Activating natural killer cell receptors: KIR recognition of a cancer-associated ligand

Lavanya Thiruchelvam-Kyle

UiO : Faculty of Medicine
University of Oslo

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Department of Molecular Medicine

Division of Anatomy

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Abbreviations

MHC	Major histocompatibility complex
NK cells	Natural killer cells
RAG	Recombinase activating gene
TCR	T cell receptor
BCR	B cell receptor
CD	Cluster of differentiation
DC	Dendritic cell
IL	Interleukin
TGF	Tumor growth factor
Ig	Immunoglobulin
T_H cell	T helper cell
V_H	Variable region of the antibody heavy chain
C_H	Constant region of the antibody heavy chain
V_L	Variable region of the antibody light chain
C_L	Constant region of the antibody light chain
V(D)J	Variable gene segment, Diversity gene segment, Joining gene segment
APC	Antigen presenting cell
DNA	Deoxyribonucleic acid
TNF	Tumor necrosis factor
PAMP	Pathogen associated molecular pattern

RNA	Ribonucleic acid
dsRNA	Double stranded RNA
TLR	Toll-like receptors
DAMP	Damage associated molecular pattern
HLA	Human leukocyte antigen
KIR	Killer cell immunoglobulin like receptor
ER	Endoplasmic reticulum
TAP	Transporter associated with antigen presentation
HCMV	Human cytomegalovirus
MCMV	Murine cytomegalovirus
ALC	Allogeneic lymphocyte cytotoxicity
CLP	Common lymphoid progenitor
BM	Bone marrow
NKDI	NK development intermediates
SLT	Secondary lymphoid tissue
LFA-1	Lymphocyte function-associated antigen 1
mNK	Mature NK cells
IFN	Interferon
p-/c-SMAC	Peripheral/central supramolecular activation center
MTOC	Microtubuli organizing center
IgSF	Immunoglobulin-like superfamily
NKC	NK cell receptor gene complex

KLR	Killer cell lectin-like receptor
LRC	Leukocyte receptor gene complex
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITAM	Immunoreceptor tyrosine- based activation motif
DAP10	DNAX –activating protein of 10 kDa
DAP12	DNAX –activating protein of 12 kDa
Clr	C-type lectin related molecules
MICA/-B	MHC class I polypeptide-related sequence A/ -B
RAET-1	Retinoic acid early transcript 1
ULBP	UL16 binding protein
H60	Histocompatibility 60

Papers included

I. Lavanya Thiruchelvam-Kyle, Sigurd E. Hoelsbrekken, Per C. Saether, Elisabeth G. Bjørnsen, Daniela Pende, Sigbjørn Fossum, Michael R. Daws and Erik Dissen. 2017. **The activating human NK cell receptor KIR2DS2 recognizes a β2-microglobulin independent ligand on cancer cells.** *Journal of Immunology* 198:2556-2567

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III. Per Christian Saether, Lavanya Thiruchelvam-Kyle, Sigurd E. Hoelsbrekken, Michael R. Daws and Erik Dissen. **Co-operative interaction of the activating transmembrane adaptor proteins DAP10 and DAP12 with NK cell receptors.** *Manuscript*

INTRODUCTION

The science of immunology aims to study and decipher the ability of organisms to resist the continuous threat of invading pathogens. These protective mechanisms consist of cells and soluble factors collectively known as the immune system.

Edward Jenner´s work in the 18th century is often considered to be the origin of the discipline of experimental immunology. In 1798 Jenner published that individuals who fell sick from the cowpox virus were immune upon recurrent exposure and also exhibited immunity towards the related disease smallpox. Jenner validated this by systematically re-exposing patients to the cowpox virus and eventually the smallpox virus. Inoculation of smallpox virus in cowpox immunized individuals resulted in only mild pathology, indicating that the immune system learns and remembers pathogen exposure. With this Jenner shaped the platform for vaccination and provided evidence for immunological memory.

The immune system has by convention been divided into two main branches: the innate immune system and the adaptive immune system. The innate system is specialized for the recognition of conserved molecular patterns from microorganisms and conveys a rapid response upon infection of the host. The adaptive immune system is specialized to cope with new antigens arising as a result of constantly evolving microbes. In multicellular organisms the immune system is also important for combating neoplastic cells arising from the host´s own cells.

The never ending host pathogen arms race

The immune system is faced with a large number of pathogens that constantly evolve new strategies to escape immune recognition and avoid elimination. Pathogens evolve rapidly as a consequence of short generation times (minutes to hours). As a result of mutations in the DNA, bacteria or viruses can rapidly evolve to escape the host defense mechanisms. Evolution of the defense mechanisms in humans is slower as a result of longer generation time (about 15 to 30 years). Our immune system thus has to use advanced strategies in order to anticipate and cope with the large variety of pathogens encountered throughout life. Examples of such advanced mechanisms are pattern recognition by the innate immune system, somatic recombination of the T cell and B cell receptor genes, somatic hypermutation in activates B cells and highly polymorphic multigene families such as MHC and some NK cell receptors. These features result in complex machinery with high potential of recognizing an almost unlimited number of microbes.

The innate immune system

The innate immune system is an evolutionary ancient form of host defense found in all organisms [1]. This system does not exhibit immunological memory (although memory-like responses have been reported in monocytes, macrophages and NK cells [2-6]) or the large spectrum of antigen recognition that is one of the defining features of the adaptive system. The multiple modules within this branch perform different functions to protect the host against infections. The skin and mucosal membranes are the main sites of contact with microorganisms, functioning as a physical barrier, and displaying specialized characteristics that protect the host from pathogens [7].

Phagocytes, such as macrophages and neutrophilic granulocytes, are innate immune cells that perform phagocytosis and are critical for the defense against both extracellular and intracellular pathogens. Monocytes migrate from blood to transform into tissue resident macrophages. Macrophages are long lived scavenger cells that phagocytose dead and degenerated cells. They also phagocytose opsonized targets, perform antigen presentation and modulate inflammation by releasing inflammatory and anti-inflammatory cytokines. In this sense, these cells are important in initiating, maintaining and ending the inflammatory process. Neutrophilic granulocytes are part of the first line of defense and are recruited to the site of inflammation by cytokines produced by e.g. macrophages. They are important for phagocytic uptake of microbes and kill pathogens through production of reactive oxygen species, release of antimicrobial peptides and by expulsion of their DNA to form neutrophil extracellular traps (NETS).

Mast cells are tissue resident cells that are particularly found in areas that are in close contact with external surfaces like skin, airways and intestines. At these locations the mast cells serve as immune sentinel cells and can participate in early recognition of pathogens and initiation as well as modulation of inflammation. They are known to play a role in bacterial and parasitic infections through phagocytosis and release of antimicrobial factors. Moreover, they are known for their role in allergic responses. Mast cells express FcƐRI that binds to IgE, often specific for parasites and allergens, in addition they express different pattern recognition receptors [8]. Basophilic and eosinophilic granulocytes protect against parasites and play a role in allergic responses [9, 10].

Dendritic cells (DC) function as a link between the innate and adaptive immune system. DCs take up pathogen molecules by endocytosis or phagocytosis, then process and present peptide antigen on MHC to T cells, bridging the innate and the adaptive responses [11-13]. DCs play an important role in presenting antigen from tumors as well as pathogens [14].

Recognition of foreign antigen by the innate cells is through recognition of microbial non-self or recognition of missing self (NK cells and complement). Recognition of microbial non-self is through a limited number of germline encoded receptors pattern recognition receptors (PRRs). These receptors bind to conserved molecular patterns called pathogen associated molecular patterns (PAMP) that are unique to microbes and not produced by the host. PAMPs are for instance lipopolysaccharides, lipoteichoic acid, RNA forms such

as 5´ triphosphate RNA or double stranded RNA (dsRNA) and unmethylated CpG-rich DNA [15].

There are several families of PRRs, such as RIG-I like receptor (RLR), NOD-like receptors (NLR) and Toll-like receptors (TLR). The best studied of these are the Toll-like receptors that are transmembrane proteins expressed mostly by innate immune cells. The human TLR family consists of ten members: TLR1, -2, -4, -5 and -6 are expressed on the plasma membrane [16-21] and TLR3, -7, -8 and -9 are found in intracellular endosomal compartments [22]. TLR4 binds to lipopolysaccharide (LPS) derived from gram negative bacteria [23, 24] and is expressed by macrophages, neutrophils, DCs, mast cells and B cells. TLR3 and TLR9 recognize dsRNA produced by many viruses [25] and unmethylated CpG rich DNA respectively [26]. The TLRs have also been shown to bind to damage-associated molecular patterns (DAMP); these are host-encoded molecules, such as heat shock proteins or host genomic DNA, that become exposed to the immune system as a result of necrotic cell death [27].

The innate immune defense also has two classes of humoral defense mechanisms which are the complement system and the acute phase proteins that are secreted from the liver upon stimulation by IL-6, IL-1 and TNFα produced by the phagocytes. Many acute phase proteins, such as C-reactive protein (CRP), function as opsonins which means that they bind to pathogens and to receptors on phagocytes, facilitating phagocytosis, [28]. The complement system consists of proteins activated in cascade reactions resulting in lysis of target cells. Some proteins of the complement system also function as opsonins, e.g. C3b [29].

The adaptive immune system

The two hallmarks of the adaptive immune system are the ability to recognize a broad spectrum of antigens and the ability to form immunological memory. Immunological memory results in a more rapid, stronger immune response upon re-encounter with the same specific pathogen [30, 31].

The adaptive immune system in mammals consists of T-lymphocytes and B-lymphocytes, [32] and has developed in jawed vertebrates as a result of acquisition of the recombinase activating genes RAG1 and RAG2. RAG1 and RAG2 together enable somatic recombination of a limited number of gene segments that encode the T- and B cell receptors [33-35]. These gene segments are called variable (V), diversity (D) and joining (J) segments. Somatic recombination of this limited number of V(D)J segments of the T cell receptor (TCR) and the B cell receptor (BCR) occurs early in their maturation and results in a myriad of different antigen specific receptors that together can identify a broad repertoire of antigens of nearly any conceivable molecular pattern [36-39]. Millions of TCRs and BCRs are generated in this process and some of these receptors will recognize some of the host´s own molecules as an antigen, potentially leading to autoimmune reactions. The potential for autoreactivity is reduced through a process called negative selection where T- and B cells expressing receptors with very high affinity for self-antigens are programmed to die by apoptosis. Negative selection takes place in primary lymphoid organs: the thymus (T cell) and the bone marrow (B cells) early in the ontogeny [40-42].

Some of the T cells that are maturing in the thymus escape deletion through negative selection and develop into to natural T-regulatory cells, nTreg [43]. Induced Tregs, iTregs, develop in the periphery to antigen stimulation [44, 45]. T-regulatory cells serve to suppress immune responses by inhibiting other immune cells and thereby preventing autoimmunity [46].

A naïve T- or B cell has not yet encountered specific antigen. Upon antigen recognition a T- or B cell will be activated and through cell division generate a large number of antigen-specific cells to fight an infection, a process called clonal expansion. T cells are subdivided based on function into three main populations: the $CD4^+$ T-helper cells, $CD8^+$ T-cytotoxic cells and $CD4^+/CD25^+/Foxp3^+/IL2R\alpha^+$ T-regulatory cells [46, 47]. In addition to a signal through the TCR, a costimulatory signal through CD28 [48] on the T cells is needed for activation and clonal expansion. CD28 binds to CD80 and CD86 expressed by antigen presenting cells (APCs) and in absence of this co-signal the activated T cell becomes anergic [49]. Activated T-helper cells are needed for initiation and persistence of the $CD8^+$ T cell response, the B cell response as well as for recruiting other immune cells. Stimulation by different cytokines promotes T-helper differentiation to either T_H1, T_H2 or T_H17 subsets [50, 51]. IFNɣ, IL-2 and IL-12 stimulation will result in a T_H1 response, IL-2 and IL-4 will result in T_H2 response while IL-6 and TGF-β induces a T_H17 response [52]. These cells will in turn produce various cytokines that influence the immune response [53, 54]. Most T cells express the αβ TCR, however subets of T cells instead

express the γδ TCR. γδ T cells are more oligoclonal and are found in the skin, intestine, lungs and the uterus [55].

Antigen recognition by B cells

In naïve B cells the BCR is based on transmembrane IgM (and its splice isoform IgD). The BCR binds a specific antigen in its native form and can induce endocytosis and processing for surface presentation of antigen-derived peptides to T_H cells in the context of MHC class II [40, 56]. B cells are normally activated with help from T_H cells. Activated B cells undergo clonal expansion resulting in formation of antibody-secreting plasma cells and memory B cells [57]. Antibodies contain both heavy chain and light chain polypeptides. The immunoglobulin heavy chain consists of a variable region, V_H, and three constant (C) regions - C_H1, C_H2 and C_H3 [38]. The heavy chain associates with a light chain containing a V_L and a C_L domain (Fig. 1). The variable domains, V_H and V_L, together determine the antigen specificity and their unique specificities are generated through V(D)J rearrangement in early ontogeny [38]. The initial effector B cells produce soluble IgM pentamers. Depending on the stimulus the heavy chain isotype can switch to α, ɣ or Ɛ [38, 58]. For instance, a T_H2 response will typically result in a switch from IgM to IgE. The heavy chain constant region isotype is responsible for the effector functions of the antibody such as opsonization where the target that is coated or opsonized by the antibody is phagocytosed. Other effector functions are mast cell degranulation and transcytosis of IgA in gut epithelium or initiation of the complement cascade [38]. In an activated B cell the rearranged immunoglobulin V(D)J region undergoes somatic

hypermutation which can generate antibodies with increased antigen affinity, designated affinity maturation [59]. Antibody is produced by plasma cells that can be long lived and hence produce immunity long after the infections are initially defeated [60, 61].

