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MOLECULAR SPECIFICITIES OF NK CELL-MEDIATED RECOGNITION OF HUMAN TUMOR CELLS

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To all members of my family, present and gone

"Alla vill till himmelen men få vill ju dö"
Timbuktu, 2005

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ABSTRACT

Natural killer (NK) cells have been implicated in tumor immune surveillance and can reject transformed cells expressing ligands for activating NK cell receptors and low levels of HLA class I. Although NK cells are well known for their ability to kill tumor cells, relatively few studies have addressed the molecular specificity of NK cell-mediated recognition of freshly isolated human tumor cells. The rational for conducting such studies is based on the fact that tumor cell lines display altered molecular expression compared to their origin.

In this thesis, we have assessed the role for NK cells in solid and hematological malignancies. We show that freshly isolated metastatic ovarian carcinoma (OC) cells express low levels of HLA class I. In one patient, we identified a genomic HLA class I haplotype loss that was associated with a HLA-A2 restricted Her2/neu specific T cell response. The low HLA class I levels, in combination with the presence of ligands for activating NK cell receptors, resulted in a significant killing of the metastatic OC cells by allogeneic NK cells, while sparing normal cells. Experiments masking activating NK cell receptors revealed a dominant role for the DNAM-1 receptor with a minor contribution from the NKG2D receptor. Studies of the receptor repertoire and functional integrity of NK cells associated to the tumor *in vivo* substantiated a role for DNAM-1 since a marked loss of DNAM-1 as well as 2B4 and CD16 were observed and resulted in significantly reduced natural cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) against autologous carcinoma cells. The DNAM-1 loss was likely caused by chronic ligand exposure, since physical interactions between the receptor and its ligand CD155 induced down-regulation. Suppressed NK cell function due to loss of DNAM-1 and NKG2D expression was also identified in the bone marrow and blood of patients with myelodysplastic syndromes (MDS). Relative to NK cells in peripheral blood, bone marrow-derived NK cells associated to the tumor cells displayed a more severe loss of the two receptors as well as a reduced effector cell function. The receptor loss was most prominent in patients with more than 5% blasts in the bone marrow, suggesting that poor NK cell function may be associated with an increased risk of progression to acute myeloid leukemia (AML). Tumor cells may also evade NK cell-mediated lysis by up-regulation of HLA-E that inhibits NK cell activity through signaling via the CD94/NKG2A receptor. Drugs have been used to manipulate the NK cell receptor ligand repertoire on tumor cells to render them more susceptible to NK cells. Selenite, a highly reactive oxidative agent, is known to selectively kill tumor cells when used in high concentrations. We show that selenite also reduced the expression of HLA-E and rendered the tumor cells more susceptible to killing by CD94/NKG2A expressing NK cells.

Given the emerging evidence for NK cell-mediated tumor immune surveillance, our data indicate that tumor progression may be promoted by perturbed activating NK cell receptor repertoires and poor function of tumor-associated NK cells. The data imply that OC could be targeted by NK cell-based immunotherapy and that MDS patients having more than 5% blasts in the bone marrow could be considered as potential candidates for NK cell-based immunotherapy. Data also indicate that selenite may be used to improve the results of NK cell-based immunotherapies by rendering HLA-E expressing tumor cells more susceptible to NK cells. Thus, a better comprehension of the molecular specificity of NK cells targeting fresh human tumor cells and the role for combinatorial treatments can hopefully advance NK cell-based immunotherapies.

LIST OF PUBLICATIONS

This thesis is based on three publications and two manuscripts. The individual papers are referred to by roman numerals.

I. Norell H, **Carlsten M**, Ohlum T, Malmberg KJ, Masucci G, Schedvins K, Altermann W, Handke D, Atkins D, Seliger B, Kiessling R. Frequent loss of HLA-A2 expression in metastasizing ovarian carcinomas associated with genomic haplotype loss and HLA-A2-restricted HER-2/neu-specific immunity. Cancer Res. 2006 Jun 15;66(12):6387-94.

II. **Carlsten M**, Björkström NK, Norell H, Bryceson Y, van Hall T, Baumann BC, Hanson M, Schedvins K, Kiessling R, Ljunggren HG, Malmberg KJ. DNAX accessory molecule-1 mediated recognition of freshly isolated ovarian carcinoma by resting natural killer cells. Cancer Res. 2007 Feb 1;67(3):1317-25.

III. **Carlsten M,** Norell H, Bryceson Y, Poschke I, Schedvins K, Ljunggren HG, Kiessling R, Malmberg KJ. Primary human tumor cells expressing CD155 impair tumor targeting by down-regulating DNAM-1 on NK cells. J Immunol. 2009 Oct 15;183(8):4921-30.

