG2D ligands can mediate shedding-induced impairment of NKG2D-mediated immune function, a potential tumour escape mechanism. These soluble NKG2D ligands can potentially induce downregulation of NKG2D receptors either by blocking NKG2D receptors or through internalisation and lysosomal degradation (248, 361). They are
detected in sera from patients with malignant diseases as leukaemia (362, 363). The mechanism(s) responsible for generating soluble NKG2D ligands have been found to be associated with translational proteolytic cleavage.

**Cytokine-based tumour therapy**

It is known that certain cytokines exert anti-tumour effects and are good candidates to augment anti-tumour effects in our approach. For instance, IL-21 may increase NK cell mediated NKG2D-dependent tumour cell lysis in vitro and rejection of grafted tumour cells in vivo (364). IFN-α appears to up-regulate NKG2D cell surface expression (A. Chalfour W. Held unpublished observation). IL-2 has been used for in vivo and in vitro expansion of NK cell and clinical trials have demonstrated that treatment of leukaemia patients with low-dose IL-2 can safely drive NK cell development and expansion (365). To augment the NK cell expansion low dose treatment of IL-2 has been administered, resulting in enhanced NK cell differentiation from bone marrow progenitors and delay in NK cell death in vivo (366). IL-15 has shown to be essential for NK cell proliferation, differentiation and cytotoxic ability as well as regulating NK cell survival (187). IL-18 has been found to significantly augment IL-12-induced NK activity in a MHC-nonrestricted manner against allogeneic lung cancer cell lines, proposing the potential of IL-18 in combination with IL-12 for clinical application in treatment of cancer (193). However, best anti-tumour results are seen when cytokines are applied in combination with other cancer therapies.

**Boosting NK cell activity by depleting Treg**

Recently, it has been shown that Tregs have the capability to suppress NK cell effector functions, i.e. proliferation, cytotoxicity and IL-12 mediated IFN-γ production in vitro and in vivo. The mechanisms behind the inhibition of NK cells in mice are still under considerations but soluble, surface-bound TGF-β and IL-10, produced by Tregs, are strong candidates (203-205). Depletion of Treg cells or blocking surface-bound TGF-β increase the proliferation and cytotoxicity of NK cells. The cytokine production by IL-12 activated NK cell has shown to be reduced in presence of Treg cells, most likely in a TGF-β-dependent manner (202, 367). Tregs might inhibit NK-cell-based tumour immunosurveillance through downregulation of the NKG2D receptors expression (368). Thus, CD4⁺CD25⁺ Treg cells can potently inhibit NK cell function in vivo, and their depletion may have therapeutic consequences for NK cell function in BM transplantation and cancer therapy (188).

Thus, all these approaches for anti-tumour treatment would be interesting to test in combination with inhibitory receptor blockade. Further basic NK cell research regarding NK differentiation and NK education to obtain NK tolerance as well as profound understanding of NK cell target recognition will hopefully result in additional novel immune-based therapies for treatment of cancer.
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REFERENCES


189. Arment, M., G. Delespesse, and M. Sarfati. 1995. IL-2 and IL-7 but not IL-12 protect natural killer cells from death by apoptosis and up-regulate bcl-2 expression. Immunology 85:331-337.


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