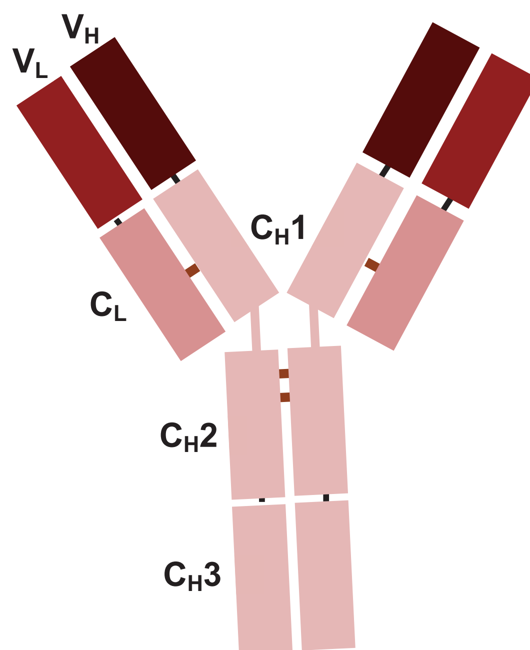

Figure 1. Schematic image of an antibody molecule, showing variable and constant regions on the immunoglobulin light and heavy chains. IgM and IgE have a fourth CH domain, not portrayed here. V_L: Variable region on the light chain. V_H: Variable region on the heavy chain. C_L: constant region on the light chain C_H: constant region of the heavy chain

MHC class I and antigen recognition by T cells

Both the adaptive and the innate immune system depend on recognition of healthy self versus altered or unhealthy self to convey appropriate immune responses. The major histocompatibility complex (MHC) molecules are crucial in the adaptive immune-recognition of non-self versus self. The MHC is a highly polymorphic chromosomal region that encodes several MHC class II molecules as well as several classical and non-classical MHC class I. This large cluster of genes is localized on chromosome 6 in the human, and was early found to be important for rejection of transplants, hence the term major histocompatibility complex. MHC class I proteins are expressed by all nucleated cells while MHC class II proteins are only expressed by APCs [62].

The αβ TCR binds antigen only when it is presented by MHC, a prerequisite designated MHC restriction [63-65]. T-helper cells recognize peptides from extracellular proteins presented by MHC class II on DC, macrophages and B cells [13]. $CD8^+$ T cells recognize intracellular peptide antigens presented by MHC class I, but also appear to need aid from activated T-helper cells to respond [66]. Cytotoxic T cells lyse infected or neoplastic target cells that are expressing an MHC class I-peptide complex that is recognized by their TCR [13, 67, 68]. Upon antigen recognition cytotoxicity is mediated by releasing perforin and granzymes through exocytosis. Perforin penetrates the target cell membrane [69-72], creating pores for granzymes to enter the cytoplasm of the target cell [73-76]. Granzymes induce apoptosis in the target cell by activating caspases [77]. Memory T cells are long lived and are generated during clonal expansion to mount a rapid and boosted response on re-encounter with the same antigen.

Invertebrates do not appear to have an adaptive immune system with the complexity found in jawed vertebrates. In jawless fish lymphoid like cells express variable lymphocyte receptors (VLR). Through a mechanism called template switching, variable arrays of leucine-rich repeat segments are randomly used as templates to generate mature VLRs [78]. This provides receptor diversity. In the sea lamprey VLRB molecules are secreted in soluble form, similar to antibodies [79].

MHC class I molecules

The most important functions assigned to MHC class I is to present peptides to T cells as well as to serve as ligands for some NK cell receptors [13, 63, 80]. MHC class I genes show the highest levels of polymorphism in the genome. This most likely reflects a selective pressure to keep up with the rapid evolution of microorganisms and to maintain the ability to present a broad repertoire of peptide antigens to the immune cells. MHC class I molecules present peptide fragments derived from intracellular proteins. In the course of an intracellular infection the MHC class I proteins can present peptides derived from pathogen-encoded proteins following proteasomal degradation. This presentation of non-self is how infected host cells can be identified and eliminated by the immune system [81]. Likewise, following malignant cell transformation, peptides from abnormal proteins are presented by MHC class I, and can be discovered by the immune system [81].

Synthesis and Structure of MHC class I

MHC class I molecules are heterotrimers consisting of the α-chain (a type I transmembrane protein), a peptide in the peptide-presenting groove of the α-chain and β2 microglobulin, an invariant and soluble globular protein non-covalently associated to the α-chain [82-84]. β2 microglobulin-deficient animals lack normal expression of MHC-I. Structurally, the extracellular part of the α-chain consists of three domains: a membrane-proximal IgSF domain (α3) preceded by the α1 and α2 domains that together make up the top of the molecule, encompassing the peptide-presenting groove. This groove is flanked and sealed off by two semi-parallel alpha-helices and supported by a beta-pleated sheet "floor". On the cell surface, stable MHC class I molecules contain a short peptide of 8-10 amino acids presented in the groove [13, 85]. In the human, MHC class I molecules are by convention termed HLA (human leukocyte antigen). HLA class I molecules are divided into classical class I molecules, HLA-A, -B and –C [86], and non-classical class I molecules, HLA-E, -F -G. In addition there are several MHC-like molecules (including the MHC I like proteins encoded outside the MHC) with sequence and structural similarity to MHC I [87]. All nucleated cells express MHC class I. In the endoplasmic reticulum (ER) the class I α-chain is retained and stabilized by chaperones such as calreticulin, ERp57 and tapasin until a peptide is loaded into the groove and the molecule has obtained correct folding. Together this is referred to as the peptide loading complex [88]. Tapasin interacts with TAP (transporter associated with antigen presentation) which translocates cytosolic peptides into the ER lumen, thereby coupling peptide delivery to MHC [89, 90]. The peptides are derived from proteasome degradation of cytoplasmic and

nuclear proteins. Viral proteins, misfolded proteins etc. are tagged for proteasomal degradation by ubiquitination [88]. After translocation into the ER, the peptide is further trimmed by the ER aminopeptidase associated with antigen processing, ERAAP [91, 92]. In infected cells these peptides may be pathogen-derived and the peptide antigen-HLA class I complex will be recognized by the immune system as foreign [93, 94].

The classical HLA class I loci are highly polymorphic. Many of these polymorphisms are located in the peptide binding groove and its pockets. The amino acids that line the pockets determine which amino acid anchor residues the pocket can accommodate, thus determining the peptide repertoire that the HLA class I molecule can bind [95, 96]. HLA-A and -B show higher surface expression levels than HLA-C. Even though HLA-C is transcribed at the same level, surface expression is hampered by several factors such as faster mRNA degradation, microRNA interference, lower association with β2-microglobulin and prolonged association with TAP because of restricted peptide binding [97-100].

The non-classical MHC Ib are less polymorphic. HLA-E presents the hydrophobic leader peptides from other MHC class I molecules and is expressed by almost all cells. HLA-E presenting normal, endogenous peptide has been identified as a ligand for the inhibitory CD94/NKG2A receptor [101].

HLA-F can be expressed with or without β2-microglobulin devoid of peptide, independent of TAP and in complex with other MHC-I in open conformation [102-104]. Reports suggest that HLA-F is predominantly retained intracellularly, but can be surface expressed on activated lymphocytes and monocytes and on cancer cells [102, 105].

HLA-G expression appears to be restricted to the fetal trophoblast cells that invade the maternal decidua early in pregnancy [106, 107].

NK cells

Another strategy of innate immune recognition is mainly conveyed by NK cells. This involves recognition of both *self*, in the form of MHC, on healthy cells, *missing self* (low or lack of MHC expression), *induced self* and potentially *altered self* on infected cells or cancer cells.

NK cells are cytotoxic innate lymphocytes that recognize and kill MHC class I disparate allogeneic cells, syngeneic neoplastic cells and cells with intracellular infections. They are also important immune modulators shaping the adaptive immune response through production of cytokines. From a myriad of germline encoded activating and inhibitory receptor genes, individual NK cell receptor repertoires are generated in a stochastic manner, resulting in a large number of different receptor repertoire combinations and accordingly a large number of NK cell subpopulations. In contrast to T cells, NK cells display cytotoxicity and cytokine production without previous sensitization and therefore NK cells can enter and defend infected tissue rapidly. NK cells express both inhibitory and activating receptors on the cell surface to discriminate between healthy and sick cells (Fig. 2). Inhibitory NK cell receptors typically recognize the presence of MHC class I in order to identify healthy, normal cells. The binding of inhibitory NK receptors to class I is largely insensitive to peptide sequence, although a degree of peptide dependent recognition has been demonstrated for some inhibitory KIR [108] and CD94/NKG2A

receptors [109]. In contrast to the adaptive immune system that learns and changes upon recurrent challenges with the same pathogen to form memory cells, the innate immune system remains essentially unchanged, depending on a limited number of germline encoded receptors. However, reports in the last decade of memory-like NK cells challenge the notion that immunological memory is solely an adaptive feature.

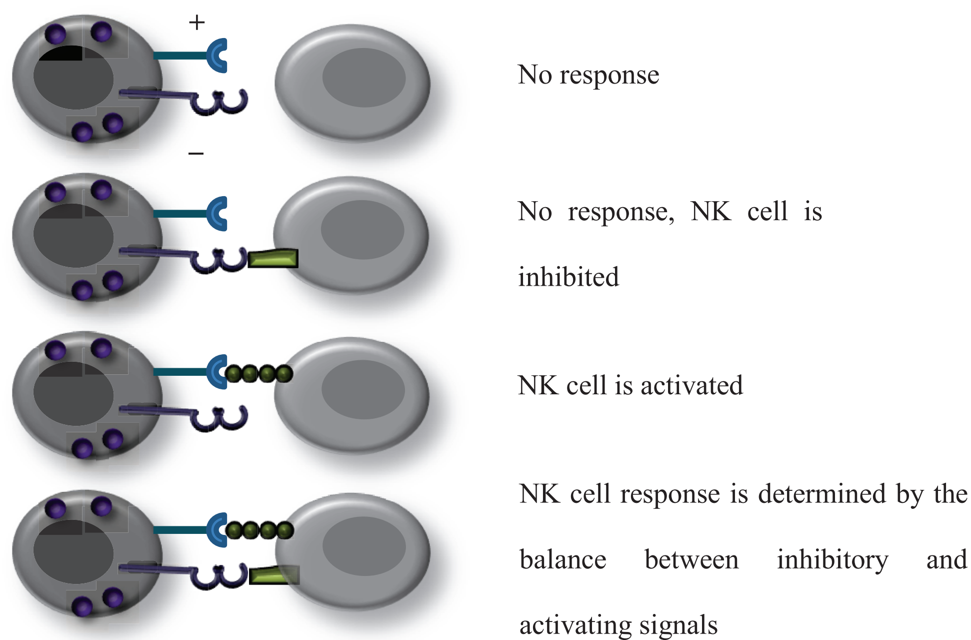

Figure 2. Target recognition by NK cells. Model portraying potential interactions and resulting responses when NK cells meet target cells and non-targets. The NK cell responses are regulated by both activating and inhibitory receptors in a delicate balance that tilts the response towards activation or inhibition.

F1 hybrid resistance

NK cells were identified in the 1970s following several independent observations. In the 1950s Snell and coworkers reported that homozygous lymphoma cells from C57BL mice grew better when transplanted into syngeneic C57BL recipients than into a F1 hybrid between C57BL and unrelated strains. In the 1970s Cudkowicz and coworkers also described that irradiated and unimmunized F1 hybrid mice rejected bone marrow from homozygous inbred parental mouse strains [110]. This resistance to parental bone marrow was termed F1 hybrid resistance. Rejection was however not observed when transplanting skin or solid organs in the same experimental setting. The phenomenon of F1 hybrid resistance violated the classical laws of transplantation which stated that grafts are accepted if the MHC haplotype is not different from the host. Snell and coworkers showed that hybrid resistance linked to MHC [111]. Cells displaying cytotoxicity without prior sensitization were observed *in vitro* for many years. This was disregarded as experimental artifacts until the 1970s when the observation of natural cytotoxicity towards tumor cells was assigned a separate cell subset, NK cells, by Kiessling and Herberman in independent publications [112, 113]. Later it was noted that there were similarities between natural cytotoxicity and hybrid resistance, indicating that the transplanted bone marrow cells were killed by NK cells [114].