IV. **Carlsten M***, Baumann B*, Simonsson M, Jädersten M, Forsblom AM, Ljunggren HG, Hellström-Lindberg E, Malmberg KJ. Poor Effector Function of Bone Marrow-Derived NK Cells in Myelodysplastic Syndromes Associated with Loss of NKG2D and DNAM-1 Expression. *Manuscript.* * *Equal contribution*

V. **Carlsten M**, Simonsson M, Nilsonne G, Hammarfjord O, Wallin R, Björkström N, Björnstedt M, Hjerpe A, Ljunggren HG, Dobra K, Malmberg KJ. Oxidative stress-induced downmodulation of HLA-E sensitizes cancer cells to NK cell recognition. *Manuscript.*

LIST OF ASSOCIATED PUBLICATIONS

This list includes publications with relevance to the thesis. The individual papers are referred to by letters in alphabetical order.

A. **Carlsten M**, Malmberg KJ, Ljunggren HG. Natural killer cell-mediated lysis of freshly isolated human tumor cells. Int J Cancer. 2009 Feb 15;124(4):757-62. Review.

B. Malmberg KJ, Bryceson YT, **Carlsten M**, Andersson S, Björklund A, Björkström NK, Baumann BC, Fauriat C, Alici E, Dilber MS, Ljunggren HG. NK cell-mediated targeting of human cancer and possibilities for new means of immunotherapy. Cancer Immunol Immunother. 2008 Oct;57(10):1541-52. Review.

C. Fauriat C, Andersson S, Björklund AT, **Carlsten M**, Schaffer M, Björkström NK, Baumann BC, Michaëlsson J, Ljunggren HG, Malmberg KJ. Estimation of the size of the alloreactive NK cell repertoire: studies in individuals homozygous for the group A KIR haplotype. J Immunol. 2008 Nov 1;181(9):6010-9.

D. Hanson MG, Ozenci V, **Carlsten MC**, Glimelius BL, Frödin JE, Masucci G, Malmberg KJ, Kiessling RV. A short-term dietary supplementation with high doses of vitamin E increases NK cell cytolytic activity in advanced colorectal cancer patients. Cancer Immunol Immunother. 2007 Jul;56(7):973-84.

E. Malmberg KJ, Levitsky V, Norell H, de Matos CT, **Carlsten M**, Schedvins K, Rabbani H, Moretta A, Söderström K, Levitskaya J, Kiessling R. IFN-gamma protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism. J Clin Invest. 2002 Nov;110(10):1515-23.

F. Rundlöf AK, **Carlsten M**, Arnér ES. The core promoter of human thioredoxin reductase 1: cloning, transcriptional activity, and Oct-1, Sp1, and Sp3 binding reveal a housekeeping-type promoter for the AU-rich element-regulated gene. J Biol Chem. 2001 Aug 10;276(32):30542-51.

G. Rundlöf AK, **Carlsten M**, Giacobini MM, Arnér ES. Prominent expression of the selenoprotein thioredoxin reductase in the medullary rays of the rat kidney and thioredoxin reductase mRNA variants differing at the 5' untranslated region. Biochem J. 2000 May 1;347 Pt 3:661-8.

H. Curbo S, Gaudin R, **Carlsten M**, Malmberg KJ, Troye-Blomberg M, Ahlborg N, Karlsson A, Johansson M, Lundberg M. Regulation of interleukin-4 signaling by extracellular reduction of intramolecular disulfides. Biochem Biophys Res Commun. 2009 Dec 25;390(4):1272-7.

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LIST OF ABBREVIATIONS

ACT	Adoptive cell transfer
ADCC	Antibody-dependent cellular cytotoxicity
AICD	Activation-induced cell death
AICL	Activation-induced C-type lectin
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
APM	Antigen-presenting machinery
Arg-1	Arginase I
B cell	Bursa of Fabricius cell
BAT-3	Human leukocyte antigen-B associated transcript 3
cAMP	Adenosine 3′,5′-cyclic monophosphate
CC	Colorectal cancer
CD	Cluster of differentiation
CDCC	Complement-dependent cellular cytotoxicity
CTL	Cytotoxic T cell
CTLA-4	CTL-associated antigen 4
Cy	Cyclophospamide
DAP	DNAX adaptor protein
DC	Dendritic cell
DLI	Donor lymphocyte infusion
DMBA	7,12-dimethylbenz[a]anthracene
DNAM-1	DNAX adaptor molecule 1
EBV	Epstein-Barr virus
ER	Endoplasmatic reticulum
Fab	Fragment, antigen-binding
FACS	Fluorescence-activated cell sorting
Fc	Fragment, crystallizable
Flu	Fludarabine
GR	Gluthatione reductase
GvH	Graft-versus-Host
GvHD	Graft-versus-Host Disease

