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Influence of Ly49 Inhibitory Receptors and MHC Class I on T Cell and NK Cell Function

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To Thomas and Linnea

"Cells let us walk, talk, think, make love

and realize the bath water is cold"

Lorraine Lee Cudmore

ABSTRACT

Natural Killer cells (NK) can kill virus infected cells and tumor cells but do not harm normal surrounding cells. The function of Ly49 receptors on NK cells is important in maintaining self-tolerance since they transmit inhibitory signals when bound to endogenous MHC class I molecules. Their role in T cells is however unclear. We showed that a Ly49A transgene expressed on all T and NK cells altered T cell development in an in vivo environment, where a ligand for Ly49A was expressed. Ly49A transgenic mice that co-expressed an MHC ligand for Ly49A, H-2D^{d}, developed a severe inflammatory disorder that resulted in death within the first weeks of age. These data indicated that the expression of Ly49A on T cells altered T cell selection and allowed survival of potentially self-reactive T cells.

Furthermore, using Ly49A/TCR$\alpha\beta$ transgenic mice, we observed a quantitative influence on cellular responses that depended upon the activating signals received through the TCR and the inhibitory signals received through Ly49A. Low activation signal resulted in T cells that were unresponsive for target cells that expressed the Ly49 ligand H-2D^{d}, but responded against target cells without H-2D^{d}. However, this inhibition could be overcome by increasing the concentration of peptide for which the T cells were specific, i.e. the activation signal. Thus, rather than behaving as simple "off" switches, our data indicated that Ly49 receptors modulate T cell signaling so that higher amounts of activating signals are required for effector-cell responses.

A prediction from the "missing-self" hypothesis is that down-regulation of MHC class I on resting haematopoietic cells should be sufficient to make them susceptible to NK cell killing. We used a method enabling kinetic and quantitative assessments of NK cell-mediated rejection responses in vivo, and showed that resting haematopoietic cells from β_2-microglobulin-deficient ($\beta_2m^{-/-}$) mice were rapidly rejected in unmanipulated C57BL/6 (B6) mice. The signaling adaptor KARAP/DAP12 was dispensable for rejection of $\beta_2m^{-/-}$ cells (lacking MHC) but critical for rejection of BALB/c cells (mismatched MHC) in unmanipulated B6 recipients while pre-activated B6 mice rejected BALB/c cells in a KARAP/DAP12-independent fashion. Loss or mismatch of MHC class I in resting cells was thus sufficient to convey susceptibility to NK cell rejection. However, activation of the effector or the target enhanced rejection and shifted the balance between different signaling pathways involved.

Using the same method we injected $\beta_2m^{-/-}$ and control B6 spleen cells into mice with a nonfunctional CD1d1 gene (CD1d1$^{-/-}$(LVK)) and hence no NKT cells. CD1d1$^{-/-}$ (LVK) mice lacked the ability to reject $\beta_2m^{-/-}$ cells in vivo after 48 hours. They were also defective in rejection of TAP1/2$^{-/-}$ cells and inhibition of RMA-S tumor outgrowth, but were able to reject allogeneic cells (BALB/c) similar to B6 mice. To determine if the CD1 molecule per se was involved we tested another CD1-deficient mouse strain (CD1$^{-/-}$). These mice rejected $\beta_2m^{-/-}$ cells as well as B6 mice, indicating that the CD1 molecule was not important for rejection of cells lacking MHC. Thus, the CD1d1-deficient mice displayed an additional defect, unrelated to the targeted CD1d1 gene. Interestingly, rejection of targets with allogeneic MHC class I molecules remained intact. Further investigation of these mice will potentially provide an answer as to how NK cells are activated to sense “normal” cells and certain tumor cells not displaying known activating ligands. Additionally, these mice strongly displays why it is necessary to critically evaluate data from genetically altered mice.

Together, these studies contribute to our understanding of how Ly49 inhibitory receptors and the MHC class I regulate T cell selection and function and NK cell activity and tolerance.

LIST OF PUBLICATIONS

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

I. Linda Fahlén, **Linda Öberg,** Thomas Brännström, Nelson K. S. Khoo, Urban Lendahl, and Charles L. Sentman. 2000.
Ly49A expression on T cells alters T cell selection.
International Immunology, 12: 215-222.

II. **Linda Öberg**, Mikael Eriksson, Linda Fahlén, and Charles L. Sentman. 2000.
Expression of Ly49A on T cells alters the threshold for T cell responses.
European Journal of Immunology, 30: 2849-2856.

III. **Linda Öberg,** Sofia Johansson, Jakob Michaëlsson, Elena Tomasello, Eric Vivier, Klas Kärre, and Petter Höglund. 2004.
Loss or mismatch of MHC class I is sufficient to trigger NK cell-mediated rejection of resting lymphocytes in vivo - role of KARAP/DAP12-dependent and -independent pathways.
European Journal of Immunology, 34: 1646-53.

IV. **Linda Öberg**, Maria H Johansson, and Klas Kärre. 2005.
Mice defective in NK cell mediated killing of targets with a "missing-self" phenotype.
Manuscript.

The following papers were not included in the thesis:

Michael R. Daws, Mikael Eriksson, **Linda Öberg**, Anders Ullen, and Charles L. Sentman. 1999.
H-2D^{d} engagement of Ly49A leads directly to Ly49A phosphorylation and recruitment of SHP1.
Immunology, 97: 656-64.

Cristina Cerboni, **Linda Öberg**, Giuseppe Terrazzano, Serafino Zappacosta, Ennio Carbone, and Klas Kärre. 2004.
Proliferative and cytotoxic response of human natural killer cells exposed to transporter associated with antigen-processing-deficient cells.
Scandinavian Journal of Immunology, 59: 159-67.

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LIST OF ABBREVIATIONS

αGalCer	α-galactosylceramide
ADCC	Antibody dependent cellular cytotoxicity
AICD	Activation-induced cell death
APC	Antigen presenting cell
β_2m	β_2-microglobulin
B6	C57BL/6
BCR	B cell receptor
BM	Bone marrow
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
ConA	Concanavalin A
DC	Dendritic cell
DN	Double negative
ER	Endoplasmic reticulum
ES	Embryonic stem cell
FACS	Fluorescent activated cell sorter
H-2	Histocompatibility-2
HLA	Human Leucocyte Antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer cell immunoglobulin-like receptor
LAK	Lymphokine activated killer cell
LIR	Leukocyte IG-like receptor
LCMV	Lymphocytic chorio meningitis virus
MCMV	Murine cytomegalovirus
MHC	Major Histocompatibility Complex
MIC	MHC-class-I-related antigens
NCR	Natural cytotoxic receptor
NK	Natural killer
NKC	Natural killer gene complex
NKT	Natural killer T
NOD	Nonobese diabetic
RAG	Recombination activation gene
SCID	Severe combined Immunodeficiency
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
ULBP	UL16-binding protein
Wt	Wild type

INTRODUCTION

GENERAL OVERVIEW

During evolution, several different mechanisms have emerged to defend organisms against attack from invading pathogens or even individuals of the same species. The innate immune system has evolved to provide early defense against pathogens and to alert the adaptive immune system. One innate system seems to be similar in plants and animals, the recognition of pathogen-associated molecular patterns (PAMPs). In mammals, toll-like receptors (TLR) recognize lipopolysaccharide derived from Gram-negative bacteria while in Drosophila the Toll receptor senses Spätzle. In plants, LRR-type proteins with similarity to TLR and Toll are involved in defense against pathogens (1). Later in evolution, specialized cells have evolved that play important parts in innate immunity.

In mammals, both innate and adaptive immunity are involved in protecting the host against pathogens. Innate immunity consists of many different components. The first line of defense is the epithelial surfaces of our body that serve as an effective mechanical and chemical barrier against pathogens. If the microorganism has managed to cross the epithelial surface, macrophages can ingest pathogens and produce bactericidal agents. Upon infection, the complement system activates complement components through several pathways that lead to recruitment of inflammatory cells, opsonization and direct killing of pathogens. Activation of macrophages through TLR leads to production of pro-inflammatory cytokines and chemokines, which leads to a local inflammatory response that brings more effector cells and molecules to the site of infection. Additionally, resident dendritic cells (DC) are activated to migrate and mature. This leads to increased expression of major histocompatibility complex (MHC) molecules as well as costimulatory molecules, aiding the stimulation of the adaptive immune response in lymph nodes (LN), where the DCs migrated. One lymphoid cell is also involved in innate immunity, the natural killer cell (NK), since it can rapidly discriminate between self and non-self and kill cells invaded by pathogens or release cytokines. The NK cell will be discussed in depth throughout this thesis.

An important step in evolution of the immune system was the capacity to randomly generate antigen-specific receptors. This is believed to have occurred when an immunoglobulin (Ig) superfamily gene was invaded by a transposable element containing genes for rearrangements (RAG1 and RAG2). What we usually define as adaptive immunity, with polymorphic receptors, RAG and the development of a MHC, do not exist in organisms older than the shark, i.e. the cartilaginous fish (2). The hallmark of adaptive immunity is the vast antigen-specific diversity of receptors and the specific selection and expansion mechanisms involved in the development of T cells and B cells with clonally expressed receptors. This allows for tolerance and memory. Antigen presenting cells (APC), such as DC, macrophages or B cells, provide the T cell with peptides presented by MHC molecules. The T cells will recognize these MHC/peptide complexes and develop into effector cells, either a cytotoxic cell or a cytokine producing helper cell, depending on which type of MHC that presents the pathogenic peptides. This will lead to the development of memory T cells able to rapidly react if the same pathogen invades a second time. B cells can ingest parts of the pathogen and function as an APC, and this allow them to receive signals leading to isotype switching and development into plasma cells that secrete antibodies able to opsonize bacteria and activate complement. Memory B cells will also provide a rapid defense against a second infection. Since both the T cell receptor (TCR) and B cell receptor (BCR) are encoded by genes that undergo random rearrangements, a vast population of receptors will be present able to recognize almost all pathogens or pathogen-derived molecules. However, there is also a possibility that receptors recognize self molecules. Therefore, it is necessary that autoreactive T cells and B cells are selected against during development. T cells are selected for their usefulness and against autoreactivity in the thymus while B cells are selected in the bone marrow. There are also peripheral selection processes available to further minimize the risk of autoimmune disease. T cells and T cell selection will be discussed further below.

MAJOR HISTOCOMPATIBILITY COMPLEX

The function of MHC molecules is to present pathogen derived peptides, either from proteins degraded in the cytosol or antigen taken up into intracellular vesicles, to T cells. The end result of this process is almost always deleterious to the pathogen since it will activate the T cells to either directly kill the infected cell, activate macrophages to kill intracellular bacteria or B cells to produce antibodies. MHC class I molecules were

discovered in the late 1930s in mice and in the 1950s in humans, while the MHC class II was not discovered until the 1970s (3). The MHC molecules were initially discovered and studied on the basis of their capacity to influence the outcome of transplantation and antibody responses. Their exact role in antigen presentation was not revealed until later in a process initiated by the discovery of the phenomenon of MHC restriction in 1973. Influenza-specific CD8 T cells from one mouse were able to kill influenza-infected cells from another mouse, but only if they shared at least one MHC class I molecule (4, 5).

Classical MHC molecules are polygenic, i.e. there are several MHC genes in each individual, and one of the most polymorphic gene families known, i.e. there are several different allelic variants of each gene. Each MHC molecule can also bind several different peptides and one peptide may also bind to different MHC molecules. In humans, the MHC is located on chromosome 6 and the genes are called Human Leukocyte Antigens, HLA. In mouse the MHC is located on chromosome 17 and the genes are named H-2.

Classical MHC class I molecules

Three class Ia genes exist in both the human and the mouse genome, called HLA-A, -B, and –C and H-2K, -D, and –L respectively. They contain a heavy chain that folds into three domains. The $\alpha 3$ domain is connected to the stalk region, spanning the plasma membrane. The $\alpha 1$ and $\alpha 2$ domains form the peptide-binding cleft (figure 1). Peptides able to bind to MHC class I molecules are usually 8-10 amino acids long. MHC class I molecules also contain a β_2-microglobulin (β_2m) molecule, noncovalently linked to the heavy chain (6, 7). MHC class I molecules present peptides from proteins within the cell. Normal cellular proteins and proteins derived from intracellular viruses and bacteria are degraded in the cytosol by the proteasome. The proteasome is a large cylindrical protease, with two subunits encoded in the MHC locus, LMP2 and LMP7 (8). The peptides are generated and transported into the ER via the TAP1/TAP2 transporter in an ATP-dependent fashion (9). The MHC α chain and β_2m are synthesized in the endoplasmic reticulum (ER) and a number of chaperon molecules are involved in the biosynthesis. After the peptide has bound in the peptide cleft, the complex is transported through the Golgi complex to the cell surface (10). Both peptide and β_2m are required for efficient cell surface expression of MHC class I. In mice

deficient for β_2m, only a low number of free heavy chains is expressed at the cell surface (11). Similarly, TAP-deficient mice have diminished expression of MHC class I on the cell surface since peptides can not be transported into the ER (12). MHC class I molecules are expressed on almost all nucleated cells. The expression level varies between cell types and tissues due to regulation by both differentiation and inflammatory stimuli (13). MHC class I molecules present viral peptides to $CD8^+$ T cells that can directly kill the infected cell, and some viruses have evolved strategies to prevent their peptides to be presented. They can interfere with antigen processing, peptide presentation, and MHC class I expression to prevent attack by cytotoxic T cells (14, 15). Although the endogenous pathway is dominant for presentation of antigens on MHC class I, exogenous antigens may also be presented by MHC class I on professional antigen presenting cells. This is probably very important in those cases where the pathogen does not infect professional antigen presenting cells (16).