Missing self

Kiessling and Herberman were the first to characterize natural killer cells, showing NK cell specificity and lysis of both syngeneic and allogeneic virus-induced leukemias

(mouse Moloney leukemia virus) without any priming [113, 115]. Further experiments demonstrated that the killer cells were different from T cells or B cells, as they did not express any of the traditional T- or B cell markers [112, 113]. Moreover, experiments showed that NK cells not only lysed tumor cells and infected cells, but also allogeneic cells from healthy individuals [116]. In 1986 Kärre showed selective rejection of an H-2 (MHC class I) deficient lymphoma in mice, and that the rejection was mediated by NK cells [117]. Based on this observation Ljunggren and Kärre formulated an alternative immune strategy operated by NK cells, subsequently known as *the missing self hypothesis* [80]. According to this hypothesis NK cells are prohibited from killing by inhibitory NK receptors recognizing MHC class I expressed on normal, syngeneic cells. In line with this, the absence or the reduction of self MHC class I that frequently occurs in infected or transformed cells would lead to killing of these cells by NK cells through loss of inhibition. In this model, HLA class I molecules presenting a "normal" endogenous derived peptide act as a marker for healthy nucleated cells. Downregulation of MHC I by cancer cells and some viruses can bypass T cell recognition. Human cytomegalovirus (HCMV) encodes several proteins that interfere with normal surface expression of MHC-I [118]. The HIV protein Nef downregulates HLA-A, -B, MIC-A and ULBP-1 and -2 [119, 120]. As an innate backup mechanism in that situation, the downregulation of MHC-I would induce a missing self response, activating NK cell effector functions.

In the original missing self model, NK cells were perceived to be activated by unspecific interaction with target cells [80]. Ly49A was the first inhibitory NK receptor to be shown to bind to MHC class I in the mouse [121, 122]. Soon after, the p58 family of MHC class

I binding receptors was cloned in the human [123, 124] and renamed “killer cell inhibitory receptors”. Although the missing self hypothesis appeared to largely explain NK cell function, contradicting reports existed where the absence of inhibitory signaling could not explain the observations. Experiments, in particular in rat models, instead indicated a role for activating NK cell receptors that could discriminate between different MHC haplotypes. This was based on observations that lymphocytes were eliminated when injected into an unprimed allogeneic host. This phenomenon was called allogeneic lymphocyte cytotoxicity (ALC). [114, 125-127]. Studies identified certain rat strains, such as PVG, as highly alloreactive towards target cells from most other rat strains. In experiments using cold targets mixed with labeled targets, polyclonal NK cells from PVG discriminated between different MHC-incompatible target cells demonstrating multiple specificities [128]. In contrast, the DA rat strain displayed poor alloreactivity independent of target cell allotype. Linkage studies mapped this genetic difference in NK cell allorecognition to the Ly-49 region in the rat NKC [129]. Conversely, susceptibility to NK cell allorecognition mapped to the MHC class I regions RT1.A and RT1.C in studies on MHC-congenic rat strains [130, 131]. These observations, together with the findings that both the Ly49 and KIR receptor families contained members with dissimilar intracellular regions, led to the hypothesis that these families contained activating members in addition to the inhibitory ones [124, 132]. Formal proof of the existence of activating Ly49 and KIR receptors came when their association with the activating adaptor protein DAP12 was discovered [133]. Accordingly, the original term “Killer cell

inhibitory receptor" was changed to "killer cell immunoglobulin-like receptor", maintaining the abbreviation KIR.

NK cell effector functions

The two main natural killer cell effector functions are cytotoxicity and cytokine production, neither of which require prior sensitization [134].

NK cells are activated at the site of infection or inflammation by cytokines secreted by resident macrophages (IL-12, IL-15, IL-18) [134]. These cytokines stimulate the production and release of many antiviral cytokines by NK cells, such as IFNγ, TNFα and GM-CSF [134, 135], which enhance the innate response and modulate the clonal adaptive response. Through NK cell crosstalk with DCs and macrophages the IFNγ from NK cells affects T cell differentiation to T_H1 cells and CTL and B cell isotype shift [136-138].

Similar to cytotoxic T cells, NK cell cytotoxicity is mediated by release of granules containing perforin and granzymes [139], that together create pores in the target cell membrane and activate caspases, resulting in initiation of apoptosis and cell lysis [77, 140]. NK cell cytotoxicity can in addition be mediated through FasL and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) on NK cells [141]. NK cell receptors may form an immunological synapse upon encountering a target cell. Immunological synapses were initially described in T cell-APC interaction [142]. The immunological synapses formed by T- and NK cells are characterized by specific organization of surface receptors, referred to as central and peripheral supramolecular activation cluster, c-SMAC and p-SMAC respectively. In NK cells LFA-1 accumulates in the p-SMAC and perforin

accumulates at the c-SMAC of activating synapses [143]. The cytotoxic granules cluster around the microtubule organizing center (MTOC), which in turn relocates to a site underneath the immunological synapse, thus polarizing and directing the degranulation towards the target [143-145]. It is thought that activating and inhibitory signals are integrated as the synapse forms, and the balance of signals controls how the synapse develops; and whether the process proceeds to MTOC polarization and degranulation [146].

An intriguing aspect of synapse formation by all lymphocytes is membrane nibbling, called trogocytosis, where parts of the cell membrane from the target cell is transferred to the lymphocyte, containing membrane molecules from the target cell or the antigen presenting cell [147]. The specific mechanism behind this phenomenon, as well as its physiological purpose is yet to be explained. Some have suggested a role of trogocytosis in immune regulation as the lymphocyte will obtain antigens from the target cell and thus gradually become targets themselves [148]. Another explanation could be that activated lymphocytes need to proliferate quickly, and production of membrane phospholipids is time consuming, thus stealing membrane is beneficial [148].

NK cell development

Initially NK cells were thought to develop in the bone marrow. Increasing evidence suggests that human NK cells can develop both in secondary lymphoid tissue and in the liver [149, 150]. NK cell precursors have been found in lymph nodes, liver, thymus and the uterus [151]. In the mouse the most studied NK cells are blood and splenic NK cells,

designated conventional NK cells, cNK cells. A population of NK cells in the liver with an immature phenotype are regarded as tissue-resident NK cells, with a $CD49a^{+}/CD49b^{-}$ phenotype. In addition, the liver contains circulating $CD49a^{-}/CD49b^{+}$ cNK cells. The tissue resident NK cells were found to reside in the liver sinusoidal space, suggesting a tissue-specific function of these cells. Tissue-resident NK cells are also present in other organs, such as the uterus. Human NK cells mostly develop from bone marrow-derived hematopoietic stem cells, through a common lymphoid progenitor, CLP [152]. The CLP population ($CD34^{+}CD38^{low}CD45RA^{-}CD10^{-}$) is capable of giving rise to all lymphocyte subset. CLP-like populations are also found outside the bone marrow, and these may represent the early precursors in T and NK cell development outside the bone marrow [153, 154]. Five developmental stages have been defined for NK cells (Fig. 3). Committed NKDI in the human acquire functional receptors in an orderly fashion: 1) CD161; 2) CD56, CD94/NKG2, NKp46 and NKG2D; and 3) KIR and CD16 [155-157]. Supporting this observation, fetal and neonatal NK cells are largely $CD94^{+}/KIR^{-}$, while mature NK cells are more often $CD94^{-}/KIR^{+}$ [158-160]. Stage 4 and stage 5 NK cells are designated mature NK cells, mNK cells. Stage 4 $CD56^{bright}$ NK cells ($CD34^{-}CD117^{+/-}CD94^{+}CD16^{-}LFA\text{-}1^{+}$) have high cytokine producing capacity and relatively lower cytotoxic capacity compared to the stage 5 $CD56^{dim}$ NK cells ($CD34^{-}CD117^{-}CD94^{+/-}CD16^{+}$). Stage 5 NK cells dominate in peripheral blood and show lower levels of monokine-induced cytokine production, but have more potent target cell-induced cytokine production and cytotoxicity [161]. $CD56^{bright}$ NK cells are more prevalent in neonatal tissue and umbilical cord blood, and the first to appear in peripheral

blood following stem cell transplantation [162]. $CD56^{dim}$ NK cells accumulate later after birth and later following stem cell transplantation. Moreover, $CD56^{dim}$ NK cells also display shorter telomeres [163]. However, $CD56^{dim}$ NK cells are not a terminally differentiated population of NK cells. The $CD57^{-}KIR^{-}CD56^{dim}$ population is able to differentiate into $CD57^{+}KIR^{+/-}CD56^{dim}$ NK cells in a unidirectional fashion. A gradual, but reversible loss of CD94/NKG2A is seen during this development. The $CD57^{+}$ population shows low proliferative ability and low responsiveness to cytokines. However, all combinations of CD94/NKG2A and KIR are found on the $CD57^{+}CD56^{dim}$ and $CD57^{-}CD56^{dim}$ populations, suggesting a non-linear differentiation scheme [164]. CD57 is seen as a marker for terminally differentiated NK cells and is also associated with CMV infection together with NKG2C as markers for adaptive NK cells [165, 166].

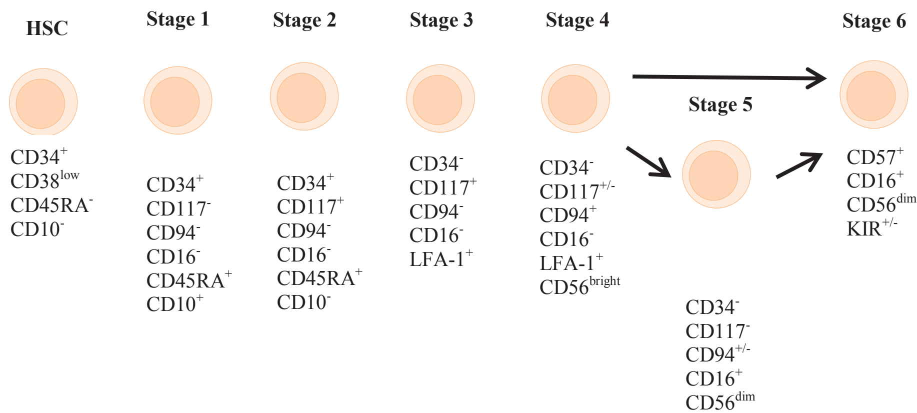

Figure 3. Schematic representation of human NK cell development. Surface antigen expression used for distinguishing the developmental stages is shown. +: expression, -: no expression.

Innate lymphoid cells (ILC) are a group of cell populations that may share some characteristics with NK cells and T helper cells. Similar to NK cells the ILC require ID2 (inhibitor of DNA binding 2) and the IL-R2γ (common cytokine receptor gamma chain) for their development. They are defined by three features: the lack of myeloid and dendritic cell phenotype markers, the lack of RAG-dependent rearranged antigen receptors and their lymphoid morphology. Three main subsets of ILCs have been identified. ILC1 are more similar to NK cells then ILC2 and ILC3. ILC1 is characterized by the expression of the transcription factor Tbet and the production of IFNγ. This subpopulation is very similar or possibly identical to liver tissue-resident NK cells. ILC2 cells express GATA3 and produce IL-4, IL-5 and IL-13, while ILC3 cells express the transcription factor RORγT and produce the cytokines IL-17 and IL-22. ILCs are involved in immunity and tissue remodeling.

Adaptive NK cells

Immunological memory refers to the ability of the immune system to respond more rapidly and effectively upon reencounter with a pathogen. NK cells possess T cell-like effector functions and originate from shared progenitor cells, but they are not considered part of the adaptive immune system. They do not undergo TCR gene rearrangement and were traditionally defined as unable to form immunological memory. This long-standing dogma has been challenged. Antigen specific NK cell memory has been reported towards haptens and viruses. In addition, NK cells can remember inflammatory cytokine milieus resulting in long lived non-antigen-specific NK cells [167-169]. Recall responses have

been described for innate cells of the myeloid lineage as well, referred to as trained immunity [2, 3, 5].

Memory-like or adaptive NK cells were recognized in particular by the identification of a long-lived NK cell subset that arises upon MCMV infection in mice [4, 170]. This subset expresses the activating Ly49H receptor that binds the MCMV ligand m157 and has been shown to be essential for immune defense towards MCMV [171-173]. Upon reencountering MCMV, this subset undergoes secondary expansion and confers protection. These cells were detectable up to six months post-infection [4]. Infection with HCMV in human is associated with expansion of NK cells expressing NKG2C or activating KIR. NK cells increase in numbers in HCMV seropositive individuals compared to HCMV seronegative individuals [174]. NKG2C^{+} NK cells also expand in allogeneic transplant patients during acute HCMV infection [175]. However, it is not established if NKGC is the receptor for a HCMV encoded ligand. A clear phenotype for adaptive human NK cells has not been defined. Amongst other characteristics the memory-like NK cells for HCMV are CD57^{hi} and lack NKG2A expression [169].

Education and self-tolerance

Similar to T cells, NK cells are educated to discriminate between self and non-self, but the mechanisms behind this process are not fully understood. In contrast to T cells, where positive or negative selection is determined through the activating TCR, NK cells are thought to be educated or licensed through the MHC class I binding inhibitory receptors. NK cells are able to lyse healthy cells that do not express self MHC class I [80, 117],

demonstrated in bone marrow transplantation where NK cells reject bone marrow from MHC disparate donors [130, 176]. Moreover, NK cells that develop in MHC class I deficient environments are hyporesponsive [177, 178]. The presence of at least one self-recognizing inhibitory receptor per cell seems important in NK cell education [179]. With regards to this, it is striking that KIR and MHC are found on different chromosomes and are inherited separately. Further complicating this matter is the fact that NK cell receptors appear to be expressed in a stochastic fashion resulting in individual NK cells that express a random repertoire of NK cell receptors. Consequently some NK cells completely lack self-recognizing inhibitory receptors [180]. This situation of potential breach of tolerance can be resolved by hyporesponsiveness [181] or, as some have suggested; by sequential expression of different inhibitory receptors by the maturing NK cell until it expresses an inhibitory receptor for self and receiving a signal that stops further receptor sampling [182].