GvL	Graft-versus-Leukemia
HA	Hemagglutinin
HEV	High endothelial venules
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cells
ICAM-1	Inter-cellular Adhesion Molecule 1
ICOSL	Inducible co-stimulator ligand
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPSS	International Prognostic Scoring System
IS	Immunological synapse
KIR	Killer cell immunoglobulin-like receptors
LAK	Lymphokine-activated cells
LFA	Leukocyte functional antigen
LGL	Large granular lymphocytes
LILR-B1	Leukocyte immunoglobulin-like receptor, subfamily B member 1
LOH	Loss of heterozygocity
LRC	Leukocyte receptor complex
LTi cell	Lymphoid tissue inducer cell
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCA	Methylcholanthrene
MDS	Myelodysplastic syndrome
MDSC	Myeloid-derived suppressor cell
Mel	Malignant melanoma
MHC	Major histocompatibility complex
MIC	Major histocompatibility complex class I-related chain
MIF	Macrophage migration inhibiting factor
MM	Multiple melanoma
NADP	Nicotinamnide adenine dinucleotide phosphate
NB	Neuroblastoma
NCR	Natural cytotoxicity receptor

NK cell	Natural killer cell
NKC	Natural killer genes complex
NKG2D	NK group 2D
NKR	NK cell receptor
OC	Ovarian carcinoma
PDI	Protein disulfide isomerase
PGE	Prostaglandin E
PI3K	Phosphoinositide 3-kinase
PLC	Peptide-loading complex
RAG	Recombination-activating genes
RCC	Renal cell carcinoma
RECIST	Response Evaluation Criteria in Solid Tumors
ROS	Reactive oxygen species
SC	Squamous cell cancer
SCT	Stem cell transplantation
SeCys	Selenocystein
SeMet	Selenomethionine
SHP	Src homology 2 domain-containing phosphatases
SOS1	Son of sevenless homolog 1
Syk	Spleen tyrosine kinase
T cell	Thymus cell
TAA	Tumor-associated antigen
TAP	Transporter-associated protein
TBI	Total body irradiation
TCR	T cell receptor
TGF	Tumor growth factor
TIL	Tumor-infiltrating lymphocytes
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor related apoptosis-inducer
Trx	Thioredoxin
TrxR	Thioredoxin reductase
ULBP	UL16 binding protein
ZAP	Zeta-chain-associated protein kinase
rADCC	Reverse antibody-dependent cellular cytotoxicity

FOREWORD

When I started medical school I thought that it was a long way to go to obtain my medical degree and to defend a thesis, but I must say that I was wrong. Why? I have realized that doing a PhD is not a job, but a lifestyle. In fact, I think that is has to be a lifestyle. Many of my friends outside the scientific community often ask me when I will finish my research and get a real job. Is that now? No, I don't think so. The world of science is so full of exciting things and offers unique contacts with persons from different backgrounds and with divergent phenotypes but with a common interest in science. These people form the Karolinska Institute, CIM and CCK to excellent science environments that made my time fly during my PhD! I would not have been here without all help, support and enthusiasm from my colleagues at the Karolinska Institute!

This thesis is a product of a genuine research interest. My ambition has been to focus on preclinical research closely linked to clinical issues. I hope that the results of this thesis to some extent can contribute to our understanding of the interactions between the immune system and cancer and hopefully advance cancer immunotherapy. My ambition is to continue with one foot in research and one foot in the clinic, although only future can tell in what format. Regardless of where I end, I'm sure that all my experiences from my time as a PhD student at the Karolinska Institute will help me in my future profession.

Mattias Carlsten Stockholm, January 12, 2010

1 INTRODUCTION

All organisms have defense systems that recognize non-self patterns of foreign pathogens such as viruses and bacteria. These systems, collectively called the immune system, have evolved throughout the history side-by-side with pathogens. Cancer is a disease that can arise in tissues of multicellular organisms and is believed to develop slowly in a multistep process that probably start decades before the actual disease appears clinically. In contrast to foreign non-self pathogens, cancer cells express self or altered-self molecules, which may be hard to discriminate from normal self-cells. What impact cancer has had on the evolution of the immune system is still an open question. In fact, it has been widely debated whether the immune system has a role at all in the protection against cancer *in vivo*. In the early 1900, Paul Ehrlich postulated a theory that the immune system recognized and eliminated spontaneously arising tumor cells and thereby protected the host from cancer (1). However, this hypothesis was first formally formulated and introduced as the "tumor immune surveillance theory" more than 50 years later by Burnet and Thomas (2-4). Since then, the existence of tumor immune surveillance has been disputed. As will be discussed in this thesis, we know that immune cells, such as thymus (T) cell and natural killer (NK) cells, can recognize cancer cells via interactions with major histocompatibility complex (MHC) and directly kill them with cytotoxic molecules. This thesis aims to delineate the molecular specificities of NK cell-mediated recognition of human tumor cells, which hopefully can contribute to advances in immunotherapy of cancer.