Classical MHC class II molecules

There are three human class II genes, HLA-DR, -DP, and –DQ, and in the mouse there are two genes, H-2A, and -E. MHC class II is expressed at the cell surface as a heterodimer consisting of an α and a β chain. In contrast to MHC class I molecules, the peptide binding cleft is composed by both the α and β chain (figure 1). The cleft in MHC class II molecules is more open, allowing longer peptides to bind (17). The peptides bound to MHC class II are exogenous, although endogenous peptides may also be presented (18). Exogenous bacterial proteins are degraded in endosomes or lysosomes by proteases activated by acidification (19). The α and β chains of the MHC class II molecules are produced in the ER. To prevent endogenous peptides to bind, the α and β chains are bound together with an invariant chain (Ii), which blocks the antigenic cleft (20). When vesicles containing MHC class II molecules fuse with vesicles containing the exogenously derived peptide fragment, the peptide binds to the cleft and the complex is transported to the cell surface. The expression of MHC class II molecules is more restricted than that of MHC class I molecules and they are found mainly on antigen presenting cells such as DC, macrophages, B cells and thymic epithelia. The function of MHC class II molecules is to present exogenous peptides to $CD4^+$ T-helper cells.

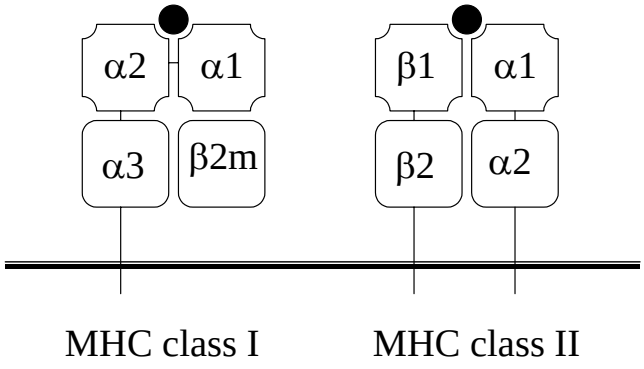

Figure 1. Schematic drawing of a MHC class I and a MHC class II molecule.

Nonclassical MHC class I molecules

Additionally, there are several nonclassical MHC class I genes. Humans express for example the class Ib protein HLA-E which can function as an inhibitory ligand for the CD94/NKG2A receptor on NK cells (21-23). There is a homologue in the mouse, Qa-1, which can do the same (24, 25). Within the MHC locus there are also genes for other MHC homologous molecules, for example the stress induced MICA and MICB in humans. There are several more distantly related MHC class I like molecules that are not expressed within the MHC locus. Examples are UL16 binding proteins (ULBPs) in humans and Rae-1 and H60 in mouse (26). The NK cell receptor NKG2D has been shown to bind to MICA, MICB, and ULBPs in human and Rae-1 and H60 in mouse (27-30).

The CD1 molecule encoded on chromosome 1 in humans and 3 in mice also belongs to the class Ib molecules. Five CD1 molecules have been identified in humans; CD1A-E. Based on sequence homology, they are divided into two groups. Group 1 consists of CD1A-C and CD1E and group 2 consists of CD1D. Two highly homologous molecules are present in mouse, CD1d1 and CD1d2, both resembling CD1D in humans. β_2m is necessary for expression and most CD1d proteins are expressed on the cell surface associated with β_2m molecules (31). Unlike the classical MHC molecules, the CD1 molecules do not present peptides but rather bind and presents microbial lipids and glycolipids. Therefore, there is no role for TAP in the presentation of CD1. Most mouse and human CD1d-restricted T cells recognize α-galactosylceramide (αGalCer), a glycosphingolipid found in marine sponges. αGalCer has therefore been widely used as an artificial ligand in studies of CD1d-restricted NKT cells (32). Recently it was shown

that a lysosomal glycosphingolipid (iGb3) can be recognized by both human and mouse NKT cells and may thus be an important patophysiological ligand (33). In mouse, CD1d1 is needed for positive selection of T cells with an invariant TCR (Vα14-Jα18), so called NKT cells, which will be discussed later.

T CELLS

T cells are responsible for the cell-mediated immune responses in adaptive immunity. All T cells express a TCR, which recognize and respond to peptide/MHC complexes. The TCR can either be formed by an α and β chain generating classical αβ T cells or a γ and a δ chain generating γδ T cells.

T cell receptor

The TCR contains of an α- and a β-chain that structurally belongs to the Ig superfamily. The TCR associates with CD3 to form a TCR-CD3 membrane complex. The CD3 consists of the γ, δ, and ε chains that make up the dimers γε, and δε. Included in the TCR-CD3 complex is also the ζζ homodimer. The cytoplasmic parts of the CD3 chains and ζζ chains contain immunotyrosine-based activation motifs (ITAM), which signal to the interior of the cell upon ligand binding (34).The coreceptors CD4 and CD8 strengthen the interaction between the TCR and MHC. This is because CD4 binds to the β2-domain of MHC class II molecules, while CD8 binds to the α3-domain of MHC class I. Associated with the cytoplasmic domain of the coreceptors is the tyrosine kinase Lck, which targets the tyrosine kinase ZAP-70 associated with the ζ chains (35, 36).

T cell development and selection

Although T cells and B cells belong to the adaptive components and NK cells are part of the innate compartment of the immune system, they both originate from a common lymphocyte progenitor in the bone marrow (BM). The common lymphocyte progenitor migrates to the thymus via the blood. Most part of T cell development takes place in the thymic cortex. The majority of the cells die within the thymus either due to failure to produce a productive TCR or because they fail to meet the criteria of thymic selection. The T cell progenitors are double negative, i.e. they lack expression of either of the coreceptors CD4 and CD8. They first rearrange the β chain of the TCR and pair with a

surrogate α chain to expresses a pre-TCR. The pre-TCR is expressed together with the CD3 complex that provides ITAMs responsible for signaling. At this stage, the T cells go through several rounds of proliferation and go into the double-positive stage. As double-positives expressing both coreceptors, CD4 and CD8, they start to rearrange the α chain locus leading to a complete TCR. After positive and negative selection, mature T cells emerge, expressing either the CD4 coreceptor or the CD8 coreceptor. These mature T cells then leave the thymus to act in immune responses (37). If the T cell population should be able to sense all different combinations of peptide/MHC molecules presented, a highly diverse repertoire of TCR must develop. This is achieved by genetic rearrangement of randomly combined gene segments allowing up to 10^{18} different TCRs to be formed.

First T cells go through positive selection. This process is considered important for establishing a "useful" receptor repertoire, i.e. one that is able to recognize MHC molecules expressed in the host (self-MHC). During positive selection, which occurs at the double-positive stage, the thymocytes interact with epithelial cells in the cortex. Only those thymocytes that can bind to self-MHC will be rescued and survive while all others will die (figure 2) (38). During positive selection, it is also decided with type of T cell the thymocyte will mature into. The duration of TCR signaling controls CD4/CD8 lineage differentiation (39). If the TCR recognizes MHC class I, the thymocyte will maintain the coreceptor CD8 and become a cytotoxic effector cell. If on the other hand, the thymocyte recognizes MHC class II molecules it will maintain CD4 instead and become a cytokine-producing helper T cell. The importance of MHC molecules for positive selection is demonstrated in mice and humans that lack MHC molecules. In for example MHC class I deficient mice, very few $CD8^+$ T cells will develop but there will be normal numbers of $CD4^+$ T cells, while the opposite is found in MHC class II deficient mice (40).

Although, the TCR need to recognize self-MHC to be useful, they should not recognize self-MHC with self-peptide too strongly since that would lead to autoimmunity. Hence, the T cell repertoire must also be selected in order to be self-tolerant. Negative selection of T cells is performed mainly by bone marrow derived APC in the medulla. T cells with too strong affinity for self-MHC/peptide will receive a strong signal leading to

death by apoptosis (figure 2). Tolerance against self-antigens is thereby achieved by eliminating T cells with self-reacting TCRs (41).

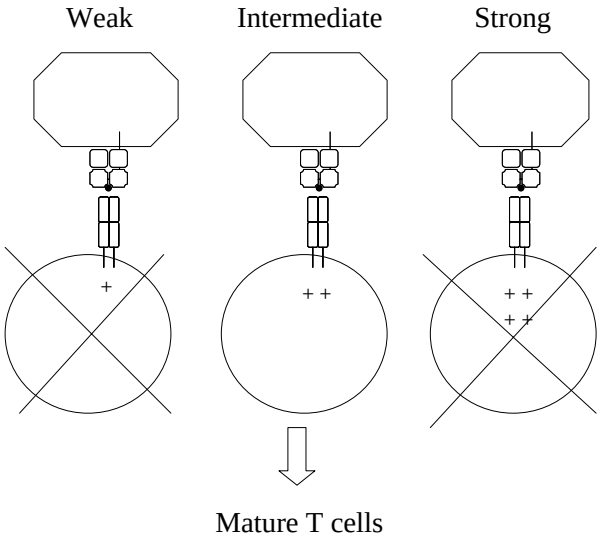

Figure 2. T cell selection. Thymocytes with weak or no binding to self-MHC + self-peptide will not proceed through positive selection. Thymocytes with too strong affinity to self-MHC + self-peptide will be deleted in negative selection. Only thymocytes with an intermediate affinity will be allowed to mature and leave the thymus.

Not all self-antigens are presented in the thymus. To solve this problem there are also peripheral tolerance mechanisms. To get activation of a T cell, the TCR signaling needs to be combined with costimulatory signals. These can only be provided by APCs. The best studied costimulatory molecules are B7-1/CD80 and B7-2/CD86, which are recognized by CD28 on T cells. Other peripheral tolerance mechanisms include down-regulation of TCR or coreceptors, and regulatory T cells (42-46).

$CD8^+$ T cell activation and function

The discovery of MHC restriction, i.e. that recognition by T cells is both antigen specific as well as host MHC specific, gave Doherty and Zinkernagel the Nobel Prize in Medicine 1996 (4). The selection process in the thymus restricts T cells to only recognize self-MHC bound to foreign peptides. However, one specific MHC may bind several different peptides, leading to that one TCR may recognize several similar MHC/peptide complexes. This is called cross-reactivity. Some peptides will induce a full T cell response and those are called agonists. Others may only induce a weak response, and they are called partial agonists. Some peptides will not induce a response

and may even actively prevent the T cell from responding to the MHC/agonistic peptide complex, and they are called antagonists (47). Different peptides may thus induce altered T cell responses. Similarly, the concentration level of the peptide may determine if the T cell will respond or not. If too little peptide is available, there will not be a strong enough activating signal transmitted and the T cell will not respond. Cross-reactivity can also occur when a nonself (allogeneic) MHC molecule binds peptide and the complex resembles the usual complex recognized by the TCR. It is estimated that approximately 1-5% of all T cells are alloreactive. As a consequence, T cells from a host can rapidly attack grafts from allogeneic donors (48).

The activation of naïve T cells occur in peripheral lymphoid organs where they meet professional antigen presenting cells. Naïve T cells express the chemokine receptor CCR7 together with L-selectin (CD62L) that enable them to attach to high endothelial venules and home to LN (49). Once in the LN, the T cell may receive two signals indicating infection. Signal one is from the MHC-TCR interaction, where the TCR recognizes a foreign peptide presented by MHC molecules on APCs. The second signal comes from CD28 that recognize costimulatory molecules, such as B7-1 (CD80) and B7-2 (CD86). These costimulatory molecules are upregulated on APCs upon infection. Once the T cell has received both signal 1 and 2, it will start to express high-affinity IL-2 receptors and to produce IL-2, that will induce proliferation and differentiation into effector T cells (50). T cell activation requires continuous intracellular signaling over several hours to take place (51). At the T cell/APC interface a 3-dimensional supramolecular structure is formed called the immunological synapse (IS). At the IS, MHC/peptide complexes can serially trigger TCRs and hence activate the T cells to form effector and memory T cells (52). After activation, T cells no longer require a second signal and will respond against cells that present the specific foreign antigen. Mature effector T cells express other chemokine receptors and integrins compared to naïve T cells, which directs homing to infected tissues (53).