NK cells lacking an inhibitory receptor are hyporesponsive. However, prolonged stimulation with IL-2 or IL-15 has been shown to result in gained expression of inhibitory receptors like NKG2A and inhibitory KIR, which can function in education [183, 184]. This mechanism is potentially beneficial in an inflammatory environment (infection, tumor) because hyporesponsive NK cells can acquire responsiveness through newly expressed inhibitory receptors [183]. NK cell education status can be altered if the MHC-I environment is altered experimentally by adoptive transfer. Transfer of NK cells from mice expressing normal MHC-I to MHC-I deficient mice results in loss of function and vice versa [185, 186].

Two prevailing models for NK cell receptor repertoire acquisition and tolerance exist, called the arming model and the disarming model [187, 188]. The arming model suggests that NK cells are hyporesponsive until they receive signals from a single or multiple inhibitory receptors, uncoupled to signals from activating receptors. This signal is thought to allow further maturation of the NK cell (licensing) [189]. The disarming model proposes that NK cells are reactive by default, but if they are constitutively activated and do not receive inhibitory signals to dampen the activating signals they are rendered hyporesponsive [181]. Thus both models explain the existence of hyporesponsive NK cells, though the disarming model is more similar to what is known about education of T cells where anergy is induced if the T cell is constitutively activated in the absence of inflammation or "danger" signals, such as tissue damage.

The natural killer cell gene complex and the leukocyte receptor gene complex

The present view on NK cell receptors has been acquired through experimental studies in several different species, from humans and other primates through rodents and cattle. NK receptors are structurally segregated into two groups, belonging to the C-type lectin-like receptor superfamily or the Immunoglobulin-like superfamily (IgSF). The C-type lectin-like receptors are clustered in a chromosomal region called the NK cell gene complex (NKC) [132, 190, 191]. Several different receptor subfamilies are located here, including NKG2D (KLRK1), NKR-P1 (CD161, KLRB), CD94 (KLRD1), NKG2A, C, E (KLRC1, C2, C3), Ly49 (KLRA), KLRE/I and KLRH1 (Table 1). Many of the IgSF receptors expressed selectively by NK cells are found in the Leukocyte receptor gene complex

(LRC) [192, 193]. Some of the subfamilies located here are KIR, LILR, LAIR and NKp46 (Table 1). NK cell inhibitory receptors from both gene complexes signal through ITIMs, while activating receptors have a positively charged amino acid residue in their transmembrane regions facilitating association with the DAP10, DAP12, FcεRIγ or CD3ζ adaptor proteins for activating signal transduction [194].

Table 1. NK cell receptors

Leukocyte receptor gene complex Immunoglobulin superfamily	NK cell gene complex **C-type lectin** superfamily	Other important NK receptors
KIR LILR (ILT, LIR, PIR, gp49B) LAIR NKp46	Ly49 CD94/NKG2 NKG2D NKR-P1 KLRE/I KLRH	NKp30 NKp44 2B4 DNAM-1

C-type lectin like receptors

The NKC encodes a group of type II transmembrane glycoproteins defined by the extracellularly oriented C-terminal end of the protein. Structurally these receptors contain C-type lectin-like domains and are expressed mainly as disulphide-linked homo- or

heterodimers. Most other C-type lectins bind carbohydrates in a Ca^{2+}-dependent manner, but this is not true for the NKC-encoded receptors (collectively termed killer cell lectin-like receptors or KLR) where the extracellular domains have been modified to bind protein ligands, and belong to the group V of the C-type lectin superfamily.

Ly49 (KLRA)

The largest receptor family in the rat and mouse NKC is the Ly49 family. In addition to extensive allelic polymorphism varying number of Ly49 genes are found in rodents. Only one Ly49 gene has been identified in the human, considered to be a pseudogene as it contains a premature stop codon [195]. Rodent Ly49 receptors form disulfide-linked homodimers on the cell surface and are expressed by NK cells and a small subset of T cells. Inhibitory Ly49 have an intracellular tail with ITIM while the activating receptors lack ITIM but contain a positively charged arginine residue in the transmembrane region which associates with DAP12 (Fig. 4) [133]. In the mouse activating Ly49 bind to both DAP10 and DAP12 [196, 197]. Interestingly, in the rat some Ly49 potentially have a dual function encompassing both a positively charged amino acid in the transmembrane region and an ITIM-bearing cytoplasmic tail [198]. The ligands for most of the inhibitory Ly49 are classical MHC class I molecules, while only some activating members have demonstrated binding to MHC class I [199]. Ly49 receptors have also been shown to bind in cis to MHC ligand, potentially restricting Ly49 recruitment to the immunological synapse and thereby lowering the threshold for activation [200, 201]. The activating

Ly49H has been shown to bind m157, a MCMV encoded MHC class I-like molecule that also binds the inhibitory Ly49I [173].

CD94/NKG2 (KLRD1/NKG2)

The CD94/NKG2 family of receptors is composed of activating and inhibitory members and is expressed by a large portion of NK cells and a subset of T cells (Fig. 4). CD94 forms a heterodimer with NKG2A, -C or –E [202]. CD94/NKG2A is an inhibitory receptor where the intracellular tail of NKG2A bears two ITIM in tandem [203]. The ligand for this receptor is HLA-E in the human and the homologous Qa-1 in the mouse [101, 204]. HLA-E is a non-classical MHC class I molecule and presents the signal peptide from other MHC class I molecules in the peptide binding groove [205]. Human NKG2C and -E lack ITIM, but associate with DAP12 through a positively charged amino acid in their transmembrane region [206]. Remarkably, the signaling from CD94/NKG2 has evolved differentially in rodents compared to mammals. Whereas human and all other studied mammalian CD94 lack signaling function, rat and mouse CD94 have a lysine residue in the transmembrane region and bind DAP10 or DAP12. Moreover, rodent NKG2C and -E seem to lack signaling function. Rodent NKG2A, like human, has a tandem pair of ITIM [207].

Although the inhibitory receptor generally shows higher affinity for HLA-E, both the activating and inhibitory receptors have demonstrated peptide-dependent binding to HLA-E. CD94/NKG2C binding has been demonstrated when HLA-E is complexed to the signal

peptide from HLA-G, but not when complexed with a peptide from the Epstein Barr virus [208].

NKR-P1 (KLRB)

The NKR-P1 family also consists of receptors with activating or inhibitory function and is expressed on the cell surface as homodimers. The inhibitory receptors signal trough cytoplasmic ITIM. The activating receptors have an arginine in the transmembrane domain that enables association with the FcεRIγ adaptor protein that contains immunoreceptor-tyrosin-based-activation motifs (ITAM) [209, 210]. The ligands for the different NKR-P1 family members belong to the C-type-lectin related (Clr) family of molecules (CLEC2). The genes encoding the Clr family are located within the NKC, intertwined with the *Nkrp1* genes. Only one NKR-P1 is found in the human, NKR-P1A, which binds to the Clr homolog LLT1. LLT1 is expressed on activated dendritic cells and B cells. NKR-P1A binding to LLT1 on the targets cells inhibits NK cell effector functions [211, 212]. NKp65 (KLRF2) and NKp80 (KLRF1) have been suggested to be part of the human NKR-P1 family based on similar lectin-like domains, cytoplasmic signaling domains and similar expression pattern of the ligands. NKp65 binds to KACL and NKp80 binds to AICL and both ligands share sequence similarity with the Clr family [213].

NKG2D (KLRK1)

NKG2D is a single-member family in human, rat and mouse (also termed KLRK1) [214-216]. This receptor does not by sequence similarity belong to the NKG2 (KLRC) family and does not dimerize with CD94, and as such the name NKG2D is somewhat

unfortunate and misleading. Virtually all NK cells in human, rat and mice express NKG2D. In the human, NKG2D is also expressed by all $CD8^+$ T cells and γδ T cells and in mice by NKT cells, γδ T cells and activated $CD8^+$ T cells [217]. The human NKG2D signals through the adapter protein DAP10 [218]. In the mouse there are two isoforms of NKG2D. The long isoform associates exclusively with DAP12, while the short isoform associates with both DAP10 and DAP12 [219-221]. Human NKG2D binds to members of the RAET1 (retinoic acid early transcript 1) family called ULPB1-4 (UL16 binding proteins) and the polymorphic MICA and MICB proteins [222-224]. The mouse orthologue binds to RAE-1(retinoic acid inducible 1), H60 (histocompatibility 60) and MULT1 (Murine ULB1-Like Transcript) [225-227], while in the rat the ligand is a RAE-1 ligand known as RAE-1L and a RAE-1 like transcript, RRLT [228]. All known NKG2 ligands are MHC class I-like proteins with a similar structure, but lacking β2-microglobulin and peptide presentation. Available crystal structures of NKG2D - ligand complexes reveal NKG2D homodimer binding to the top of the α1/α2 platform [229, 230]. The ligands for NKG2D are upregulated under viral infections and following DNA damage or malignant transformation [231].

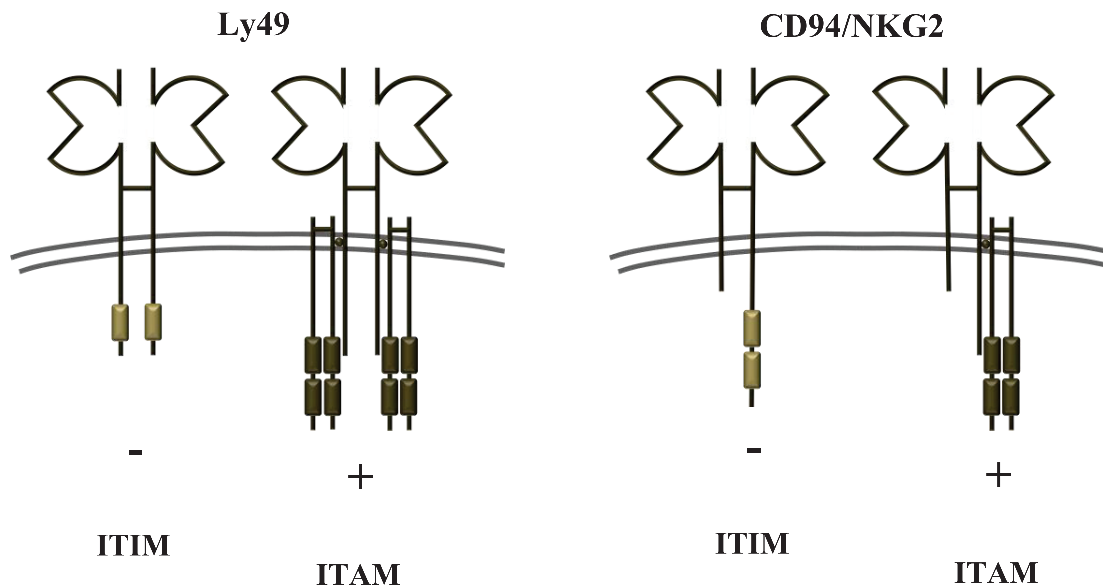

Figure 4. To the left is a schematic illustration of inhibitory and activating Ly49 receptors. The inhibitory Ly49 signal trough ITIM motifs, while the activating Ly49 associate with the activating adaptor protein DAP12 containing ITAMs. To the right is a schematic illustration of inhibitory and activating human CD94/NKG2 heterodimers, respectively. Human CD94/NKG2A is an inhibitory receptor signaling trough ITIMs, while CD94/NKG2C is an activating receptor that associates with DAP12 for signaling with ITAMs, similar to the Ly49 receptors.

Immunoglobulin-like receptors

The IgSF receptors encoded by the LRC contain immunoglobulin-like domains and are type I transmembrane glycoproteins defined by an extracellular N-terminus. Like the NKC, the LRC contains families of receptors with activating and inhibitory members.

NKp46, NKp44 and NKp30 are called natural cytotoxicity receptors (NCR). NKp46 has been identified in human, mouse, rat and cattle and in these species is almost exclusively

expressed by NK cells, and is used as a marker for NK cells. NKp46 can also be expressed by IL-22 producing ILC3. NKp46 signals through association with heterodimers of CD3ζ / FcεRIγ [232]. NKp46 has been shown to recognize a yet unidentified ligand on tumor cells, and has also been shown to bind to the hemagglutinin protein encoded by influenza virus [233, 234]. However, the matter of NKp46 ligand remains unsettled. NKp44 has been shown to bind a truncated isoform of the mixed lineage leukemia-5 (MLL5) protein on malignant cells [235]. NKp30 has been described to bind two different ligands, BAG6 (formerly named BAT3) and B7-H6 that belongs to the B7 family of proteins [236, 237].

Killer cell immunoglobulin like receptors, KIR

In striking contrast to the Ly49 family that is expanded in rodents, the KIR family is expanded in primates (and cattle) but is represented by only one locus in the rat and two loci in the mouse [192, 238, 239]. The physiological significance of rodent KIR is as yet unknown. Mouse KIR3DL1 is expressed on all NK cells, but at very low levels, and ligands have as yet not been identified. Rat KIR3DL1 has not been studied functionally [192, 238].