Cytotoxic T cells contain lytic granules that can be released in response to foreign antigen. The lytic granules contain at least two proteins, perforin, which polymerizes to form pores in target cell membranes, and granzyme, which are serine proteases that induce apoptosis. In perforin knockout mice, almost all killing capacity by $CD8^+$ T cells is lost, and this results in increased susceptibility to viral infections and delayed

tumor clearance (54). However, as will be discussed later, NK cells also use the perforin/granzyme killing pathway, and some results of susceptibility in perforin$^{-/-}$ mice may be due to lack of NK cell killing. Both CD8^{+} and CD4^{+} T cells can also kill target cells via Fas-FasL pathway. Ligation of Fas on the target cell to FasL on T cells induces activation of caspases, which leads to apoptosis in the target cell (54). Cytotoxic T cells can also secrete cytokines, such as IFN-γ, TNF-α and TNF-β. IFN-γ acts by inhibiting viral replication and induces increased expression of MHC class I and molecules involved in peptide loading (55). IFN-γ also activates macrophages, recruiting them to sites of infection and augments their capacity to destroy ingested bacteria.

When the antigen is no longer around, the IL-2 production declines. This and other changes in stimuli will lead to apoptosis in the activated T cells. When a T cell becomes activated, expression of another receptor for B7 molecules will start to be expressed, CTLA-4. CTLA-4 has a 10-20 fold higher affinity to B7-1 and B7-2 than CD28 and will transduce an inhibitory signal. This will contribute to terminate the immune response (56).

Although most T cells die after exerting their effector functions, some T cells differentiate into memory cells. These memory cells remain in the body for a long time, sometimes for life, and they can rapidly be reactivated in case of reinfection. Mice infected with LCMV keep LCMV specific memory T cells for at least a year (57). A population of effector CD8^{+} T cells that express high levels of CD127 (IL-7Rα) has been shown to be the precursors of memory T cells (58). Memory T cells can be divided into two subsets based on their expression of the LN homing receptors CCR7 and CD62L. The CD62L^{Hi}CCR7^{Hi} subset is named central memory T cells (T_{CM}) and is found in spleen, blood and LN while the CD62L^{Lo}CCR7^{Lo} subset is called effector memory T cells (T_{EM}) and is found in spleen and blood but not LN. It is the T_{CM} subset that confers the most effective protective immunity following challenge with virus or bacteria. It is possible that the T_{EM} cells can differentiate into T_{CM} cells and hence are part of the differentiation pathway from naive→effector→T_{EM}→T_{CM} (59). Memory T cells can express Ly49 receptors and this expression increases with time as the mouse ages (60).

NK CELLS

NK cells are large granular lymphocytes that constitute 5-10% of the peripheral blood lymphocytes in humans and approximately 5% of mouse spleen lymphocytes. They were first described in 1975 as a cell population able to spontaneously kill certain tumor cells, i.e. YAC-1 (61, 62). NK cells do not express rearranged antigen-binding receptors, typical of T or B cells. RAG-deficient and SCID mice that can not rearrange Ig or TCR genes have normal NK cell development while they lack B cells and T cells (63). Although NK cells express certain "typical" NK cell markers, such as NKR-P1, DX5, Ly49 and CD94/NKG2, also subsets of T cells can express these markers. Therefore, to define an NK cell requires a combination of criteria. For example, the lack of expression of CD3, TCR or BCR combined with the expression of NKR-P1 (NK1.1) or DX5. In humans NK cells may be defined as $CD3^{-}CD56^{+}$ lymphocytes.

NK cell development

Similar to all immune cells, NK cells are derived from oligopotent cells in the BM. In contrast to T cells however, NK cells do not need the thymus to develop while they critically need the BM to differentiate (64). Since NK cells and T cells share several functional and phenotypical characteristics, it has been suggested that they originate from a common precursor (65-67). The NK cell precursors express IL-2Rβ, but lack many of the typical NK cell markers (68). These immature NK cells gradually acquire surface markers and several functions of NK cells. NKRP-1 and CD2 appear early, followed by DX5. The NKG2 family of receptors precede the Ly49 families and they require interaction with stromal cells (69, 70). Several different cytokines may play sequential and essential roles in NK cell development, for example IL-15 and IL-21 (71, 72). IL-15 knockout mice lack functional NK cells, strongly implying a role for IL-15 in NK cell differentiation (73).

NK cell receptors

A balance between inhibition through inhibitory receptors and activation through activating receptors determines the NK cell response. Most of the inhibitory receptors recognize classical MHC class I molecules. The main MHC-recognizing NK cell receptors found in humans and in rodents belong to different protein gene families. Human NK cells use Killer Cell Immunoglobulin-like receptors (KIR) while the corresponding receptors in mice and rats are C type lectin-like Ly49 receptors. Why

evolution has led into two separate paths is not known. In humans only a pseudogene of a Ly49 receptor can be found, Ly49L (74, 75). However, Ly49 genes are also used in horses, while cats, dogs, pigs, cows and nonhuman primates have Ly49 genes and KIR genes (76-79). Regardless of species and receptor type, diversification and amplification of the receptor family has occurred independently and in parallel. Both humans and mice express C-type lectin like receptors such as CD94/NKG2, while mice in addition express Ly49 receptors. These receptors are encoded in the NK cell gene complex (NKC) located on chromosome 6 in mouse (80).

In both groups of receptors, there are inhibitory as well as activating receptors. Inhibitory receptors mainly mediate their effect through immunotyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domains. Upon ligand binding, the ITIM becomes phosphorylated by a SRC-family tyrosine kinase, which then recruits and activates phosphatases, such as SHP-1 and SHIP (81-83). These phosphatases then block the function of protein tyrosine kinases in the activation pathway. Activating receptors lack ITIM but usually contain a charged amino acid in the transmembrane domain. These receptors can then, upon ligand binding, associate with adaptor proteins, such as CD3ζ, FcεRIγ, or KARAP/DAP12, which all contain ITAMs (84, 85) or to DAP10 (figure 3). This leads to tyrosine phosphorylation of the ITAM, which recruits and activates Syk family tyrosine kinases and downstream signaling pathways. The DAP10 signaling pathway includes Grb-2, PLC-γ2, SLP-76 and PI3K but the signaling pathway seem to converge with ITAM signaling pathway to a common cytotoxic cascade (86, 87).

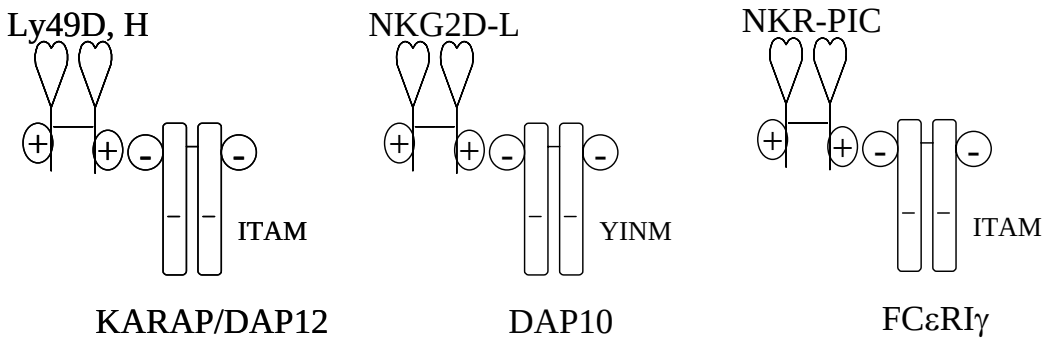

Figure 3. Activating receptors signal through different adaptor proteins.

The Ly49 family

Ly49 receptors are C type lectin-like receptors expressed as disulphide-linked homodimers. Each receptor is expressed on a fraction of NK cells and independently of other receptors expressed by the same NK cell. This leads to expression of Ly49 receptors on partly overlapping NK cell subsets. Ly49 receptors are also expressed on some T cells (discussed below). Ly49A was the first receptor to be described using the monoclonal antibodies YE1/48, YE1/32 and A1 (88-90). Ly49A was first suggested to be a T cell receptor but it was later shown that Ly49A is an inhibitory NK cell receptor. It is expressed on approximately 20% of B6 NK cells and mediates inhibitory signals upon recognition of H-2D^{d}, and H-2D^{k} (91, 92). It may also bind weakly to H-2D^{p} and H-2D^{b} (93-95). Since the discovery of Ly49A as an NK cell receptor, the Ly49 family has grown rapidly. In B6 mice the total chromosomal region containing Ly49 genes has been sequenced. In a cluster of approximately 600kb in the NKC on mouse chromosome 6, there are 15 uninterrupted Ly49 genes. Additionally, there are also three pseudogenes and one more distal but functional gene. The B6 Ly49 haplotype thus contains genes for Ly49A-N, Ly49Q, Ly49X (96). In 129 mice, 18 genes have been found in the Ly49 gene cluster, but there are both allelic differences and strain-specific genes in the two haplotypes (97, 98). There are new Ly49 receptors being discovered continuously in different strains of mice as well as in different species. The majority of Ly49 receptors are inhibitory and bind to one or more MHC class I alleles.

Two other well studied inhibitory Ly49 receptors are Ly49C and Ly49G2. Ly49C is expressed on a major subset of NK cells (30-50%) and is quite promiscuous in its binding to MHC class I molecules (93, 99, 100). H-2K^{b} inhibits NK cell responses through binding to Ly49C (101). The 5E6 antibody used to discover Ly49C has later been found to also bind Ly49I, which complicates the interpretation of early results (102). Another antibody can be used, which selectively recognizes Ly49C, 4LO3311 (103). Ly49G2 is expressed on nearly 50% of NK cells. It can, similarly to Ly49A bind H-2D^{d} (104). In order to bind to H-2D^{d}, Ly49A needs a peptide to be presented in the MHC class I molecule, but any peptide will do, i.e. binding is peptide-dependent but not peptide-selective (105, 106). This does not seem to be a general feature for Ly49 receptors however. Ly49C does appear to be both peptide-dependent and peptide-selective (107).

There are a few activating Ly49 receptors with short cytoplasmic tails that lack ITIM motifs. Ly49D recognizes the same MHC class I molecule as Ly49A and Ly49G2, H-2D^d (108, 109). There is evidence that the Ly49A and Ly49D receptors recognize different parts of the H-2D^d molecules (110). Structurally related inhibitory and activating receptors recognizing the same ligand are commonly found among human and rodent natural killer cell receptors. The role of activating self-reacting receptors is not known but since inhibitory receptors usually have higher affinity for their ligand than activating receptors they counteract activation signals to prevent autoimmunity (111). One hypothesis is that activating receptors have evolved to recognize viral protein or stress-induced ligands rather than MHC class I molecules. For example, Ly49H associates with the adaptor molecule KARAP/DAP12 and recognizes the MCMV encoded protein m157 (112, 113). The paired inhibitory receptor to Ly49H is Ly49I. In some strains where only Ly49I is expressed, recognition of the viral protein results in immune evasion of the virus (112). It is hence, possible that the activating receptor Ly49H evolved from the inhibitory receptor Ly49I in response to the evolutionary pressure of the virus to escape immune recognition.

Cell surface levels of Ly49 receptors are influenced by the MHC class I expression of the host. If a host expresses a MHC class I molecule that the L49 receptor can bind to, the receptor expression level on the NK cells is down-regulated (114). As a consequence, Ly49A expression is low on NK cells from H-2D^d expressing mice strains. On the other hand, in β_2m-deficient mice, which do not express MHC class I molecules, expression of all Ly49 molecules are high (115). The receptor calibration model suggest that expression of Ly49 receptors with specificity for self is calibrated to optimal levels by host MHC class I molecules to sense small alterations in MHC class I expression (116). Both MHC class I molecules on the same cell as well as on surrounding cells can calibrate the Ly49 expression level (117). According to the calibration model, this would ensure that target cells with only a partial loss can be detected and killed by corresponding NK cell, since higher levels of MHC class I is needed to inhibit NK cell function if the Ly49 expression is low. Indeed, Ly49^{high} and Ly49^{low} NK cells recognize tumor targets expressing low levels of H-2D^d differently (118). However, mice transgenic for Ly49A do not downmodulate the Ly49A receptor to the same level in the presence of the ligand H-2D^d (119). Another study showed that Ly49A expression can be upregulated when few cells expressing the ligand is present

in the surrounding (120). These studies argue against a calibration of receptors to a useful level and rather suggest that the expression level is just a consequence of the frequency of ligand interactions. Receptor level modulation has also been found in rat Ly49 receptors, but not in KIRs in humans (121, 122). CD94/NKG2 receptors in humans may however be regulated similarly while the killer cell lectin-like receptor G1 (KLRG1) in mice has an opposite modulation pattern (122, 123). It therefore seems like receptor modulation is characteristic for C type lectin-like receptors.

CD94/NKG2 and NKG2D

At birth, murine NK cells express hardly any Ly49 receptors. The NK cell activity is instead regulated by another C type lectin-like receptor, CD94/NKG2A (70, 124). The receptor is a heterodimer between CD94 and NKG2A but CD94 may also form heterodimers with NKG2C or NKG2E. The CD94/NKG2 receptors are specific for the nonclassical MHC molecules Qa-1 in mice and HLA-E in humans (21, 23, 25, 125). Qa-1 and HLA-E are dependent on β_2m for expression but they bind and presents peptides derived from leader sequences of classical MHC class I molecules (22). Therefore, CD94/NKG2 receptors are able to monitor the status of the classical MHC class I molecules in a cell by using HLA-E or Qa-1 as surrogates. In addition, CD94/NKG2 can recognize HLA-E molecules with peptides derived from viruses, while HLA-E binding of heatshock protein 60 derived peptides may interfere with CD94/NKG2 binding (126, 127). CD94/NKG2A is an inhibitory receptor that contains ITIM motifs in the cytoplasmic domain (128). CD94/NKG2C and CD94/NKG2E are activating receptors that associates with the adaptor molecules KARAP/DAP12 (129).

NKG2D is not very homologous to the other NKG2 proteins even if the gene is located next to the other NKG2 genes in the NKC (128, 130). NKG2D is not expressed together with CD94 but instead as a homodimer. Murine NKG2D recognizes retinoic acid early transcript 1 (Rae-1), histocompatibility 60 (H60) and mouse UL16-binding protein-like transcript 1 (Mult 1) (29, 30, 131). In humans, NKG2D recognizes MHC class-I-chain-related protein A and B (MICA and MICB) and ULBPs (27, 28). These ligands are not expressed at all or only at very low levels on normal cells but are upregulated upon stress, infection or tumor transformation (132, 133). NKG2D mRNA can be alternatively spliced, resulting in two different forms of NKG2D, NKG2D-L (long) and NKG2D-S (short). The long form associates with DAP10 while the short

version associates with KARAP/DAP12. NKG2D provides costimulation or activation to NK cells (134).

Other NK cell receptors

NKR-P1 is another C type lectin-like receptor family with at least five members, NKR-P1A, -B, -C, –D, and -F (135-138). NKR-P1C is an activating receptor without known ligand and it is recognized by the commonly used antibody, NK1.1 (134, 139). Recently, however, it was shown that two members of the NKR-P1 family (NKR-P1D, and NKR-P1F) interact with C type lectin-related (Clr) molecules expressed on dendritic cells and osteoclasts (137, 140). Interestingly, the gene for the Clr ligand is located closely to the gene for the receptor in the NKC. Another commonly used antibody for NK cells is DX5. This antibody recognizes CD49b, which might be involved in NK cell activation (141).

In humans, KIRs can be either activating (with short cytoplasmic tail) or inhibitory (long cytoplasmic tail). The inhibitory forms contain ITIM motifs as the inhibitory Ly49 receptors and similarly, the activating forms of KIRs may associate with KARAP/DAP12. KIRs recognize HLA-A, HLA-B, and HLA-C alleles (142). KIRs are expressed on subsets of NK cells, partially overlapping, similarly as Ly49 receptors in mice. There are thus several similarities between the human KIR family of receptors and the murine Ly49 family. This makes it relevant to study Ly49 receptors in mice despite that humans do not contain functional genes for this family of receptors.

The ILT/LIR/MIR family of receptors contains both inhibitory as well as activating members. ILT1, ILT2/LIR-1 and ILT5 are expressed on NK cells and can interact with HLA molecules and the viral encoded class I-like molecule UL-18 (143, 144). The murine counterparts to these receptors are called paired Ig-like receptors (PIRs) but they have not been found on NK cells (145).

Natural cytotoxic receptors (NCRs) have been described in humans, namely NKp30, NKp44 and NKp46 (146). A murine homologue of NKp46 has been described (147). There are also several coreceptors expressed on NK cells. One is CD59, which functions physically associated with NKp46 and NKp30 (148). The ligands for NCRs are currently unknown and once revealed may give further understanding to how NK

cells can reject targets which do not express any known activating ligand. Recently, CD69 a common activation marker was shown to be able to inhibit NK cell activity against tumors (149).

The missing-self hypothesis

The missing-self hypothesis was coined by Klas Kärre in his doctoral thesis in 1981 (150). It is proposed that, in contrast to T cells, NK cells kill target cells because they lack MHC class I molecules present in the host. This was shown by injecting a MHC class I deficient tumor, RMA-S, into B6 mice. This tumor was readily rejected while the parental MHC class I expressing tumor cell line RMA was not. Treatment of the mouse with anti-asialo GM1, which remove NK cells, resulted in tumor outgrowth of both tumors, indicating a role for NK cells in rejection of MHC class I deficient tumors (151, 152). Two models were proposed to explain the missing-self hypothesis. One, referred to as the "target interference" model, suggested that expression of self MHC class I molecules masked potential activating ligands on the target cell. The other model, referred to as "effector inhibition", suggested that self MHC class I molecules delivered inhibitory signals to the NK cell, after initial triggering by activating receptors recognizing broadly expressed ligands. Confirmation of the effector inhibition model for the missing-self hypothesis came when the Ly49A receptor was shown to mediate inhibition when bound to its ligand H-2D^d (91). Later it was shown that it is not only the total absence of MHC class I that can trigger NK cells to kill but more specifically that the target cells lack one or more MHC class I molecules present in the host environment of the NK cell (figure 4). NK cells in D8 mice (B6 mice transgenic for H2-D^d), rapidly reject bone marrow and lymphoma grafts from B6 mice (153). As a consequence, the altered expression of MHC class I during many virus infections or tumor transformation, opens the possibility that the affected cells may become targets for NK cells. Inhibition of NK cells may not only go through MHC class I molecules. It has been shown that 2B4 (CD244), which binds to CD48, can inhibit NK cell function (154).

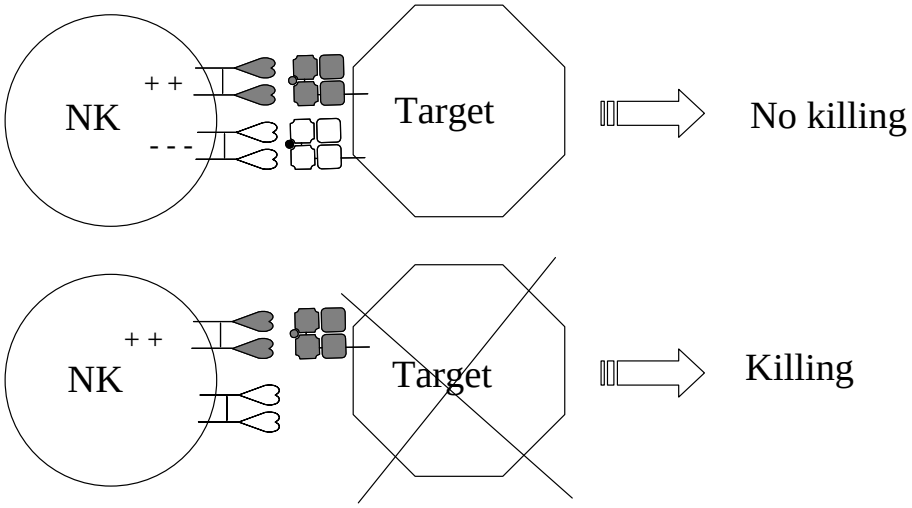

Figure 4. Missing-self. NK cells express inhibitory receptors that recognize MHC class I on target cells, which overrides activating signals and therefore prevents killing.

Although NK cells sense the lack of or reduced levels of MHC class I molecules, they do not attack all cells without MHC expression. One example is erythrocytes that have no MHC class I expression. Therefore it should be noted that NK cells need initial activating signals to be able to kill a target, as indeed proposed in the effector inhibition model of the missing-self hypothesis. How NK cells are activated were a mystery for a long time. Now, many activating receptors on NK cells are known. Some of them may bind viral derived proteins, as described for Ly49H above (112, 113). It is believed that all NK cells need to express at least one self MHC class I molecule to prevent autoimmunity. In most cases the inhibitory signal will override the activation signal, preventing cells expressing self MHC class I molecules from being killed (155, 156), although a balance been inhibition and activation probably determines the outcome.

In vitro and in vivo activity of NK cells

Cytokines affect the development and activation of NK cells. IL-2, IL-12, IL-15, IL-18 and IL-21, as well as IFN-γ can induce proliferation and activation of NK cells (157-159). IL-15 is crucial for NK cell development and for homeostatic expansion (160). The combination of IL-12 and IL-18 is particularly effective in augmenting NK cell activity (161). In experimental systems, NK cells can be activated in vivo by interferon type I inducers, such as poly I:C or Tilorone (162). In vitro, high doses of IL-2 are commonly used as well as IL-12 in combination with IL-18, to stimulate NK cells prior to functional assays.

Since their discovery, NK cells have been analyzed experimentally mainly through their capacity to kill tumor cell lines in vitro and in vivo. But NK cells have been shown to execute other functions too. They produce different cytokines, especially IFN-γ that can regulate the development of acquired immunity (163, 164). For example, secretion of IFN-γ is important in the early clearance of MCMV in mice (165). There is evidence indicating that NK cells play a role in resistance to MCMV, HSV, influenza virus and Coxsacki virus in mice and HSV, EBV, HCMV and papilloma virus in humans (166). The best evidence supporting a role in defense against human viruses comes from studies of individuals that lack NK cells or NK cell activity. The first clinical observation was in a young woman that developed severe varicella, HCMV and HSV infections (167). She was shown to lack NK cells but it is still not clear whether it was a genetic defect. Patient with mutations in the FCγ receptor III (CD16) have partial NK cell defects. CD16 binds the Fc portion of some antibodies, and if crosslinked it mediates antibody dependent cellular cytotoxicity (ADCC) (168). These patients suffer from recurrent viral infections (169, 170).

NK cells can reject bone marrow grafts from allogeneic as well as semisyngeneic (parental) donors. Rejection in the latter situation is called F1 hybrid resistance. F1 offspring will reject marrow grafts from either of its parents (171). Since the F1 will express the combined MHC class I haplotype of both its parents, T cell mediated allorecognition should not be involved according to the rules of transplantation. Instead it is the absence of “self” MHC class I that paves the way for the NK cells in the F1 to reject either parental graft. This phenomenon has been explored in bone marrow transplantations of patient with leukemia. When grafts with some degree of KIR ligand mismatch were used, leukemia patients suffered from fewer relapses and displayed less graft versus host disease. In mouse systems it was shown that NK cells were able to kill leukemia cells and also host bone marrow derived APCs preventing presentation to grafted T cells. The NK cells were also able to prevent host versus graft reactions since they killed of any remaining T cells in the host (172, 173). Recent studies have identified NK cells as possible killers of autologus antigen presenting cells (APC), i.e. DC, and syngeneic neurons in vitro (174-177).

Interestingly, NK cells are also found in the uterine mucosa. They accumulate at the placental implantation site, where they are in close contact with placental trophoblast cells. However, the exact function of uterine NK cells is unknown. Uterine NK cells express high levels of CD56 as well as KIRs but they do not express the CD16 receptor. Uterine NK cells have lower cytotoxic activity and probably act through secreting cytokines (178). NK cells have been implicated in recurrent pregnancy loss as well as in the development of pre-eclampsia (179, 180).

NK cells mediate cytotoxicity mainly through perforin and granzyme, but they may also use FasL and TNF-related apoptosis inducing ligand (TRAIL) to kill target cells (54). Both FasL and TRAIL can induce apoptosis when bound to receptors on the target cells. TNF-α, which is produced by NK cells in response to various cytokines, may also mediate activation-induced cell death (181, 182).

NKT CELLS

Although many of the NK cell receptors described above, such as Ly49, NKR-P1, DX5, 2B4 are associated primarily with NK cells, some T cells may also express these markers (60, 183-185). NKT cells are found in many organs but mainly in the spleen (1% of splenocytes), among mature thymocytes (10-20%) and in bone marrow and liver they account for 20-30% of the T cells. In all these organs the absolute numbers are fairly similar, approximately 1 million cells per organ (186).