The cattle KIR family consists of more than 10 members, some are presumed to be activating because they lack ITIM and have a positively charged residue in the transmembrane region [192, 240]. Ligands for cattle KIR have not been identified, but appear not to be widely expressed on normal, resting cells (Boysen and Dissen, unpublished observations). The extracellular regions of KIRs consist of two or three Ig-

like domains followed by a short stalk region. The cytoplasmic regions of inhibitory KIR are long and typically contain two ITIM motifs, while the cytoplasmic regions of activating KIR are short and lack ITIM (Fig. 5). Instead, activating KIR have a positively charged lysine residue that facilitates association with the DAP12 adaptor molecule [241, 242]. Although they are structurally unrelated, Ly49 receptors in rodents are considered functional homologues for KIR in human, because both families include activating and inhibitory receptors that use similar signaling mechanisms and the inhibitory variants bind specifically to allelic variants of classical MHC class I [208, 243]. However, because ligands for activating KIR and Ly49 remain mostly unidentified, this view remains provisional.

The human KIR family consists of 15 KIR loci clustered on chromosome 19 (19q13.4), two of which are pseudogenes. This region displays extensive allelic polymorphism and gene content variability [123, 124, 244]. Two main haplotype groups, group A and group B, have been identified. Four framework genes are found in both haplotypes, with some exceptions; these are KIR2DL4, KIR3DL2, KIR3DL3 and KIR3DP1. KIR3DL3 is placed in the centromeric end, KIR2DL4 in the middle and KIR3DL2 at the telomeric end [245] (Fig. 6). The group A haplotypes typically display little variation in gene organization, and is made up of eight genes including the framework genes. The B haplotypes contain activating KIR and are defined by the presence of one or more of the genes KIR3DS1, KIR2DS1, -2, -3, in addition to the inhibitory KIR2DL5A/B and KIR2DL2 [245]. The haplotype diversities are a result of different recombination events, resulting in different gene content, and the picture is further complicated by allelic polymorphisms [244].

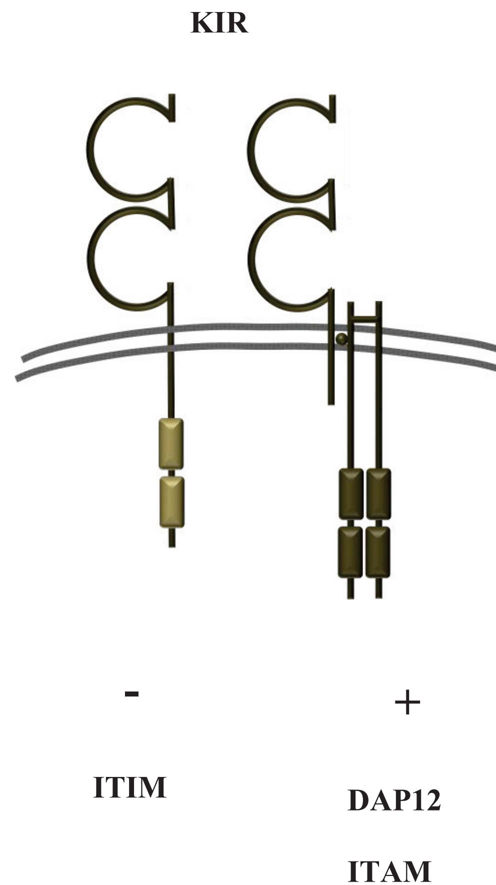

Figure 5. The inhibitory KIR have a long intracellular tail with ITIMs for inhibitory signaling. The activating KIR typical have a short intracellular tail which associates with the adaptor protein DAP12 which contains ITAMs for activating signaling.

KIR Nomenclature

The KIR nomenclature is based on the structure of the molecules, denoting the number of extracellular IgSF domains as well as the length/signaling function of the cytoplasmic region. Hence the KIR having two or three extracellular Ig-like domains are accordingly

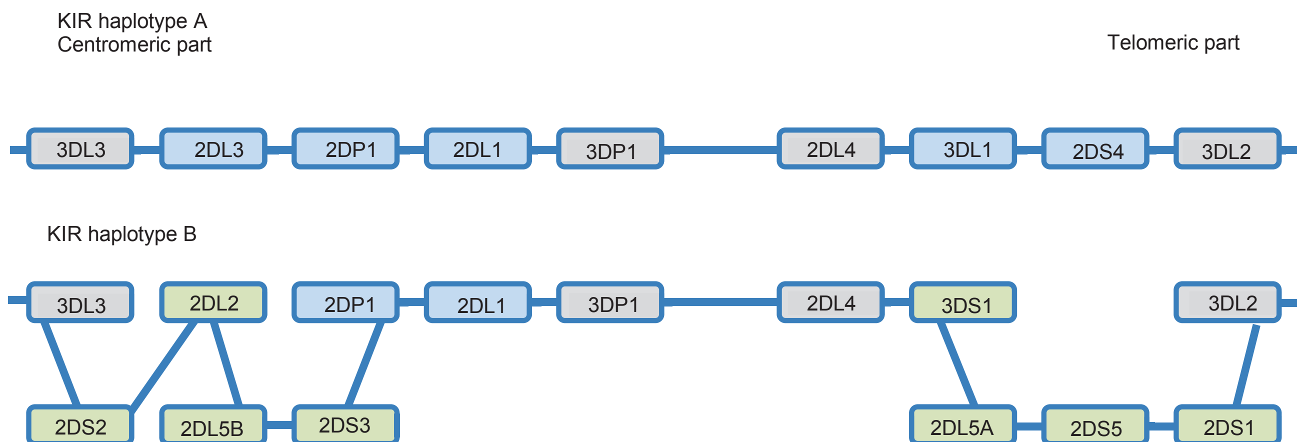

Figure 6. KIR haplotype A and B. Framework genes are shown in grey. Genes that can occur in both haplotypes are shown in blue. Genes that only occur in haplotype B are shown in green.

termed 2D or 3D, respectively, followed by S" or "L" corresponding to a short (activating) or long (inhibitory) cytoplasmic tail (Fig. 5). Pseudogenes are named with a "P". The last digit refers to the locus number within a group of genes sharing a similar structure (e.g. KIR2DS1, KIR2DS2. Next, the KIR alleles are designated digits where the first three digits distinguishes allelic exon sequence that give non-synonymous changes, the following two digits indicate differences in exon sequences that give synonymous changes, and the last two digits encode DNA substitutions in non-coding regions (i.e. KIR2DL1*0030202). (IPD KIR database)

Target cell recognition by NK cells

NK cell effector functions are regulated by the balance between activating and inhibitory signals. Based on the close sequence similarity between the ligand binding parts of the activating and inhibitory receptors within the same family, it was assumed that the activating receptors would bind similar ligands to the inhibitory receptors. Over the years the search for ligands for the activating NK receptors has been challenging, and the general view is that functionally relevant ligands for activating Ly49 and KIR receptors are largely unidentified. Therefore the functional role of activating KIR and Ly49 has been disputed, and some have argued that they are obsolete remnants that have lost their ligands, or even exist as "accidents of evolution". One piece of evidence has led many researchers to propose that activating KIR and Ly49 receptors have a rapid turnover in evolution, and that activating receptors arise from inhibitory receptors by gene duplication followed by acquisition of activating function as a result of a recombination event: Ly49H, an activating Ly49 in the mouse, binds a mouse CMV-encoded MHC class I-like ligand, m157. A popular hypothesis suggests that the Ly49H receptor became an evolutionary advantage after MCMV evolved m157 as a decoy for the inhibitory receptor Ly49I. Similarly, HCMV has developed ways to avoid NK cell mediated lysis. Classical MHC-I is usually downregulated during HCMV infection. Peptides derived from the HCMV encoded UL40 have been shown to bind HLA-E, maintaining HLA-E surface expression on infected cells and thereby mediating inhibition of NK cells through CD94/NKG2A [246]. However, some of the UL40 derived peptides are recognized by CD94/NKG2C and activate NK cell lysis of the target cell [247].

In summary, target cell recognition by NK cells is mediated through both inhibitory and activating receptors, and the decision to kill or to spare an encountered cell depends on a delicate balance between activation and inhibition. Exemplifying this principle, simultaneous engagement and signaling from an inhibitory Ly49 and the activating NKG2D receptor can shift the signal towards activation [146]. Accordingly, NK cell killing can be initiated by loss of MHC class I expression but also by increased expression of ligands for activating receptors that are capable of overruling inhibitory signals.

NK cell receptors binding MHC class I or MHC class I-like molecules

Different receptor families binding MHC class I molecules have been identified in human and rodents. These are the rodent Ly49s, the human KIR and the CD94/NKG2A,-C,-E heterodimers, which all have activating and inhibitory members. Physiologically functional, multimember families of Ly49 receptors have only been found in a few mammalian species in addition to rodents and horse. More than 15 different Ly49 receptors have been described in the mouse, while 26 Ly49 loci have been found in the rat [192, 198].

Murine CMV (MCMV) encodes several MHC-I homologs that may function to deceive NK cells. m157 is an MHC-I like protein encoded by MCMV and is recognized by the inhibitory Ly49I receptor in some mouse strains, potentially hampering NK cell activation. However, other mouse strains express an activating receptor that binds m157, Ly49H, on NK cells. The *Ly49h* locus conveys resistance to MCMV infection [173, 248, 249]

A well characterized and conserved receptor shown to bind MHC class I-like molecules expressed by stressed cells is the activating receptor NKG2D. NKG2D appears to be promiscuous, recognizing different and somewhat unrelated ligands within the same species, binding MICA/-B and ULBP in human, RAE-1, H60 and MULT1 in mice and RAE1 L and RRLT in the rat [217, 228].

The CD94/NKG2 heterodimers bind HLA-E in the human and the HLA-E orthologue Qa-1 in the mouse [204, 250, 251]. HLA-E and Qa-1 present peptides derived from the leader regions of other MHC class I molecules, thus providing NK cells a way of monitoring the MHC expression level on a target cell with the use of a single receptor [101, 205, 250]. Interestingly, in the mouse lemur, the CD94 and NKG2 gene families are expanded, and only a single KIR and Ly49 are present. Furthermore, the mouse lemur genome lacks an HLA-E orthologue, suggesting that in these animals, CD94/NKG2 may play a more general role in MHC recognition as a functional equivalent of KIR or Ly49 [252].

Table 2. Genes, their alternate name and ligands for KIR.

Gene	Alternate names	Ligand
KIR2DL1	CD158a, NKAT1	HLA-C2
KIR2DL2	CD158b1, NKAT6	HLA-C1 >> HLA-C2
KIR2DL3	CD158b2, NKAT2	HLA-C1 >> HLA-C2
KIR2DL5A	CD158f	-
KIR2DL5B	KIR2DL5.2	-
KIR3DL1	NKB1, NKAT3	HLA-Bw4, some HLA-A, HLA-F
KIR3DL2	CD158k, NKAT4	Some HLA-A, HLA-F
KIR3DL3	CD158z	-
KIR2DL4	CD158d	HLA-G
KIR2DS1	CD158h	HLA-C2 (weakly)
KIR2DS2	CD158j, NKAT5	HLA-C1? HLA-A11?
KIR2DS3	NKAT7	-
KIR2DS4	CD158i, NKAT8	HLA-F
KIR2DS5	CD158g, NKAT9	-
KIR3DS1	CD158e2, NKAT10	HLA-F

-: ligand not established

MHC class I Molecules as Ligands for KIR

NK cells recognize and kill cancer cells and cells with intracellular infections through mechanisms yet to be fully disclosed. From an elevated perspective, NK cell recognition can be explained by recognition of altered self, induced self or the absence of self MHC class I molecules (missing self). While the archetypical example of altered self is MHC class I presentation of pathogen-derived peptides other potential alterations of MHC class I could conceivably be more generic and include e.g. alterations in glycosylation, peptide lengths, post-translational modifications of peptides, loss of peptides recognized by inhibitory KIR etc. The class I-like molecules ULBP1-4 and MICA and -B are examples of induced self, becoming expressed on cells following stress, heat shock, DNA damage etc. [217]. Missing self is seen as reduced or abrogated expression of class I, and is seen as an inherent viral strategy in several viral infections and in some cancer cells [253-255]. Because the immune system to such a large extent relies on MHC class I to report intracellular status, surface expression of class I molecules is tightly regulated. Misfolded class I molecules or molecules lacking peptide are held back in the endoplasmic reticulum (ER) by chaperones and subsequently degraded. Despite this quality control, misfolded or "open" class I molecules can be expressed at the surface at very low levels, albeit with a short half-life [256].

Members of the highly diverse family of HLA class I molecules are ligands for the inhibitory KIR (Table 2). Crystal structures have revealed that the inhibitory variants -2DL1 and -2DL3 bind the top of HLA class I, to a surface composed of peptide and both alpha-helices, towards the C-terminal end of the presented peptide [257]. All inhibitory

KIR tested show a low degree of selectivity for the peptide bound to MHC class I [258-263].

NK cells have receptors that recognize HLA-A, -B, -C but also HLA-E, -F and -G (Table 2). Some alleles of HLA-A and -B act as ligands for the inhibitory KIR with three Ig-like extracellular domains, while most inhibitory KIR with two Ig-like domains are restricted to allelic variants of HLA-C [244]. The HLA-C alleles can be distinguished into two groups, HLA-C1 and HLA-C2, based on the amino acid in position 80. The C1 group is defined by an asparagine residue in position 80 and is bound by KIR2DL2 and KIR2DL3. The C2 group, defined by a lysine residue in the same position, is bound by KIR2DL1[264]. Across this delineation, some C2 group alleles can bind to KIR2DL2 (and to a lesser extent, KIR2DL3) [265].

HLA-G is the ligand for KIR2DL4, a receptor expressed on all NK cells. KIR2DL4 has an intracellular ITIM motif but has activating signaling capacity through a charged transmembrane amino acid, and appears mostly to cluster in endosomes and signal from intracellular compartments [107, 266, 267].

Although there can be more than 95% sequence identity between the extracellular region of inhibitory KIR and their activating siblings, activating KIR are strikingly unable to bind to the same class I ligands. Notably, substituting a single amino acid of KIR2DS2 for the corresponding residue in KIR2DL2 and -2DL3 (Y45->F) in a KIR-Fc fusion protein experiment enabled binding to C1 group ligands [268]. Several observations indicate the ability of KIR2DS1 to bind weakly to certain alleles of HLA-C [269, 270]. Activating

KIR2DS2 has been reported to bind HLA-C [271], and also, in a peptide-dependent manner, to HLA-A11[268, 272]. However, this remains to be corroborated. KIR2DS3, -4 and -5 are orphan receptors that have as yet not been found to engage in significant interaction with HLA class I.

KIR3DL1, -2 and KIR3DS1 have all been shown to bind to HLA-F open conformers. KIR3DS1 showed the strongest affinity to HLA-F open conformers, compared with the inhibitory KIR3DL1 and KIR3DL2. [103, 273, 274] .

Paired receptors

The functional relevance of the co-existence of highly similar paired activating and inhibitory receptors is yet to be revealed. Constitutive expression of activating receptors potentially recognizing endogenous ligands may increase the risk of autoimmunity. On the other hand, this situation also enhances the number of cells "ready-to-kill" immediately upon infection or malignant transformation resulting in changes in endogenous proteins. Inhibitory receptors appear to be required for recognition of missing-self, to identify healthy-self and for NK cell licensing as NK cells lacking any inhibitory signal are rendered hyporesponsive [80, 275]. There is evidence of evolutionary selection pressure to preserve the homology between the ligand-binding domains of activating and inhibitory family members. Evidence indicates that the activating receptors closely follow the inhibitory sibling mainly by exchange of genetic material. [276, 277]. Both the Ly49 and KIR families show extreme allelic polymorphism, as well as considerable heterogeneity among haplotypes with regards to the number of

receptor loci [278]. Presumably, this is needed to keep up with the highly polymorphic and rapidly evolving MHC and rapidly evolving pathogens [279]. Thus, NK cell receptor diversity appears to be maintained through rapid mutations and recombination events between the loci within a receptor family [280].

DAP10 and DAP12 adaptor proteins

Activating NK cell receptors lack intracellular signaling motifs. To convey activating signals they associate with DAP10, DAP12, FcεRIγ or the CD3ζ adaptor proteins through a charged amino acid in the transmembrane region [241, 281]. In this system, the ligand binding and the signal transduction domains are associated with different subunits [282]. One advantage of this system could be that it allows the ligand binding proteins to diversify, e.g. KIR and Ly49, by mutations in the gene while the signaling function can be preserved and shared between different families of receptors [276, 277]. The DAP10 and DAP12 signaling subunits are highly conserved in evolution [283]. The DAP10 and DAP12 genes are found on chromosome 19 in the human. The small extracellular regions of DAP10 and DAP12 have a conserved cysteine, involved in the formation of a disulfide-bonded homodimer [283]. These homodimers pair with the associated receptors via ionic interaction between an aspartic acid residue centrally located in their transmembrane region, and a positively charged amino acid located in the transmembrane region of the receptor. DAP12-associated receptors were identified functionally by expression cloning from different cDNA libraries and screening for their ability to bring DAP12 to the surface in 293T cells, “DAP trapping” [284-286]. Human NKG2D was the

first receptor identified to associate with DAP10: one NKG2D homodimer associates with two DAP10 homodimers [218]. DAP10 and DAP12 have different signaling pathways. Tyrosine phosphorylation of the conserved signaling element in DAP10, YINM, recruits phosphatidylinositol-3 kinase (PI3K) and the Grb-Vav1-SOS1 complex. DAP12 has a conserved ITAM motif, D/ExxYxxL/I$x_{6\text{-}8}$YxxL/I, which upon tyrosine phosphorylation may recruit Syk or ZAP70 tyrosine kinases [283]. The signaling capabilities of DAP10 and DAP12 are not precisely known DAP12 has been shown to induce both cytotoxicity and IFN-γ secretion [220, 283, 287-289]. DAP10 associated to NKG2D has been shown to mediate costimulation in activated T cells [220, 290].

Aims of the study

The fundamental aim of my work was to study the function of activating NK cell receptors, particularly the activating KIR and to identify their ligands. Furthermore, based on recent observations in our laboratory [207] , we wished to investigate whether the activating receptors and the adaptor proteins DAP10 and DAP12 can be promiscuous in their associations, thereby contributing to an increased understanding of how NK cell activation is regulated.

1. **Identification of ligands for activating KIR.** While HLA class I molecules were rapidly identified as ligands for inhibitory KIRs, ligands for most of the activating KIR remain to be identified, although the extracellular regions of the inhibitory and the activating receptors are highly similar. A lack of understanding of the function of activating KIR hampers the use of NK cells in e.g. bone marrow transplantations and in cancer therapy. Whereas epidemiological evidence points to a role for activating KIR in several infections (HIV, hepatitis C) and diseases ranging from autoimmune disorders and cancer to pre-eclampsia, these observations cannot easily be explored experimentally due to lack of knowledge about what is recognized by activating KIR. Several studies indicate that KIR family members function either to protect against or to increase susceptibility to infections, autoimmune disease and cancer. We hypothesized that activating KIR recognize MHC class I or class I-like ligands that have undergone molecular modifications as a result of intracellular stress or infection. This could be a mechanism by which "sick" cells signal the immune system, reporting that the

internal homeostasis is somehow disrupted, potentially also when the cell is unable to undergo apoptosis due to neoplastic transformation of the cell. *Based on this hypothesis we aimed to investigate stressed cells as well as multiple human cancer cells lines for their ability to bind to activating KIR.*

2. **Determination the specificity of the interaction between the adaptor proteins DAP10 and DAP12 and activating NK cell receptors, and whether they can form heterodimers**. Unlike the inhibitory receptors that signal through ITIMs in the intracellular region, activating NK cell receptors do not have endogenous signaling motifs. Instead, the activating receptors have a charged amino acid in their intracellular region with which they associate with DAP10, DAP12, FcƐRIγ or CD3ζ. Mouse NKG2D with a long intracellular tail associates with DAP10 but not DAP12, while mouse NKG2D with a short intracellular tail is able to associate with both DAP10 and DAP12. NKG2D in human has only been shown to associate with DAP10. Current data indicate that there is little overlap between receptors that associate with DAP10 and receptors that associate with DAP12. DAP10 and DAP12 have previously only been shown to form homodimers. In contrast, FcƐRIγ and CD3ζ associate to some of the same receptors and can form heterodimers as well as homodimers. For instance, human NKp30 binds to either CD3ζ homodimers or heterodimers of FcƐRIγ and CD3ζ, while human NKp46 bind to either FcƐRIγ homodimers or to heterodimers of FcƐRIγ and CD3ζ. *We therefore aimed to investigate the ability of DAP10 and DAP12 to form heterodimers.*

Moreover, we wanted to systematically re-evaluate the ability of different NK cell receptors to bind to DAP10 and DAP12 in mouse, rat and human.

Summary of results

Paper I In paper I we report that the KIR2DS2 reporter cells recognized a ligand expressed by cancer cell lines. All cancer targets recognized by KIR2DS2 were also recognized by KIR2DL2 and KIR2DL3 reporters. Trogocytosis of membrane proteins from the cancer targets was observed with responding reporter cells, indicating the formation of KIR2DS2-ligand specific immunological synapses. The recognition was C1 and C2 independent for target cells that were recognized by both KIR2DS2 and KIR2DL2/-3, while targets cells that were only recognized by KIR2DL3 expressed C1 group alleles. As expected anti-HLA class I antibodies blocked KIR2DL3 responses towards C1 expressing targets, but did not block recognition of target cells that were recognized by both KIR2DS2/KIR2DL3/-L2 siRNA knockdown of β_2-microglobulin reduced the expression of class I heavy chain on the cancer targets by more than 97%, but did not reduce the KIR2DS2 reporter responses, indicating a β_2-microglobulin independent ligand for KIR2DS2. Importantly, KIR2DL3 responses towards some KIR2DS2-ligand-expressing cells were also undiminished after β_2-microglobulin knockdown, and were not blocked by anti-HLA I antibodies, suggesting that KIR2DL3, in addition to the traditional HLA-C ligands, can bind to the same β_2-microglobulin-independent ligand as KIR2DS2. These observations indicate the existence of a novel, presently uncharacterized ligand for the activating NK cell receptor KIR2DS2. Molecular identification of this ligand may lead to improved KIR-HLA mismatching in hematopoietic stem cell transplantation therapy for leukemia, and new, more specific NK cell-based cancer therapies.

Paper II In paper I we reported that the activating KIR2DS2, as well as its inhibitory siblings KIR2DL3 and KIR2DL2 recognize a β_2-microglobulin-independent ligand expressed on the surface of several carcinoma cell lines. In paper II we demonstrate that a soluble ligand in the supernatant from the prostate cancer cell line PC-3 is recognized by KIR2DS2 and KIR2DL3. The OVCAR-3, SK-BR-3 and DU145 cell lines also produced a soluble ligand that activated the KIR2DS2 and KIR2DL3 reporter cell lines, but in lower concentrations, while supernatant from the KIR2DS2 ligands negative cell lines T47D and WM9 did not activate an EGFP response in any of the KIR reporters. Proteinase K treatment of PC-3 supernatant abolished the response by KIR2DS2 reporter cells, whereas digestion with Heparinase II, DNAse I and PNGase F had no effect, indicating that the ligand is a protein. The soluble ligand produced by PC-3 cells could be purified by size exclusion chromatography and ion exchange chromatography, and maintained the ability to activate the KIR2DS2 reporter cell lines after purification. Ongoing activities aim to identify candidate ligands by mass spectrometry. Soluble ligands for the activating NK cell receptors NKG2D and NKp30 are believed to represent an immune evasion strategy that provide the cancer cells with a survival advantage. Our finding that a similar mechanism involves KIR2DS2 ligand in cancer cell lines merits further investigation in cancer patients, as well as the molecular identification of the cancer cell KIR2DS2/-2DL2/-2DL3 ligand.

Paper III Contact between a NK cell and a target cell is often controlled by several different activating and inhibitory receptors acting simultaneously side by side in the immunological synapse. The outcome, either activation or inhibition of the NK cell, is presumably dependent on the sum of activating versus inhibitory signals. Activating receptors signal indirectly by association with short transmembrane adaptor proteins. The association between the activating receptor and adaptor protein depends on an intramembrane ionic bond formed between a positively charged residue (lysine or arginine) and a negatively charged residue (aspartic acid) in the transmembrane region of the receptor and adaptor, respectively. The activating adaptors DAP12 (KARAP), CD3ζ and FcεRIγ contain cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAM). In contrast, the adaptor DAP10 lacks ITAM and instead activates NK cell effector functions through a YxNM motif. Based on our finding that mouse and rat CD94 readily bind to both DAP10 and DAP12, we decided to more closely investigate the ability of these two adaptors to bind to human, mouse and rat NK cell receptors. In paper III we report that in transfection systems, several NK cell receptors in human, mouse and rat can bind to both DAP10 and DAP12, whereas others bound to only one of them. DAP10-DAP12 heterodimers were identified in complex with NK cell receptors. Moreover we show that DAP10 and DAP12 are able to form higher order complexes. The data indicate the possibility that in some instances, both signaling mechanisms can be evoked simultaneously by the same receptor. Higher order homo- or heterocomplexes of DAP10 and DAP12 could in theory provide a molecular mechanism for receptor clustering, possibly bringing different receptors into the same cluster.

METHODOLOGICAL CONCIDERATIONS

This section provides a discussion of the methods used, why they were considered as appropriate for this project, their advantages, limitations and potential problems. Detailed information on materials and methods is found in the individual papers.

One of the project aims was to identify ligands for the activating KIR. In a deeper sense, for a molecule to be regarded as a physiological ligand it must bind to the receptor under physiological conditions (pH, salt concentration, normal folding and glycosylation). The affinity in the interaction must be sufficient to induce intracellular signaling at relevant expression levels of receptor and ligand. Notably, the avidity is often considerably increased in immunological synapses, where a large number of receptors are clustered.

With careful consideration of advantages and limitations, three methodological approaches were chosen to search for ligands recognized by activating KIR. These three methods include a cellular reporter system, a cytotoxic NK cell system and soluble KIR fusion proteins. The section will also include methods that were tested, but which did not yield results.

Cellular reporters

Cellular reporters were generated using BWN3G, a mouse T cell line transfected with EGFP (enhanced green fluorescent protein) under the control of three copies of the NFAT promoter element. This cell line was again transfected with chimeric receptors containing the extracellular region of a KIR fused to the transmembrane region of CD8 and the cytoplasmic region of CD3ζ, containing its three ITAM signaling motifs. The advantages

of the reporter system is that it mimics the *in vivo* situation with regards to receptor-ligand interaction, incorporating avidity changes that result from receptor and ligand clustering in synapses. Moreover, the molecules are expressed with correct topology in the membrane, with correct folding and post-translational modifications such as glycosylation. Importantly, the system is well suited to monitor whether a ligand binding to KIR in a cell-to-cell contact is capable of inducing signal activation on the effector cell side. Alongside the advantages of the reporter cell system, a few potential problems require attention and the use of proper controls. Unspecific activation through other receptors on the mouse T cell line, possibly facilitated by certain cytokines has been observed. In our hands, co-incubation with two mouse dendritic cell lines was found to induce EGFP expression (unpublished observations) independent of the binding of the chimeric receptor, demonstrating the need for receptor-negative controls at all times. Although the system is sensitive, it may fail to detect ligand binding if unknown co-receptors are required.