Definition

It is not straightforward to define an NKT cell. Originally, NKT cells were characterized in mice as cells expressing both a TCR and NK1.1. The majority of these cells are selected by the nonclassical MHC molecule CD1 in the thymus and express an invariant TCR (Vα14-Jα18) paired with Vβ8.2, Vβ7 or Vβ2. These "classical" NKT cells are usually $CD4^-CD8^-$ or $CD4^+CD8^-$ (187). Although they usually express NK1.1, this marker is downregulated upon activation (188, 189). There are also $NK1.1^-$ NKT cells in the thymus, which are immature precursors to NKT cells (190, 191). There are several other categories of NKT cells described. Some are CD1-dependent but express a more diverse TCR repertoire (192-194). Then there are T cells that are not CD1-restricted but have an unbiased TCR repertoire and express both NK1.1 and DX5. Many of these cells are actually T cells acquiring certain NK cell markers after

activation or differentiation into memory T cells and should not be termed NKT cells (187, 195-197). When I discuss NKT cells during this thesis I refer to "classical" NKT cells unless otherwise stated (see Table 1).

	Classical NKT cells	Non-classical NKT cells	CD1d-independent T cells
CD1d dependent	Yes	Yes	No
TCR repertoire	Restricted Vα14-Jα18, Vβ8.2, Vβ7 and Vβ2	Semi-diverse Some Vα3.2-Jα9, Vα8, Vβ8.2	Diverse
NK1.1 expression	+ (resting and mature) -/low (immature or post-activation	+/-	+
Subsets	$CD4^+$ and DN	$CD4^+$ and DN	$CD4^+$, $CD8^+$and DN

Table 1. Categories of mouse NKT cells. Partly adapted from Godfrey et al. (197).

CD1 restriction

Several studies in different knockout mice provided clues to how and where NKT cells arise. NKT cells are not found in β_2m-deficient mice since expression of CD1d is β_2m dependent (198). NKT cells develop normally in TAP-deficient mice since CD1d does not present peptides as MHC class I molecules do, but rather presents lipids (199). Definite proof for a role of CD1 molecules came from CD1-deficient mice, which lack all classical NKT cells (200, 201). Until recently, the endogenous ligand recognized by NKT cells in mouse was not known. In experimental models, a synthetic glycosphingolipid called α-galactosylceramide has often been used to stimulate NKT cells (32). Zhou et al. has recently though presented evidence that a lysosomal glycosphingolipid (iGb3) can be recognized by both human and mouse NKT cells. In mice deficient for this lipid, NKT cells do not develop, suggesting that iGb3 is involved also in the development of NKT cells (33).

Function

NKT cells respond rapidly to TCR stimulation and are able to activate a number of immune responses. Activated NKT cells can produce both Th1 cytokines, (IFN-γ) and Th2 cytokines (IL-4) (202, 203). NKT cells can stimulate many cell types. After initial activation, NKT cells produce IFN-γ and IL-2 that in combination with IL-12 produced by DC can lead to activation of NK cells and memory $CD4^+$ and $CD8^+$ T cells (204-208).

In tumor surveillance, NKT cells may contribute by modulating NK cell function through IFN-γ production (209). NKT may also suppress anti-tumor immunity of cytotoxic T cells by producing IL-13 in some circumstances (210).

In bacterial infections, several studies have suggested roles for NKT cells. In Pseudomonas aeruginosa infection, CD1d-restricted NKT cells may contribute to bacterial clearance by activating macrophages and recruitment of neutrophils (211). CD1d-deficient mice were shown to be susceptible to the spirochete Borrelia burgdorferi while wt mice were resistant, indicating a role for NKT cells in modulating B cell activation and antibody production during infection (212). NKT cells seem to respond to Mycobacterial lipids and may have some effect in the immune response against Mycobacterium tuberculosis and M. bovis although some studies have shown no susceptibility difference in CD1d-deficient mice (213-215). During some infections, NKT cells may not have a beneficial effect but rather the opposite, as in Salmonella choleraesuis (216). NK cells have also been shown to be beneficial in several virus, fungal and parasitic infections (31).

NKT cells have been implicated as important in autoimmune reactions. In several human autoimmune diseases, reduced levels of NKT cell frequency in the periphery correlated with disease (217). In NOD mice, which spontaneously develop autoimmune diabetes, reduced levels and functional deficiencies of NKT cells have been found (218-220). By reconstituting NKT cells, diabetes could be prevented (221, 222).

CD1d-independent T cells with Ly49 receptors

As described above, a subpopulation of $CD8^+$ T cells express NK cell markers such as Ly49 receptors. During viral infections or other activation stimuli, T cells with

expression of both inhibitory and activating Ly49 receptors emerge (184, 195, 223, 224). Memory T cells also express Ly49 receptor and these increase over time as the mouse ages (60). These data indicate that Ag-mediated signals may initiate expression of NK cell receptors on $CD8^+$ T cells. Apart from controlling NK cell activation, inhibitory Ly49 receptors may also down-modulate T cell effector functions in vitro and in vivo (185, 225-227). It has been suggested that one role of Ly49 receptors on $CD8^+$ T cells, and why they are upregulated, could be to maintain peripheral tolerance of T cells that may otherwise respond to self antigens (228). But exactly why and which T cells that express Ly49 receptors are still unclear.

USE OF TRANSGENIC AND KNOCK-OUT MICE

Most of the studies in this thesis are based on well established methods, which will not be discussed here in detail, with one exception; the use of transgenic and knock-out mouse strains. The ability to change the mouse genome by either introducing new genes or direct targeting of a gene to remove it or replace it by a mutated copy has many advantages. However, there are pitfalls that need to be taken seriously when evaluating results obtained from genetically altered mice. Many of the phenotypes examined in transgenic or knockout models are influenced by the genetic background in which they are studied. Allelic variations between background genes in different mouse strains may significantly alter the outcome of gene targeting. One example comes from the IL-18-deficient mouse. IL-18-deficiency on the 129xCD1 or DBA/1 background resulted in higher susceptibility to Leishmania major infection. However, IL-18 deficiency on the B6 background had very little effect (229). Sometimes, it is not only the genetic background that may alter results, also environmental factors may shift one response towards another (230). This may lead to misinterpretations of the results obtained, although sometimes it may give us clues to how a phenotype is influenced by other modulating factors (231).

Another problem with transgenic mice is that the transgene is randomly inserted into the genome. Depending on where it is inserted, different levels of the transgene may be expressed. It is therefore advisable to examine several different founder lines before drawing conclusions. Yet another problem is caused by the generation of the mice since it is easier to generate transgenic mice by using hybrid strains. This is because hybrid strains generally reproduce better and have higher quality embryos for microinjection

(232). These hybrid founders are then backcrossed to the mouse strain of choice, usually B6.

When performing gene targeting in embryonic stem cells (ES), the most common ES cell line used is derived from the 129 mouse strain. As there are many different 129 substrains, comparing results from laboratories using different strains become difficult. Problems may arise when the gene targeted founder line is backcrossed to the preferred strain. If few backcrosses are made a significant portion of the 129 genome will be present in the gene targeted mouse. In each backcross, the genetic composition will become more and more similar to the wanted strain but it takes six generations of backcross breeding to get genetic backgrounds that are > 99% homogenous (233). There will still always be genes from the 129 background flanking the targeted gene. Another problem is mutations. Mutations can occur already in the ES cell line used (234), or during backcrossing and they are very difficult to account for as they appear spontaneously.

From a practical standpoint, transgenic and knockout mice are usually studied long before congenic strains are generated. It is then very important to include perhaps both littermate and inbred (129 and B6 in most cases) mice as controls and be aware of the possibility that the phenotype seen in the mouse tested may not be the result of the specific gene that is being examined. If possible, experiments using independently generated gene altered mice should be used as well as mice on different backgrounds.

AIMS OF THIS THESIS

When I started my thesis work, it was known that some T cells express NK cell markers, but it was not known whether these receptors could change the development or activity of T cells.

Our first aim was to study in vivo whether the expression of an inhibitory Ly49 receptor on T cells could alter T cell development in the absence or presence of the specific MHC class I ligand.

Secondly, we aimed at resolving how inhibitory signals from a Ly49 receptor could influence T cell responses and specifically if it was possible to titrate the activating and inhibitory signals to quantitatively shift the balance of the response.

Many studies have verified the missing-self hypothesis both in vitro and in vivo. However, these studies all had their limitations due to the use of tumor cells or rapidly proliferating cells.

Our aim was to critically examine missing-self rejection in vivo of healthy resting cells in unmanipulated hosts. In addition we aimed to study the role of the KARAP/DAP12 signal transduction pathway in rejection of different types of MHC mismatched cells.

During the study it became clear that one strain of CD1d-deficient mice had an unexpected defect in rejection of cells displaying a missing-self phenotype.

In the last paper our aim was to characterize the defect in missing-self rejection in one strain of CD1d$^{-/-}$ mice further and in particular resolve if NKT cells are involved in missing-self rejection or not.

RESULTS AND DISCUSSION

In the first two papers we examined how an inhibitory NK cell receptor, Ly49A, could affect the development, selection and function of NK cells and T cells. We generated Ly49A transgenic mice using an altered human CD2 promotor, which generated high expression of Ly49A on all T cells, NK cells and thymocytes from an early stage of development.

PAPER I: LY49A EXPRESSION ON T CELLS ALTERS T CELL SELECTION

Paper I describes he generation of Ly49A transgenic mice. To avoid problems with background strain and flanking genes, the transgene was produced using B6 mice as both egg donors and fertile males. Although this may be less efficient than using other mouse strains as donors, we were able to obtain several founder lines expressing high levels of the transgene Ly49A.

Is H-2D^b a ligand for Ly49A?

In cytotoxic assays using IL-2 stimulated spleen cells (lymphokine activated killer (LAK) cells), we verified that the Ly49A transgene was able to recognize its MHC class I ligand, H-2D^d, and mediate inhibition. In most respects, Ly49A transgenic mice on a B6 (H-2^b) background exhibit similar characteristics as non-transgenic littermates. However, they differ in some in vitro assays for cellular immune responses. We observed that LAK cells from Ly49A transgenic mice killed the normally rather NK resistant target RMA much better than non-transgenic LAK cells. Indeed the Ly49A transgenic LAK cells killed RMA much better than the TAP-deficient variant RMA-S and the "standard" NK cell target YAC-1, while the opposite was seen in nontransgenic mice. Depletion of T cells or NK cells prior to the assay showed that T cells were responsible for killing of RMA cells while NK cells killed RMA-S and YAC-1. Even if these T cells could have been tumor specific, this indicated that autoreactive T cells might have been generated in the Ly49A transgenic mice, since they killed tumor cells with an autologous MHC phenotype (H-2^b). How can T cell selection be altered in H-2^b mice that do not express any strong ligand for Ly49A? Ly49A binds strongly to its ligands H-2D^d and H-2D^k, but may also bind weakly to H-2D^b (93, 94). The level of Ly49A expression is also lower in B6 mice compared to β_2m-deficient mice suggesting

a ligand in B6 (H-2^b) mice capable of down modulating Ly49A (226, 235). Since we obtained Ly49A transgenic mice with very high levels of transgene expression, a weak ligand such as H-$2D^b$ may have been enough to slightly alter the T cell selection, allowing autoreactive T cells to mature. We reasoned that for these T cells to become autoreactive in the periphery strong activation stimuli were needed, and hence the effect could only be seen after in vitro activation with IL-2.

Can Ly49A inhibitory signaling alter T cell selection?

Ly49A may be able to modulate TCR signaling by recruiting SHP-1. When Ly49A binds its ligand, the ITIM motif will become phosphorylated and recruit SHP-1 (81, 83). SHP-1 has many substrates and can regulate the activity of ZAP-70 to suppress TCR signaling (82, 236). Ly49D/DAP12 transgenic mice have been developed. On a H-2^b background, no significant differences in T or NKT cell development was seen, consistent with the fact that Ly49D do not bind to H-2^b MHC class I molecules. However when they crossed these mice to a H-2^d background, a severe reduction in NKT cell development was seen. Coexpression of a Ly49A transgene could partially rescue NKT cell development (237). In order to test the hypothesis that the Ly49A transgene expression lead to altered T cell selection and autoreactive T cells, we crossed our Ly49A transgenic mice to a H-2^d background (D8 or BALB/c). We found that the thymus was extremely reduced in size and peripheral lymphoid organs contained reduced numbers of T cells. Although Ly49D and Ly49A have opposite activities, both seem to influence positive and negative selection of T and NKT cells in the thymus. In Ly49D/DAP12 transgenic mice this would result in fewer self-reacting CD1-restricted NKT cells in the periphery since additional positive signaling would result in that more NKT cells failed negative selection. This is likely since NKT cells probably have rather high avidity for self to start with. Autoreactive NKT cells have been demonstrated (238). Potentially, there might also be more useless T cells that cannot sense self-MHC, which would be allowed to mature in Ly49D/DAP12 transgenic mice. In our Ly49A transgenic mice, the opposite would occur. On a H-2^d background, there would be increased amounts of negative signaling during T cell selection. T cells with a low but usually sufficient affinity for self-MHC would die by neglect due to failed positive selection since the inhibitory signal from Ly49A would reduce the threshold below what is needed for positive selection. Conversely, inhibition from Ly49A would allow autoreactive T cells to mature since the threshold for negative

selection would be altered (figure 5). As a result, there would be fewer T cells able to mature but these might exhibit an autoreactive phenotype. One may reason that this would be of no consequence since the peripheral T cells would also express Ly49A, and could hence be inhibited. Why this may not be the case will be discussed below.

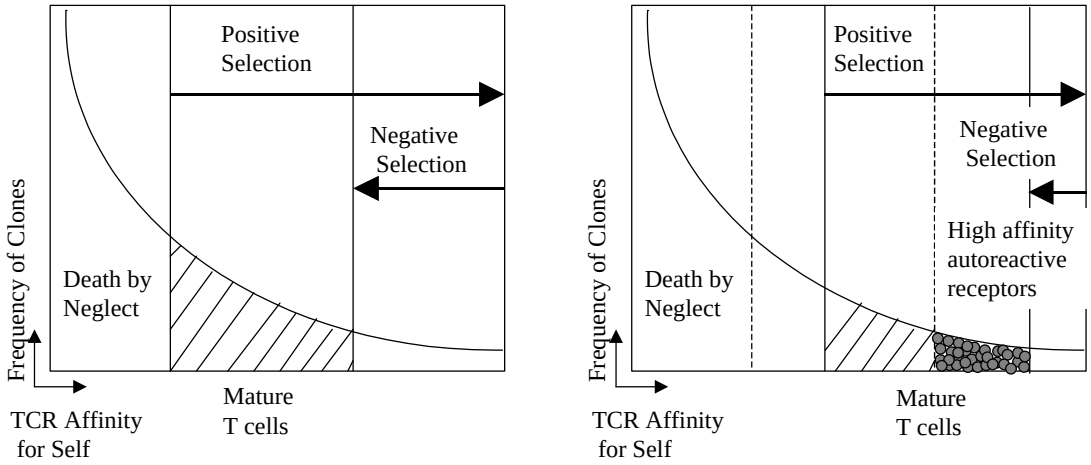

Figure 5. Model of thymic selection. Activation of the Ly49A transgene would be expected to shift the receptor threshold for both positive and negative selection as seen in the right figure. Note that the T cells that mature will be both quantitatively as well as qualitatively different from the normal situation. Adapted from Pauza et al (239).

Why do Ly49A transgenic mice on a H-2^d background develop disease?

When we crossed the Ly49A transgenic mice to a H-2^d background (D8 or BALB/c), introducing a strong ligand for Ly49A, the offspring did not only show reduced T cell numbers but also developed a severe inflammatory disorder with a high death rate within the first weeks of age. Histological examinations displayed a massive cellular infiltration of the heart and the liver, consisting mainly of lymphocytes, granulocytes, fibroblasts, and plasma cells. These findings were very similar to findings in another Ly49A transgenic mouse strain published at the same time as our study (239). As the authors of that study pointed out, the disorder resembled the picture seen in graft-versus-host disease (240, 241).

Since we had indications that T cell selection may be altered we examined if Ly49A could interfere with superantigen induced negative selection. Some antigens that bind to MHC class II molecules are not presented as peptides in the peptide binding cleft.

Rather they simultaneously bind to the Vβ domain of the TCR and to the MHC molecule. These viral or bacterial proteins are called superantigens as they can activate large number of T cells. In mice, superantigens also induce negative selection of entire Vβ-families. If the superantigen is present in the thymus, either as exogenous antigen or endogenously expressed, it will induce apoptosis of the majority of T cells expressing the specific Vβ-chain the protein binds to (41, 242). BALB/c mice endogenously express a viral superantigen that specifically binds to Vβ chains 3, 5, and 11, and these subsets are thus deleted in the thymus (243-246). In contrast, $CD4^+$ T cells with these TCR chains were not depleted in Ly49A transgenic mice crossed to BALB/c. This data indicated that the selection process had been altered by the presence of the inhibitory receptor. In the human system, MHC class I specific inhibitory receptors can affect superantigen-induced responses (247). An altered T cell selection process as described above and in figure 5 may thus explain how autoreactive T cells are allowed to be selected and mature in the thymus. But it does not explain why these T cells are not controlled in the periphery by the Ly49A-H-$2D^d$ inhibition.

Why does not peripheral tolerance via Ly49A control autoimmunity?

Failing to negatively select autoreactive T cells in the thymus would not cause any problem if the Ly49A transgene encoded receptor modulated the TCR signaling also in the periphery. Previous studies have shown that mature peripheral T cells and thymocytes have different thresholds for activation (248, 249). Immature thymocytes are more sensitive to TCR stimulation than mature T cells allowing T cells recognizing low-affinity MHC/peptide complexes to be positively selected. In the periphery, these self-complexes will not elicit any response since the mature T cell requires more stimulation. It is therefore even more puzzling that Ly49A inhibition could not turn the autoreactive T cells off in the periphery. However, Ly49A expression levels are lower on peripheral T cells than in the thymus, which may result in less inhibition in the periphery. Both positive and negative selection as well as activation of mature T cells is influenced by costimulatory signals from CD28/B7 interactions (250-252). It is possible that these costimulatory signals affect immature and mature T cells differently and that they may also interfere with Ly49A inhibition. Autoreactive T cells allowed to escape negative selection recognize self-antigen. It is therefore also possible that persistent T cell activation may override Ly49A inhibition. Not all Ly49A transgenic mice on a H-2^d background developed the inflammatory disorder and the reason for this

is not clear. But it probably depends on the exact level of inhibition versus activation received by the thymocytes during selection and in the periphery. A similar disease has been observed in Ly49G2 transgenic and an even more pronounced disease was seen in Ly49A/Ly49G2 double transgenic mice (253). However, there are also Ly49 inhibitory receptor transgenic mice that do not display this phenotype (119, 254, 255). It is likely a question of level of transgene expression combined with receptor-ligand interaction strength that determines if the inhibitory signal from Ly49 receptors is high enough to significantly alter T cell selection.

PAPER II: LY49A EXPRESSION ON T CELLS ALTERS THE THRESHOLD FOR T CELL RESPONSES

How can NK cell receptors modulate T cell responses?

Expression of Ly49 receptors can be found on a subpopulation of $CD8^+$ T cells. Especially memory T cells express inhibitory Ly49 receptors and these increase over time as the mouse ages (60). However, the TCR repertoire of $Ly49^+$ $CD8^+$ T cells is polyclonal and not restricted as in NKT cells. Analogous to the regulation in NK cells, Ly49 receptors are capable of regulating T cell responses. Using a T cell hybridoma, Ly49A was shown to inhibit TCR/CD3-induced IL-2 production as well as activation-induced cell death (AICD) (227). The effect was due to signals mediated by the intracytoplasmic domain and not by Ly49A interfering with binding between the coreceptor CD8 and MHC class I. It has been suggested that the Ly49A binding site partially overlaps with the CD8 binding site on MHC class I molecules (256, 257). Other studies have revealed roles for Ly49A on T cells in both viral and tumor settings (225, 226). During infection with LCMV few antigen-specific T cells express Ly49 receptors while CD94/NKG2 is upregulated. However, engagement of CD94/NKG2 with its ligand $Qa\text{-}1^b$ does not seem to affect the T cell response (224, 258). About 2 % of the T cells after LCMV infection express Ly49G2. Although these T cells are able to kill target cells pulsed with the LCMV peptide Gp33-41, this killing is inhibited if the target cells express the ligand for Ly49G2, $H\text{-}2D^d$ (259). The exact rules for which T cells that express Ly49 receptors and why are still unclear.

An important question was whether inhibition through Ly49 receptors could completely abolish T cell activity or whether it was a matter of quantitative balances

that determined the outcome. Inhibitory receptor signaling has been shown to override activating receptor signaling in NK cells in many studies although there are exceptions (110, 260). To evaluate this, we crossed our Ly49A transgenic mice with TCR transgenic mice (TCR318). The TCR318 mice are transgenic for the Vα2-Vβ8.1 TCR specific for an LCMV derived peptide presented by H-2D^b (Gp33-41) (261). To avoid the autoimmune like disease observed in Ly49A transgenic mice on a H-2^d background (paper I), we only used mice from H-2^b (B6) background. This allowed us to set up an in vitro system where the activation signal could be modulated by addition of different amounts of the specific peptide. Additionally, we used two different founder lines expressing slightly different levels of the Ly49A transgene. Both founder lines have sufficient expression level of Ly49A to make it functional and able to mediate inhibitory signals upon ligand binding (262).Thus we could modulate the activation as well as the inhibitory signal and hence examine the balance between inhibition through Ly49A and activation from the TCR.

How does Ly49A modulate T cell selection in a TCR transgenic mouse?

Similar to what was seen in paper I, the expression of Ly49A on the transgenic T cells lead to an altered T cell selection. We detected reduced number of T cells in the periphery and in the thymus of T cells expressing the transgenic TCR specific for the LCMV peptide Gp33-41 (Vα2-Vβ8.1). In the thymus the reduction in TCR usage could not be seen among CD8$^+$ CD4$^+$ thymocytes. It was only found when we looked into the single-positive compartments, suggesting that the Ly49A inhibition caused many of these T cells to fall beneath the level for positive selection (see figure 5, Paper I). We saw the strongest effect on T cell selection in the mice expressing the highest level of Ly49A, suggesting that T cell selection is based on a balance between activation and inhibition. As described in the discussion of Paper I, T cell selection could be altered in H-2^b mice since Ly49A has a weak ligand (H-2D^b) in these mice. (94). It is also possible that Ly49A interfered with the binding between MHC class I and the TCR since Ly49A may interact with the coreceptor CD8, and therefore blocked TCR activation signals. However, similar altered selection was observed for CD4$^+$ T cells in Ly49A/TCR double transgenic mice with a TCR specific for MHC class II (239).

Is Ly49A a ”fail-safe” mechanism that always provides a global inhibitory signal?

In order to quantitatively investigate how Ly49A inhibitory signals may effect T cell proliferation we pulsed spleen cells from H-2^b or H-2^b/H-$2D^d$ with increasing doses of the agonistic peptide Gp33-41 and observed how well TCR transgenic T cells were able to proliferate. T cells expressing Ly49A proliferated dramatically less when H-$2D^d$ expressing cells were used as stimulators in vitro. Similarly, the cytotoxic response against target cells expressing the ligand H-$2D^d$ was reduced for T cells expressing Ly49A. However, this was dependent on the concentration of the peptide added. At high concentrations and hence strong activation stimuli, the T cells could respond as strongly to H-$2D^d$ expressing cells as to cells lacking the specific Ly49A ligand. This indicated that even in the presence of strong inhibition from the Ly49A receptor, TCR signaling could override this inhibition if enough positive signals were provided. We also observed a difference between the two founder lines of Ly49A used. T cells with the highest level of Ly49A required more activating signal to override the inhibitory signal. Thus, both the amount of antigen given and the expression level of the inhibitory receptor could influence the activation balance and determine the response. Quantitative balancing of activation thresholds has been shown previously for CD2 in T cell activation (263). Still the possibility remains that Ly49A might affect the binding between TCR and MHC class I due to interaction with the coreceptor CD8 and therefore affect the response.

A possible function for the Ly49A inhibitory receptor on T cells in nontransgenic, physiological conditions, could be to prevent crossreacting self-peptides from eliciting an immune response. The majority of T cells recognizing self-peptides are eliminated in the thymus but if the self antigen is not presented in the thymus, peripheral tolerance mechanisms must exist. Signals from inhibitory receptors could potentially lower the activation signals received from a TCR-MHC/self-peptide interaction below the threshold for response and hence prevent cross-reactivity. We tested this hypothesis using the TCR/Ly49A double transgenic mice. Proliferation against B6 spleen cells pulsed with the strong agonist Gp33-41, which is recognized by the transgenic TCR, was high and consistent but was low against D8 (H-2^b, H-$2D^d$) spleen cells. We then used a crossreacting, partially agonistic self peptide, mDBM (264), and observed proliferation when the peptide was pulsed on B6 spleen cells but it required a higher

dose of peptide compared to GP33-41. When we pulsed D8 spleen cells with mDBM no or extremely low proliferation could be seen even at high peptide dosages (figure 6). This suggests that Ly49A on T cells could possibly prevent T cells from crossreactivity.