Reporter Assays

Cellular reporters were co-incubated with various target cell lines overnight, and the reporter cells were analyzed for EGFP production by flow cytometry. We aimed to standardize handling of the cells as much as possible, with special attention to a few points: We routinely detached adherent tumor cells by careful flushing with PBS containing 2 mM EDTA. EDTA will chelate divalent cations that are necessary for cadherin-mediated cell adhesion, and several other cell surface proteins rely on divalent

cations such as Ca^{2+} and Mg^{2+} to maintain proper folding. Thus, exposing cells to EDTA could lead to partial denaturation of cell surface proteins, which in turn could affect our experiments as we are searching for a potential protein ligand. However, we expect that the cell surface proteins reassume their native conformation when resuspended in culture medium supplemented with 10% serum, since serum proteins represent a large buffering reservoir of Ca^{2+} and Mg^{2+} ions. Furthermore, the results can be affected by cell counting errors due to a miscalibrated or malfunctioning cell counter. Cell viability is an essential parameter, and best practice culturing techniques were applied.

Cell Culture

Culture conditions such as cell confluence, pH of the medium, number of apoptotic or dead cells in the culture and unrecognized infections can profoundly influence the phenotypic characteristics of cells, resulting in unwanted experimental variation, but sometimes also revealing important keys to biological functions. In our cell culture, we have aimed to keep most conditions constant in all experiments, while some other conditions (cell densities, variations in medium compositions etc.) have on occasion deliberately been varied to explore the effects. Special attention has been given to using the same batches of reagents, to cell confluence and to regular exchange of culture medium. The cell lines used have regularly been tested for *Mycoplasma* infection using a PCR-based protocol.

Cell Lines

Most of the experiments in our studies were based on cell lines. Some were derived from tumors and adapted for *in vitro* culture, others have been immortalized by viral transformation. The advantages of cell lines are many: they are easily available, exist in multiple variants, from different tissues and species, can be cultured for longer periods of time and are robust with regards to culture conditions. Moreover, most commonly used cell lines are phenotypically stable, allowing experiments to be repeated and also to be replicated and confirmed by other investigators. In contrast, primary cells often have a limited lifespan in culture, are less easily obtained and importantly tend to be more susceptible to culture conditions. The obvious problem with cell lines is that they tend to be physiologically less representative than primary cells. This is particularly true for cancer cell lines that are genetically unstable. Therefore, observations in cell lines will often ultimately require corroboration by experiments in primary cells or *in vivo*. Another problem with cell lines is that they have often been cultured for many cell divisions, and passed through several laboratories, posing a risk for contamination with other cell types as well as unrecognized infection. Our cells were authenticated by genotyping at the Oslo university hospital cell typing laboratory.

siRNA Knockdown

To optimize knockdown efficiency, various cell concentrations, siRNA concentrations and different cationic lipid formulations were tested. In our hands, using 293T cells as the pilot cell line, Lipofectamine RNAiMAX performed significantly better than

Lipofectamine and Lipofectamine 2000. Polyethyleneimine (PEI) performed almost as well as Lipofectamine RNAiMAX, but the latter was preferred for slightly higher efficiency combined with lower concentrations of siRNA, possibly reducing off-target effects of the siRNAs. Contrary to the manufacturer´s instructions we added antibiotics to the media, as no reduction in viability or transfection efficiency was observed. With all cell lines tested, knockdown (measured as reduction in cell surface staining of β2 microglobulin or HLA class I) peaked between 72 and 96 h following transfection start. A disadvantage in this system would be off-target effects of the siRNA transfection. To control for more general effects, all experiments included irrelevant control siRNA.

Soluble KIR Fusion Proteins

Soluble purified KIR fusion proteins were also generated as an alternative approach to demonstrate KIR-ligand engagement. The fusion proteins consisted of the extracellular region of KIRs fused to the Fc-region of human or mouse IgG. A similar approach is the use of KIR tetramers, previously applied by some investigators. These tetramers are expressed in bacteria; thus they lack normal glycosylation and require refolding in vitro to obtain correct conformation. Our Fc fusion proteins are expressed in eukaryotic cells, and are more likely to attain correct tertiary and quaternary structure. Moreover, they can easily be purified by affinity chromatography using protein A or protein G.

A clear advantage of this method is the ability to work with a purified receptor. Moreover fusion proteins can be applied in several methods, including flow cytometry, ELISA and blocking assays. The most obvious disadvantage is lack of sensitivity due to limited

avidity compared to cell-cell contacts. In addition, Fc fusion proteins are probably more prone to incorrect folding than the membrane-bound receptors expressed on reporter cells. To improve avidity, we also generated KIR-Fc multimers, as explained below.

Using PCR and standard recombinant DNA methods, we generated expression vectors for soluble KIR-Fc fusion proteins in an expression vector with a CMV promoter driving expression of an open reading frame consisting of an N-terminal signal peptide followed by the KIR ectodomain fused to the CH2 and CH3 domains of human IgG1. Primers were chosen to include the C1 and C2 IgSF domains, as well as the stalk region of the KIRs. PCR primers for cloning were designed to include two different lengths of the stalk region, including a total of 726 or 735 base pairs, yielding two alternative constructs per KIR.

To produce KIR Fc fusion proteins, 293T cells were transiently transfected with expression constructs using a PEI protocol. The PEI transfection medium was aspirated after 24 h and replaced with a serum-free medium AIM-V (Invitrogen). After approximately 96 h of incubation in AIM-V, the supernatants were collected by centrifugation, sterile filtered, and adjusted to pH 7.0. The harvested supernatants were passed through protein A affinity columns overnight, the columns were washed with 20 mM sodium phosphate buffer pH 7.0 and the fusions proteins were eluted using a pH 3.0 sodium citrate buffer and collected in 1 ml fractions. Positive fractions were identified by spectrophotometric analysis at 280 nm, pooled and dialysed overnight against 1 l of PBS. Protein yield was again measured as $A_{260\ nm}$, and the purified supernatant were stored at 4 °C in PBS containing 10 mM NaN_3 as a preservative.

In binding assays the Fc fusion proteins were incubated with various target cells at a concentration of 20 µg/ml in PBS containing 2% FCS and 10 mM NaN_3 for one hour at 4 °C, before washing and staining with a phycoerythrin-conjugated secondary antibody towards human IgG1. Phycoerythrin was chosen for its bright fluorescent properties, because Fc fusion proteins often bind weakly in flow cytometry. Receptor-ligand interactions are typically non-covalent and thus reversible, and binding is dependent on affinity and avidity. The binding of fusion proteins depends on correct conformation of the protein, which again affects the affinity and avidity of the binding. Affinity is the strength of binding of one receptor to its ligand, defined by the dissociation constant, K_d. K_d is the relative concentration of ligand needed to occupy half of the receptors. Avidity is the combined strength of multiple receptor-ligand interactions, and if the affinity is low, avidity can be increased by increasing the number of KIR ectodomains coupled together (for Fc fusion proteins, two ectodomains are coupled together in a dimer). We first generated fusion proteins with mouse IgG1-Fc, purified on protein G columns and eluted at pH 2.7. Barely detectable binding led us to generate new constructs based on the human IgG1 Fc region combined with protein A purification and elution at pH 3.0. We have attempted several modifications to increase the signal in flow cytometry KIR-Fc experiments. We first tried reducing the washing steps during the experiment. Secondly we tried fixing the samples with variable concentrations of formaldehyde immediately following primary incubation with fusion protein. Aldehyde fixation can increase unspecific binding and, in our hands, did not lead to an increase in specific binding as measured by flow cytometry.

Fusion Protein Multimers

Fusion protein multimers were generated to investigate whether increased avidity would enhance binding and specificity. KIR-Fc fusion proteins were first bound to protein A-biotin; then the Fc-protein A complexes were incubated with streptavidin, in this case coupled to the small fluorochrome Alexa Fluor 647 to allow direct visualization by flow cytometry. One streptavidin molecule has four binding sites for biotin, thus allowing for a considerable increase in avidity.

Cytotoxic Natural Killer Cell lines

We wanted to investigate if a putative ligand is capable of inducing a physiological effector function in NK cells. To address this, we made use of the rat NK cell line RNK16, originally a NK cell leukemia from an F344 rat. This cell line is cytotoxic and can be transfected to generate stable clones expressing native KIR receptors, with their natural cytoplasmic tails and signaling motifs. The advantage of using a rat NK cell line is that we have better control of the receptors involved, since (to our knowledge) RNK-16 lack inhibitory as well as activating receptors for human HLA molecules. This allowed us to investigate effector function of a single receptor, and the resulting effector response of co-expressing activating and inhibitory receptors that recognize the same ligand.

We transfected the cytotoxic rat NK cell RNK16 with untagged full-length cDNA constructs representing KIR2DL3, KIR2DS2 and KIR2DS4, respectively. In redirected cytotoxicity assays, specific anti-KIR antibodies induced redirected inhibition with the KIR2DL3 transfectants, and activated cytotoxicity with the KIR2DS2 and –DS4

transfectants. A major problem, however, encountered with this system is a generally low propensity of RNK16 cells to react with human target cells. This is presumably due to lack of appropriate adhesion molecules, hampering the establishment of an immunological synapse necessary to initiate lysis. One methodological approach to this obstacle could be fusion of our cancer cell lines with the rat myeloma YB2/0, to generate hybridomas possibly carrying sufficient adhesion molecules as well as maintaining expression of KIR ligand. An alternative approach is to use human NK cells, and so we transfected NKL cells (using the Neon transfection system) expressing KIR2DS2. Unfortunately, the particular subline of NKL cells used for the transfection showed overall poor cytotoxic potential against standard targets, and thus were not of value.

Gel filtration for purification of soluble ligand

To exclude serum proteins from later mass spectrometry analysis PC-3 cells were washed with PBS and grown for 4 hours in PBS without serum. The supernatant was collected, centrifuged and filtered using 0.2 µm pore filters. 0.2 % of the zwitterionic detergent CHAPS was added to the supernatant to reduce the formation of unspecific protein complexes and 300 µL of the supernatant was run through a Superdex 200 column for size exclusion chromatography. 24 fractions were collected and tested for reactivity with the reporter cells, and the fraction giving the strongest reporter reactivity was separated further by ion exchange chromatography. The advantage of size exclusion chromatography is that this is a simple way of separating proteins based on size and reduces the number of contaminating proteins in the fraction where our protein of interest

is accumulated. However, fraction analysis by SDS-PAGE revealed that the number of different proteins present in the fractions was still high and that there was a significant level of overlap between the fractions, reflecting the moderate discrimination capability of this technique.

Ion exchange columns

To increase the purity of the protein ligand after gel filtration, we attempted anion or cation exchange column chromatography. The fractions eluted from the columns were tested for reactivity with the KIR2DS2 reporter cell line. A clear disadvantage was the fact that we did not know the pKa of the protein of interest. For the anion exchange column we used Tris buffers at pH 8.5 and 9.0. This resulted in negative charge of the protein of interest, since it then bound to the anion exchange column. However, the protein of interest did not achieve high negative charge as it dissociated from the column at low salt concentrations, and was spread throughout multiple elution fractions tested in reporter assays. Subsequently cation exchange column was tested. Again, we did not know at what pH the protein would achieve a suitable positive charge to bind strongly to the column. At pH 4.6 the protein ligand lost the ability to activate the reporter cells. The cation exchange column was run at pH 6.0, apparently giving a sufficient positive charge of the protein of interest, as it was eluted in three consequent fraction and at higher salt concentrations. Even after serial chromatography the sample will still contain contaminating proteins. However, the combination of different methods allowed significant enrichment of the protein of interest, since different proteins in the supernatant

will be eluted in different fractions with each method. Following gel filtration and ion exchange chromatography, the fraction containing the highest concentration of the KIR2DS2 ligand was analyzed by mass spectrometry.

Mass spectrometry

Mass spectrometry (MS) analysis to identify the soluble ligand for KIR2DS2 in the supernatant from the PC-3 cell line was performed at two different core facilities at the University if Oslo.

Before MS analysis the proteins in the sample were digested with trypsin. Trypsin digestion creates peptides of various lengths, and their composition can be identified from their molecular mass, determined by MS. In MS/MS the peptides are further fragmented and run through a second round of MS. This is used to sequence proteins because the fragments can be used to match predicted peptide sequences that are found in protein databases. The sequenced fragments can then be organized in silico into full-length sequence predictions.

One advantage of this method is the sensitivity because small amounts of protein can be detected. However, the signal from small amounts of proteins can be lost if there is high degree of contamination in the sample. Proper sample preparation is critical for the result. When the ionized proteins are run on MS, the more abundant species have a tendency to suppress signals from the less abundant proteins. Moreover, the mass spectrum from a complex mixture of multiple proteins is difficult to fully analyze because of the overwhelming number of components. A pure sample will give high quality and

reproducible results. A critical problem in our system is that the supernatant from the cells contained multiple contaminating proteins and we do not know the relative abundance of the protein ligand compared to the contaminating proteins.