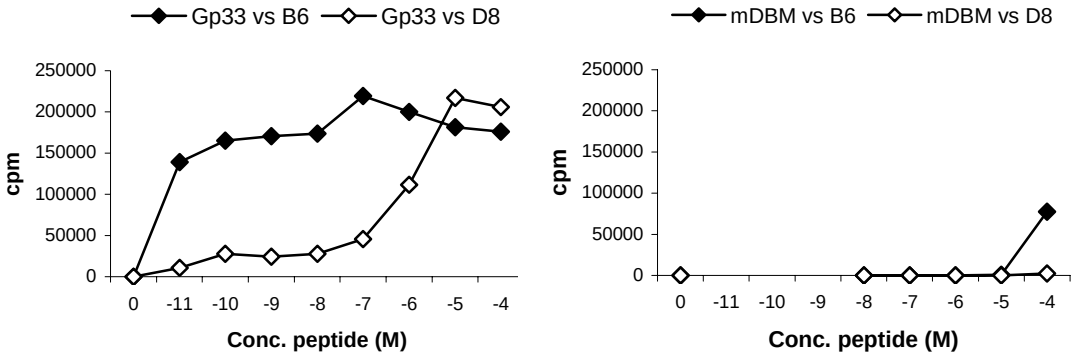

Figure 6. Proliferation of TCR/Ly49A T cells against spleen cells pulsed with the strong agonist Gp33-41 or the cross-reactive selfpeptide mDBM.

PAPER III: LOSS OR MISMATCH OF MHC CLASS I IS SUFFICIENT TO TRIGGER NK CELL-MEDIATED REJECTION OF RESTING LYMPHOCYTES IN VIVO - ROLE OF KARAP/DAP12-DEPENDENT AND -INDEPENDENT PATHWAYS

Several studies have supported that NK cells can reject cells with a missing-self phenotype. NK cell rejection has been extensively studied using bone marrow grafts. However, BM cell rejection assays have their limitations. They require irradiation of the recipient mouse and measures mainly rejection of activated proliferating cells. Tumor cell based assays are also widely used to examine NK cell activity as many tumor cells display high sensitivity to NK cell killing, probably due to enhanced expression of activating ligands (265, 266). Although still used extensively, questions regarding the natural role for NK cells in tumor surveillance have been raised. Assays using radioactively labeled tumor cells have been used to evaluate NK cell cytotoxicity in vivo (153, 267, 268). These assays are limited to cells that can be radioactively labeled and require radioactivity. We wanted to study the in vivo role of NK cells under normal physiological conditions; i.e. what happens when normal cells in the body for one reason or another loose MHC class I expression?

In vivo NK cell cytotoxicity assay-the method.

To be able to avoid irradiating the recipient mouse or use tumor cells we used fluorescently labeled cells (figure 7). We used and further developed a method that had recently been described for T cells (269). By labeling target cells (for example normal spleen cells or bone marrow cells) and control cells with different amounts of carboxyfluorescein succinimidyl ester (CFSE), we were able to track rejection rate of target cells compared to control cells in vivo. The advantages using this method are that it measures NK cell rejection in vivo, in an unmanipulated host, and of nonactivated naïve cells (spleen or bone marrow cells).

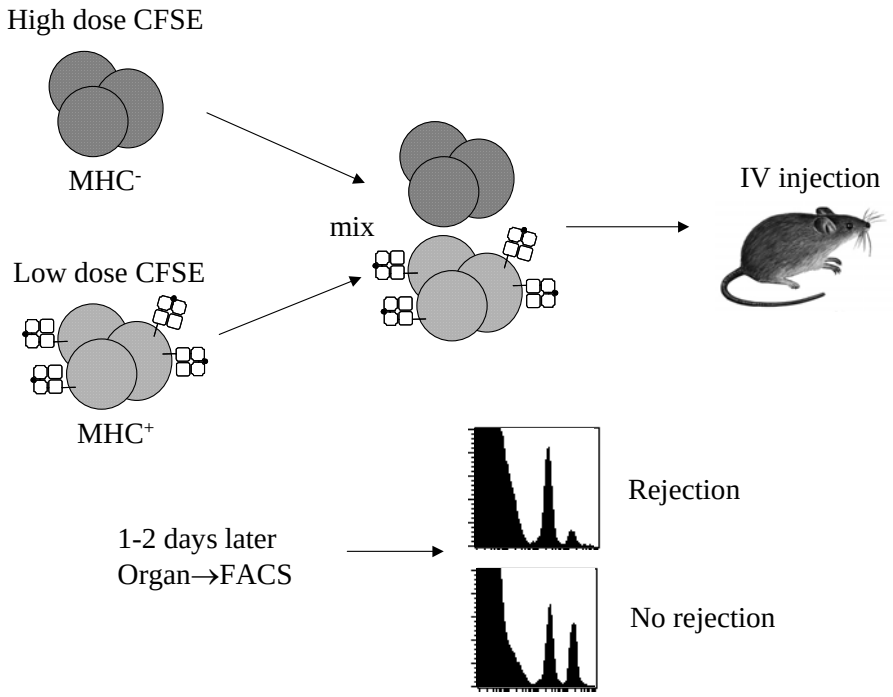

Figure 7. The CFSE in vivo method. MHC^- and MHC^+ cells are labeled with different concentrations of CFSE, mixed 1:1 and injected i.v. into mice. The ratio of remaining $CFSE^{high}$ and $CFSE^{low}$ cells is determined by FACS.

NK cells are relatively radiation resistant compared to T cell or B cell and hence there are a higher proportion of NK cells in a mouse after irradiation, even if the absolute numbers do go down (270). If NK cells require help from another lymphocyte population this could hamper the NK cells effect in irradiated mice. However, data suggested that irradiating the host prior to injecting the cell mix, did not affect the NK cells ability to reject MHC^- bone marrow cells during a short time frame (figure 8).

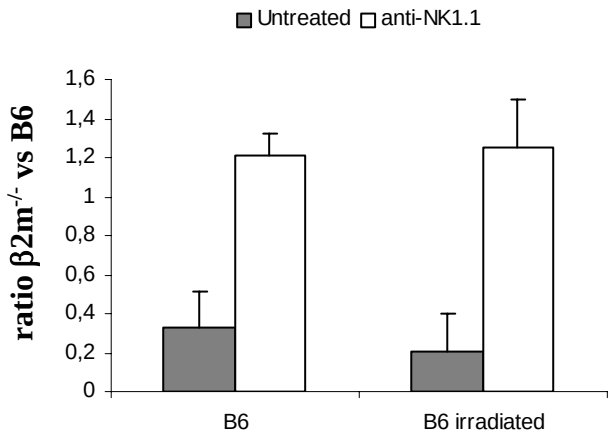

Figure 8. In vivo rejection of β_2m-deficient bone marrow cells in untreated and irradiated B6 mice. Irradiation with 800-900 rad was done prior to the experiment and cytotoxicity was evaluated 1 day after i.v. injection of BM cells. Summary of 2 experiments.

Does organ homing or number of cells influence in vivo NK cell cytotoxicity?

We found that the BM cells mainly distributed to the liver and spleen cells to the spleen. But both cell types could be found in blood, lung, BM, spleen, lymph node and liver. It is probably the size of different cell types, and differential expression of adhesion molecules and chemokine receptors that explains their homing pattern (271, 272). When tumor cells are injected i.v. they tend to localize to the lungs, probably due to the large size of these cells, leading to arrest in the first capillary bed they encounter.

An advantage of the CFSE method is that it is possible to relate killing potential to the amount of target cells present in an organ. We observed that an increased number of target cells in an organ reduced the relative killing of $\beta_2m^{-/-}$ vs. B6 cells. Our interpretation is that the number of NK cells in a specific organ is constant and each NK cell probably can reject only a certain number of target cells during a specific time period. This is similar to what can be observed in in vitro cytotoxicity assays where the effector:target ratio influences the result. It is therefore important to take the number of target cells in account when comparing killing in different organs. Since NK cells in

β_2m-deficient mice and TAP-deficient mice are tolerant towards cells with reduced MHC class I expression, it was possible that increased number of MHC$^-$ target cells in an organ could tolerize the NK cells (273-275). We have however failed to reduce in vivo killing of $\beta_2m^{-/-}$ or RMA-S cells by priming the mice with either $\beta_2m^{-/-}$ cells or RMA-S tumor cells prior to experiment. This suggests that exposure to target cells in vivo did not rendered the NK cells tolerant. On the other hand, we did not observe any increased killing capacity in these mice either, indicating that the NK cells did not have enhanced capacity for missing-self rejection after exposure to MHC class I deficient cells.

Does activation or MHC status alter NK cell rejection?

In many NK cell cytotoxicity assays, there is a requirement for activation of the NK cells. In vitro killing assays are often done with IL-2 activated cells (LAK) since it is difficult to observe direct ex vivo killing against many targets. Target cells are also usually not normal cells, but activated in some way. Many tumor cells express higher levels of stress induced activating ligands, such as Rae-1, which makes them more susceptible to NK cell lysis (265, 266). When we compared the NK cell rejection of naïve spleen cells to the rejection of in vitro stimulated ConA blasts or to tumor RMA-S cells, activated cells and tumor cells were always rejected more efficiently by NK cells. However, since neither $\beta_2m^{-/-}$ ConA blasts nor RMA-S express any known activating ligands for NKG2D, other mechanisms must exist for these cells to be better recognized and killed by NK cells (265). One possibility is that tumor transformation and strong in vitro activation upregulate still unknown activation ligands on these target cells.

Ly49 receptors display a diverse expression pattern, resulting in expression on partially overlapping subsets of NK cells. NK cells in D8 mice are inhibited when they sense H-2D^d through Ly49A and Ly49G2 (91, 104). When we injected B6 cells into D8 mice, they were rejected but with a slower kinetic than $\beta_2m^{-/-}$ cells. Many NK cells in D8 mice are tolerant against themselves because they express Ly49A and/or Ly49G2 but they also coexpress other Ly49 receptors. It has been shown that approximately 6% of NK1.1$^+$ cells in D8 coexpress Ly49A and Ly49C. Coexpression of Ly49G2 and Ly49C could be seen in 29% of the NK cells in D8 mice (115). Ly49C interacts strongly with H-2K^b expressed on the B6 cells (101). It is therefore likely that a proportion of NK

cells in the D8 mice were inhibited by the interaction of Ly49C with H-2K^b when B6 cells were injected. All NK cells in the D8 mice could probably participate in the rejection of $\beta_2m^{-/-}$ cells and hence the kinetic were faster. Since both B6 cells and $\beta_2m^{-/-}$ cells were injected simultaneously into D8 mice, it suggests that a strong inhibition of an NK cell does not prevent it from acting against other targets during the same time. Simultaneous inhibition and killing has been shown in vitro where an NK cell bound to a resistant target were able to bind to and kill a susceptible target at the same time (276).

Can activated NK cells use alternative signaling pathways?

There are different adaptor molecules used by different activating receptors to transmit signals into the NK cells. Ly49D binds to H-2D^d and signals via the adaptor molecule KARAP/DAP12 (85, 277). KARAP/DAP12-deficient mice have been generated that have a nonfunctional ITAM and therefore cannot transmit activation signals (278). It has been shown that KARAP/DAP12$^{-/-}$ mice can kill tumor targets lacking MHC molecules, such as RMA-S, indicating that KARAP/DAP12 is not involved in missing-self recognition (278). We confirmed this by injecting $\beta_2m^{-/-}$ cells into KARAP/DAP12-deficient mice. There was no difference in the ability to reject naïve $\beta_2m^{-/-}$ spleen cells in B6 or KARAP/DAP12$^{-/-}$ mice. However, the rejection of BALB/c cells was impaired in KARAP/DAP12$^{-/-}$ mice. The most likely explanation is that NK cells expressing Ly49D recognize H-2D^d on BALB/c cells, which activates the NK cells (279). Since BALB/c mice also lack MHC molecules present in B6 mice (H-2^b), it could be assumed that the NK cells would also sense missing-self. If an alternative way to activate the NK cells existed, some degree of missing-self recognition would have been seen despite the KARAP/DAP12 defect.

When we activated the NK cells prior to challenge with CFSE labeled cells by treatment of the mice with an IFN inducer (Tilorone), even mice deficient for KARAP/DAP12 signaling were able to reject BALB/c cells. There are at least three possible explanations for this observation. First, increased levels of KARAP/DAP12 independent activating receptors that recognize ubiquitously expressed non-MHC ligands could shift the signaling balance so that BALB/c cells were now rejected. Second, an unknown activating, KARAP/DAP12 independent, receptor that cross-reacts with one or several H-2^d locus products expressed on BALB/c cells could be

upregulated by interferon induced by the Tilorone. Third, after activation an alternative signaling pathway may be used by the Ly49D receptor. Alternative signaling pathways have been detected for another activating receptor, NKG2D, which can use either DAP10 under naïve situations or KARAP/DAP12 when the NK cells are activated (134).

PAPER IV: MICE DEFECTIVE IN NK CELL MEDIATE KILLING OF TARGETS WITH A "MISSING-SELF" PHENOTYPE

Do NKT cells participate in the rejection of targets lacking MHC molecules?

During the study of paper III, we wanted to test the role of CD1-restricted NKT cells in rejection of $\beta_2m^{-/-}$ naïve cells. We observed that in contrast to wt B6 mice, $CD1d1^{-/-}$ (LVK) mice were incapable of rejecting both $\beta_2m^{-/-}$ and $TAP^{-/-}$ spleen cells. However, since NK1.1 treatment abolished in principle all killing capacity in B6 mice, it did not appear plausible that NKT cells were involved. Although some NKT cells do express NK1.1, the expression level is usually lower and not high enough for anti-NK1.1 antibodies to deplete them (280, 281). We tested in paper I the 3A4 antibody that recognizes the SW3A4 determinant of NK1.1 (282). It only depleted NK cells and not $CD3^+$ $NK1.1^+$ cells. To verify that NKT cells were not involved in the rejection of $\beta_2m^{-/-}$ target cells, we repeated the in vivo rejection experiments with another, independently generated CD1-deficient mouse strain, as well as with the $J\alpha281^{-/-}$ mice (which lack the specific TCR used by NKT cells), and $RAG^{-/-}$ mice (which lack all T cells and B cells) (201, 283, 284). All of these mice were fully capable of rejecting $\beta_2m^{-/-}$ naïve spleen cells, indicating that NK cells solely mediated the process. Why $CD1d1^{-/-}$(LVK) mice could not reject missing-self targets is not known and will be discussed further below. However, these findings clearly emphasize the importance of maintaining a skeptical attitude when analyzing data obtained from transgenic or gene targeted mice, since the results may have nothing to do with the gene that has been altered.

Why cannot the $CD1d1^{-/-}$(LVK) mice reject MHC-deficient targets?

There are several possible explanations for why the $CD1d1^{-/-}$(LVK) mice were unable to reject targets with a missing-self phenotype. First, they may not have any NK cells at

all, or the NK cells may have a global functional defect making them unable to reject regardless of target type. However, we found that CD1d1$^{-/-}$(LVK) mice had normal numbers of NK cells and were able to kill allogeneic BALB/c cells as well as YAC-1 tumor cells. Another possible explanation is that the NK cells in CD1d1$^{-/-}$(LVK) mice lack a general activating receptor or its signaling pathway to be able to sense normal cells. In that case, the absence of MHC class I molecules would be of no importance since the NK cells would not receive any initial triggering to kill.

As was shown in paper III, rejection of allogeneic BALB/c cells in B6 mice require functional signaling through KARAP/DAP12. This is probably due to the activating receptor Ly49D, which binds to H-2D^{d} present on BALB/c cells and signals via KARAP/DAP12. KARAP/DAP12$^{-/-}$ mice have very little expression of activating Ly49 receptors indicating that other signal adaptor molecules cannot substitute for KARAP/DAP12 (285). On the other hand, we detected normal levels of activating receptors on NK cells from CD1d1$^{-/-}$(LVK) mice. The CD1d1$^{-/-}$(LVK) mice also displayed an opposite rejection pattern to KARAP/DAP12$^{-/-}$ mice. They were able to reject BALB/c cells but not MHC^{-} cells. This indicates that signaling through KARAP/DAP12 was functional in CD1d1$^{-/-}$(LVK) mice and a defect in this pathway could not be the answer to why these mice were incapable of rejecting targets with a missing-self phenotype.

How are NK cells activated to sense missing-self?

Although much focus has been on the inhibitory Ly49 receptors role in missing-self rejection, NK cells must also need some kind of initial activation to kill target cells. Some activating NK cell receptors bind glycoproteins that are induced during transformation or viral infection. Other NK cell receptors directly recognize viral ligands expressed on infected cells. Additionally, many activating NK cell receptors have probably not been discovered yet or have no known ligand (286). It is possible that the main ligands of the activating Ly49 receptors are not self proteins, but rather viral proteins, and that interaction with MHC class I molecules represents crossreactivity. Some viruses try to interfere with NK cell activation by blocking expression of activating ligands. One example is MCMV, which encodes viral proteins that interfere with expression of NKG2D ligands on the infected cells (287-289). There are also natural cytotoxicity receptors, such as NKp30, NKp44 and NKp46 with so far

unknown endogenous ligands, which may be implicated in antiviral, antitumor and missing-self responses (147, 290-292).

We observed defects in rejection of $\beta_2m^{-/-}$ and $TAP^{-/-}$ naïve spleen cells, and of MHC-deficient tumor targets in the $CD1d1^{-/-}$(LVK) mice. One reason for these rejection defects could be that an activating receptor or signaling pathway used in missing-self recognition of normal cells is defective. How NK cells are initially activated to sense naïve spleen cells such as $\beta_2m^{-/-}$ cells is not known. A type of NK cell tolerance develops in $\beta_2m^{-/-}$ mice, whereby NK cells avoid the killing of autologous (MHC class I deficient) haematopoietic cells, but the mechanisms behind this are not known. Although some cellular responses are similar in $\beta_2m^{-/-}$ mice and $CD1d1^{-/-}$(LVK) mice, there are also differences. Ly49 receptors are normally downmodulated on NK cells after they have bound to their ligand and transmitted inhibitory signals (114). In $\beta_2m^{-/-}$ mice this is not observed since there are no MHC class I molecules that the Ly49 receptors can bind to. However, in $CD1d1^{-/-}$(LVK) mice we observed normal downmodulation of inhibitory Ly49 receptors. This downregulation is usually interpreted as the consequence of engagement of inhibitory Ly49 receptors on the NK cell, and it may be speculated that this would only occur when initial activation via triggering receptors has taken place. One possible interpretation of our results is therefore that the defect lies downstream of the critical interplay between inhibitory and activating receptors. Naïve spleen cells probably express no or very low levels of stress induced ligands. In humans, ULBPs have been found on B cells from peripheral blood, but not on NK cells or T cells (133). Several tumor cells have been shown to express NKG2D ligands but RMA-S tumor cells or $\beta_2m^{-/-}$ ConA blasts do not, implying that other unknown activating pathways must exist (265). $Fyn^{-/-}$ mice display defects reminiscent of the ones we observed in $CD1d1^{-/-}$(LVK) mice (293). Target cells that lack NKG2D ligands and MHC class I could not be killed in the absence of Fyn. Fyn is a member of the src-related protein tyrosine kinase family implicated in many activating pathways (294). Direct interactions between Fyn and NK cell receptors such as CD2, have been reported (295). However, the receptor that signals through Fyn is not known. We do not know if Fyn is affected in our $CD1d1^{-/-}$ mice. It is possible that Fyn, another molecule in the same signaling pathway, or the actual receptor is affected resulting in the similar phenotype seen in the two knock-out mice. Other candidates for

activating receptors involved in the activation part of missing-self rejection are NKp46 and in humans other NCRs, and DNAM-1, that recognizes PVR and Nectin-2 (296-298). One may also consider LFA-1 in this context (299).

Further genetic analysis of CD1d1$^{-/-}$(LVK) mice.

Since the CD1 deficiency could not explain why the CD1d1$^{-/-}$(LVK) mice were incapable of rejecting targets with low expression of MHC, we have started to search for a genetic cause for the defect. The CD1d1$^{-/-}$(LVK) mice were generated on a 129/SvEv background and only backcrossed 5 times to B6. This opens the possibility that background genes and/or flanking genes may influence the phenotype. We observed that 129/SvEv mice were defective, similarly as CD1d1$^{-/-}$(LVK) mice, in rejection of β_2m$^{-/-}$ and TAP$^{-/-}$ cells. 129 mice have been shown by many groups to have differences in immunological responses as compared with B6 mice. Interestingly though, one group has showed that their 129 mice were capable of rejecting TAP-deficient bone marrow grafts. The reason for our opposite results may lie in that different substrains of 129 mice were used or the different methods to measure rejection. We speculate that a gene variant causing lack of rejection may have been carried from the 129/SvEv genome into the CD1d1$^{-/-}$(LVK) mice.

To further try to dissect the genetic component responsible for the rejection defect in CD1d1$^{-/-}$(LVK) mice, they were crossed with B6 mice to generate F1 mice. If the gene causing the defect was dominant, also F1 mice would be unable to reject β_2m$^{-/-}$ cells. This was not the case, indicating a recessive trait. However it is possible that the defect in CD1d1$^{-/-}$(LVK) as well as in 129/SvEv mice is that they lack an activating receptor or signaling molecule expressed in B6 mice, that is needed in at least one copy (as in the F1 mice) and hence dominant. When we backcrossed the F1 mice to B6 to generate F2 mice, we observed both rejection and defective rejection in F2 mice with a functional CD1d1 gene. Similarly, F2 mice with a nonfunctional CD1d1 gene also seemed to segregate into rejecting and nonrejecting groups (figure 9). Hence, we reason that it is unlikely that the gene responsible for the defect is linked to the CD1d1 gene. However, it should be noted that so far only few mice have been tested of both the F1 and F2 mice. Further experiments are needed to verify these data. It is not clear either whether the defect is caused by a single gene or multiple genes, and this needs further investigation.

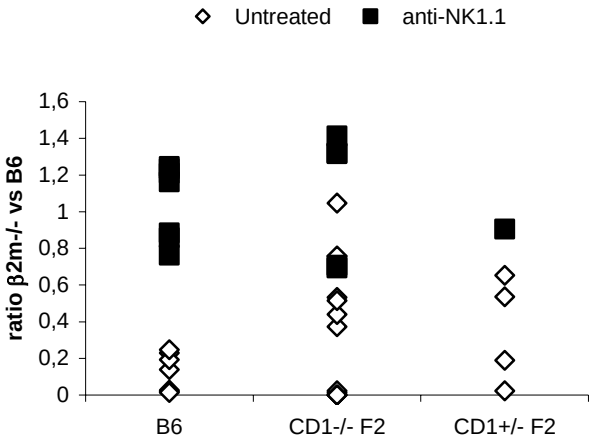

Figure 9. Rejection of $\beta_2m^{-/-}$ spleen cells in F2 mice with a functional or a nonfunctional CD1d1 gene. The figure displays individual mice from at least 2 experiments.

Although it might seem likely that the defect is caused by a gene trait from the 129SvEv mouse strain, other possibilities exist. Mutations can occur either already in the ES cell line used to generate the knock-out mice, or spontaneously during the backcrossing to B6. In that case, the defect in CD1d1$^{-/-}$(LVK) and 129/SvEv mice may be unrelated. We are currently crossing CD1d1$^{-/-}$(LVK) with 129/SvEv mice to elucidate the possibility of complementation between genes. If the genes do not complement each other, it may be possible to identify the gene by generating B6x129 F2 mice and genetically search, with microsatellite markers, for a genetic region of 129/SvEv origin that correlates with the phenotype seen in the CD1d1$^{-/-}$(LVK) mice.

Unfortunately, we do not have the answer to why the CD1d1d$^{-/-}$(LVK) mice are unable to reject targets with a missing-self phenotype while other types of NK cell rejection pathways seem intact. Further research will hopefully give us an answer to whether the defect lies in an activating receptor, a signaling molecule downstream of an activating receptor or another yet undefined type of molecule. Irrespectively of the detailed mechanism, these mice are useful tools to test whether missing-self rejection is important in various NK cell mediated in vivo responses and natural resistance phenomena.

CONCLUDING REMARKS

Both NK cells and T cells are part of the immune system designed to protect us against invading pathogens. However, NK cells are part of the rapid, innate immune defense while T cells belong to the adaptive immune system. Despite this, several receptors and signaling pathways are shared between these cells.

NK cell receptors such as Ly49 are expressed on T cells and we show that this expression may alter both T cell selection and T cell function. Furthermore we verify that Ly49 inhibitory receptors can shift the balance between activating and inhibitory signals, rather than acting as a decisive signal always turning cells off.

The missing-self hypothesis has been challenged and verified many times. Although bits of the puzzle have been added gradually during the years, the picture is still not complete. We showed that the missing-self hypothesis is valid also for naïve cells in a naïve mouse. Furthermore we added pieces to the puzzle by studying how both activation and different MHC status influenced the rate of NK cell rejection. We showed that the signaling adaptor KARAP/DAP12 was not needed for rejection of $\beta_2m^{-/-}$ cells but critical for rejection of BALB/c cells in unmanipulated B6 recipients. Intriguingly, we also displayed that an alternative activation pathway, not using KARAP/DAP12 signaling, can be used by activated NK cells to sense allogeneic BALB/c cells.

Finally, we used CD1d1-deficent and other gene targeted mice to exclude that NKT cells play a part in missing-self rejection. However, during this process it became clear that the CD1d1$^{-/-}$(LVK) mice have a defect in NK cell rejection of MHC^{-} targets, despite normal numbers of NK cells and normal expression pattern of the most well known receptors. Why these mice cannot reject targets displaying a missing-self phenotype is not clear and will need further investigation.

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