How well the protein is cleaved by trypsin can influence if a protein is available for detection in MS. In native form, our protein of interest is poorly cleaved by trypsin, demonstrated by a relatively robust KIR2DS2 reporter assay response after trypsin digestion. We have no method for investigating the trypsin efficacy of the SDS-treated, denatured form of the ligand. Alternative enzymes such as Glu-C, Lys-C, Proteinase-K and pepsin can be used for digestion alone or together with trypsin in the case of poor digestion with trypsin.

Protein glycosylation will influence the calculated mass that is used to sequence and identify proteins in MS. This is a pitfall as we do not know how heavily our protein of interest is glycosylated. Deglycosylation of the proteins in the sample can improve the MS analysis, but complete deglycosylation of all N- and O-linked sugars is difficult to achieve.

GENERAL DISCUSSION

This PhD project aimed to shed light on the long standing enigma of NK cell activating receptors. This essential part of the NK cell physiology is yet not fully understood, almost 25 years after the molecular identification of the natural killer cell receptors. Our experimental data have been discussed in detail in Paper I, II and III. This section aims not to repeat elements already discussed, but instead to evaluate the observations in a broader context.

Identifying ligands for the activating KIR has proven to be a difficult task. Healthy, normal cells most likely do not express strong ligands for activating NK receptors in order to prevent autoimmune reactions [291]. Moreover, transformed or infected cells expressing ligands for activating NK cell receptors are likely to be rapidly eliminated *in vivo [292]*. Consequently, cancer cell lines would possibly not be ideal targets to search for ligands for activating NK cell receptors, as they are likely to have overcome immune recognition in order to survive and establish tumors. A second problem with using cancer cell lines to screen for KIR ligands is the propensity of cell lines to undergo changes *in vitro*. Thus the physiological significance of ligands expressed on cell lines *in vitro* is difficult to evaluate.

Several laboratories have been intrigued by the paucity of functional ligands for activating KIR, and the lack of success in this area does not appear to reflect a lack of resources spent to resolve this research task. Although the high degree of sequence similarity between activating and inhibitory KIR are suggestive of HLA class I molecules as ligands for activating KIR, this has been notoriously difficult to demonstrate experimentally.

Recent observations suggest that the glycosylation machinery is different in activated NK cells compared to resting cells [293]. Thus, differential glycosylation of KIR and other activating NK cell receptors might serve as a safety mechanism to only allow functional affinity levels in situations where the NK cells are activated by parallel inputs. This hypothesis also offers one of many possible explanations for the difficulties experienced in identifying ligands for activating KIR

Taken together, the data in Paper I and Paper II indicate that ligands for activating and inhibitory KIR are not restricted to normal HLA class I molecules expressed together with β_2-microglobulin

Functional implications of paired activating and inhibitory NK cell receptors

Pairs of inhibitory and activating receptors are common in both the NKC and LCR. As previously described, the activating and inhibitory pairs of receptors may exhibit high levels of sequence similarity in their extracellular ligand binding domain, while the intracellular regions differ in their signaling properties. NK cell inhibitory receptors have an ITIM motif in the intracellular signaling region, while the activating receptors associate with transmembrane adaptor proteins to signal.

The functional implications of paired receptors may be many and are still a topic of discussion.

1. Several activating and inhibitory receptors share binding specificity where the activating receptor binds the same self-molecule as the inhibitory receptor.

Examples are KIR2DL1 and KIR2DS1 binding to HLA-C, activating and inhibitory members of NKR-P1 in rat and mouse that bind the same Clr, CD94/NKG2 binding to HLA-E and some pairs of Ly49 binding to mouse MHC class I [250, 294, 295]. For these paired receptors the activating receptor typically binds with lower affinity compared to the inhibitory receptor, suggesting that inhibition will dominate under such circumstances. One implication of this phenomenon can be that pairs of activating and inhibitory receptors are able to assess MHC class I ligand or other self ligands with higher sensitivity than either receptor member alone. However, a more intuitive implication of the low affinities displayed by activating receptors is that other ligands with higher affinity remain to be identified. Such ligands could have structural homology to, or represent structurally altered versions of the self-ligand, expressed on stressed cells (infected cells, malignant cells) shifting the balance toward activation. Activating receptors binding to molecules expressed by normal cells could also be an unavoidable side-effect of the evolutionary pressure to maintain homology within the pair of activating and inhibitory receptors. And the functional implications of this homology could result in the effects discussed under the point number 3, below.

2. Many activating members of paired NK cell receptors do not bind the same ligand as the inhibitory receptors; hence the functional ligands for these activating receptors remain unknown. This supports the notion that activating receptors are needed to bind other ligands than the inhibitory receptors, such as stress or damage induced ligands, virus encoded ligands, self-ligands that are altered under infection

or malignant transformation (e.g. MHC presenting abnormal peptide, altered glycosylation or altered conformation of self molecules). Supporting this is the fact that the activating NKG2D receptor binds to DNA damage- or stress-induced self encoded ligands [217], KIR3DS1/ KIR3DL1, KIR3DL2 and KIR2DS4 have been demonstrated to bind to HLA-F and MHC-I open conformers (also induced self) [273, 274] and Ly49H binds to a virus encoded MHC-like molecule (m157) [173, 249].

3. Recently HLA-F in open conformation was identified as a ligand for KIR3DS1. Interestingly the inhibitory receptor KIR3DL1 as well as KIR3DL2 were also shown to bind open conformers of HLA-F, but with lower affinity. Moreover, HLA-F was shown to be expressed by HIV infected CD4^{+} T cells and KIR3DS1^{+} NK clones were able to suppress autologous HIV infected CD4^{+} T cells [274]. KIR3DS1 has been associated with many diseases, including slower disease progression in HIV [296]. HLA-F has previously been shown to be expressed by activated lymphocytes and monocytes, and some cancer cells. KIR3DS1 displays low polymorphism similar to HLA-F, supporting the concept that KIR evolve in pace with their MHC ligand. Interestingly, here HLA-F is demonstrated to be a high affinity ligand for KIR3DS1, and the inhibitory KIR3DL1 as well as KIR3DL2 binds with low affinity, therefore the balance is likely to shift towards activation. The functional implications of the inhibitory receptors 3DL1 and 3DL2 binding to the same ligand as the activating receptor 3DS1, albeit with low affinity, are not clear. Some potential theories explaining this could be:

a. Cancer cells and infected cells downregulate or abrogate inhibitory ligands on the cell surface. HLA-F could be upregulated in cancer cells as a mechanism of immune evasion by inhibition via KIR3DL1/-2. However, the immune system has evolved to counteract this through an activating receptor, KIR3DS1.
b. HLA-F open conformers can be a strategy for the stressed cells to flag to the immune system that they are infected or malignant. The formation of a low affinity/low avidity, but functional inhibitory synapse can keep the activation “under control”. Keeping activation under control can be advantageous to avoid unwanted off-target effects, moreover, a low level of inhibitory signal can ensure that the NK cells are not over-activated and thereby not rapidly becoming hyporesponsive or anergic. This will allow the NK cells to keep on killing for a longer time, and ultimately form memory cells.
c. Alternatively, the inhibitory receptors have low affinity and will not form a stable immunological synapse and convey an inhibitory signal, and the balance is shifted to activation.

A β2-microglobulin independent ligand for KIR2DS2 and KIR2DL3 on cancer cells

In spite of evidence pointing to normal HLA-C molecules as functional ligands for KIR2DS1, we hypothesize that all activating KIR2D receptors are primarily selected for their ability to discover disease. The data in Paper I provide some support for this

hypothesis, as they indicate that KIR2DS2 can recognize one or several ligands expressed on certain cancer cells. KIR2DL3, previously demonstrated to bind strongly to group C1 alleles (and weakly to at least some C2 alleles) recognized some tumor cell lines in a fashion that was blocked by anti-HLA-ABC antibodies as well as by siRNA knockdown of β2 microglobulin. Other tumor targets engaged KIR2DL3 in a fashion that could not be blocked with the same antibodies, and that appeared not to depend on co-expression of β_2-microglobulin. Thus, we interpret our data as indicative of two different types of ligands for KIR2DL3: The first type of ligand fits well with a normal C1 group allele, and explains our observations with T47D cells. The second type of ligand is not readily explained in the context of normal HLA-C expression, and requires additional models:

<u>Model 1:</u> Upon DNA damage, normal cells first attempt to repair the damage. If this repair is unsuccessful, mechanisms are initiated that lead the cell into apoptosis [297, 298]. On the other hand, the hallmark of cancer is the accumulation of DNA damage, but without apoptosis [299]. Thus, DNA-damage-induced apoptosis is not a failsafe mechanism. Another strategy to eliminate cells that have lost control of DNA integrity involves the immune system. The activating NK cell receptor NKG2D binds HLA class I-like molecules induced on the cell surface of target cells by DNA damage or cellular stress [222]. We speculate that activating KIR may in a similar fashion recognize markers of intracellular disequilibrium. Incorrectly/incompletely folded class I molecules could escape the ER and reach the cell surface in cells that have lost fundamental mechanisms of quality control [300, 301]. This would be true for cancer cells (Paper I). Thus, surface

expression of class I molecules lacking β_2-microglobulin might serve as a signal of intracellular lack of integrity. It is conceivable that an as yet uncharacterized molecule could substitute for β_2-microglobulin in stabilizing the class I structure, sufficient for release from ER chaperones and transport to the cell surface [302]. As a candidate example, the class I-assembling ER chaperone calreticulin has been shown to reach the cell surface of cancer cells, and to be important for the immune response towards the tumor [303]. Conceivably, a fraction of the HLA-C molecules on cancer cells may be expressed together with an ER-derived accessory molecule (such as calreticulin) [300], and this accessory molecule may serve as a "superantigen" to increase affinities between activating KIR and HLA-C. This fraction would thus be inaccessible to common anti-HLA-ABC antibodies. Model 1 would to a large extent explain the observations in Paper I.

Model 2: several HLA class I-like molecules exist, some of them expressed in a β2 microglobulin-independent fashion, summarized below The HLA class I-like molecules listed in Table 3 all have a groove-like structure formed by α1 and α2 domains. The groove-like structure can hold potential ligands [304, 305]. Some of these molecules serve known functions unrelated to the immune system, and are expressed by healthy cells (e.g. HFE - that modulates iron internalization by transferrin receptor; FcRn – that transports IgG across epithelial cells). However, they should not be disregarded as candidates since small changes in their structure could potentially lead to recognition by NK cells.

Table 3.

MHC class I-like molecules	Associated light chain
Neonatal Fc receptor – FcRn	β2m
Hereditary hemochromatosis protein – HFE	β2m
CD1	β2m
Zinc-α2-glycoprotein – ZAG	PIP
MHC class I chain related proteins – MICA, -B	-
ULBPs	-

DAP10 and DAP12

NK cells express the signaling adaptor proteins DAP10, DAP12, FcƐRγ and CD3ζ. FcƐRγ and CD3ζ heterodimers and homodimers have been shown to exist in NK cells and to associate with certain NK cell receptors. DAP10 and DAP12 are generally believed to form homodimers in NK cells.

Many immune receptors have been shown to associate with either DAP10 or DAP12, whereas others, like mouse NKG2D and some activating Ly49 can be expressed together with both these adaptors. It is not yet understood what determines these processes. Our data indicated the formation of homotrimers of DAP10 and homotrimers of DAP12 in transfected cells. We also observed formation of DAP10 and DAP12 heterodimers. In

addition, SDS-PAGE analysis indicated the formation of heterotrimers (DAP10/DAP12/DAP12 or DAP12/DAP10/DAP10). However, it is yet unclear what regulates the formation of heterodimers and multimers of DAP10 and DAP12 and if these exist in vivo in NK cells. In transfected cells it is possible that relative overexpression of adaptor proteins facilitates formation of heterodimers and multimers. It is also possible that in transfection systems, the receptors and adaptor proteins become promiscuous, which could explain our observation in paper III, where both DAP10 and DAP12 were co-precipitated with human NKG2D. However, under the same conditions only DAP12 was precipitated together with the activating KIR2DS2 and only DAP10 with rat NKG2D. Only DAP12 co-precipitated with NKG2C in the NK cell lines NK-92 and NKL. Recruitment of both DAP10 and DAP12 by the same receptor can potentially result in a stronger activation of both cytotoxicity and cytokine production, and receptors that are able to associate with both adaptor proteins will then be more potent activators of the NK cell These observations have led to several questions: What factors regulate the selective association of the adaptors to some receptors, if DAP10 and DAP12 have different affinity to different receptors or if adaptor-receptor association and heterodimerization of DAP10 and DAP12 are different in activated versus resting NK cells.

Although the formation of heterodimers consisting of DAP10 and DAP12 has not been documented in vivo in NK cells; DAP10/DAP12 heterodimers have been demonstrated in osteoclasts in association with MDL-1. In immunoprecipitation from transfected 293T cells we have identified heterodimers of DAP10 and DAP12 and have shown that these heterodimers can associate with NK cell receptors. DAP10/DAP12 heterodimers were not

detected in the human NK cell lines tested. The question still remains if DAP10/DAP12 heterodimers can be found in primary NK cells and associate with NK cell receptors. Structurally these two distinct signaling molecules seem to readily form heterodimers in transfected non-NK cells. If this dimerization process is regulated in NK cells, it is interesting to ask what mechanisms regulate the formation of such heterodimers, and under which conditions they operate.

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