1.1 THE IMMUNE SYSTEM

The human immune system consists of immune cells and soluble molecules such as cytokines and antibodies. All immune cells arise from hematopoietic stem cells (HSC) in the liver, thymus and the yolk sac during fetal life and in the bone marrow after birth (5, 6). They are continuously being renewed and enter the circulation where they stay or migrate to specific tissue sites. As far as we know today, immune cells are being produced throughout the lifespan of humans, although hypocellularity is observed in the bone marrow of elderly (7). The cellular immune system can be divided into the innate and adaptive arms. Innate immune cells recognize invaders with germline-encoded receptors, whereas adaptive immune cells generate and clonally expand cells with specificity for foreign epitopes, providing immunological memory. With respect to the massive knowledge and complexity of the immune system, all aspects and components of the system will not be discussed in this thesis. Instead, aspects of the immune system that relate to the data presented in this thesis will be introduced, whereas non-related immunological issues can be studied elsewhere (8, 9).

1.1.1 Components of the immune system in humans

Immune responses to pathogens and transformed cells are orchestrated by signals from cell surface receptors on immune cells that are engaged by cell-bound ligands or soluble factors. This section will focus on the cytokines and tissue antigens involved in the regulation of cellular activity and migration.

1.1.1.1 Cytokines and chemokines

Cytokines are polypeptides that are involved in the regulation of cellular activation, differentiation, proliferation and survival and act by inducing intracellular activation signaling

through specific cell surface receptors, selectively expressed by subsets of immune cells. Examples of cytokines are the interleukins (ILs), the tumor necrosis factors (TNFs) and the interferons (IFNs). IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 are type I cytokines belonging to the common cytokine receptor γ-chain (γ_c) family that all share the same IL-2Rγ subdomain (10). These cytokines are crucial for development and proliferaton of multiple cell lineages of both the innate and adaptive immune systems. IL-2 was the first cytokine described in this family and is known to be important for proliferation and survival of B cells, T cells and NK cells and can also enhance the killing capacity of T cells and NK cells (11). IL-2, together with IL-1, IL-12, IL-15 and IL-18, belongs to a group of cytokines called proinflammatory cytokines due to their involvement in the induction of inflammation. TNF-α is also a proinflammatory cytokine that is released by inflammatory cells in response to infections (12). The type I interferons, IFN-α, IFN-β, can be expressed by almost all cells in response to viral infections and up-regulate immune related molecules for enhanced viral clearance (13). IFN-γ belongs to the type II interferon group and is primarily released from immune cells (13). In contrast to the immune stimulatory cytokines, IL-10 and tumor growth factor (TGF)-β mediate inhibition of immune responses and modulate the expression of immune receptors (14, 15).

Chemokines are cytokines that control the migration of leukocytes to specific sites in the body, a process also called chemotaxis. This is critical to induce proper immune response at the site of the disease. Chemokines exert their biological effects via G protein-linked transmembrane receptors that are selectively expressed by subsets of immune cells (16). Examples are chemokine (C-C motif) ligand (CCL) 8 that is an attractor for many immune cells (17) or CCL19 and/or CCL21 that recruit CCR7 expressing immune cells to the lymph node, chemokine (C-X-C motif) ligand (CXCL) 10 that is secreted by several cell types in response to IFN-γ (18), chemokine (C-X3-C motif) ligand (CX3CL) 1 that is primarily expressed by activated endothelial cells and promotes strong adhesion of leukocytes to activated endothelial cells (19, 20) and chemokine (C motif) ligands (XCL) that belong to a small family of chemokines that seem to be involved in attracting T cells.

1.1.1.2 The major histocompatibility complex

The MHC was first identified in the 1950ies as tissue antigens involved in the rejection of transplants in mice (21). Today, MHC molecules are known as antigen-presenting proteins that are essential for the discrimination of normal, altered-self and non-self cells by presenting endogenous peptides to the T cell receptor (TCR) (22, 23) on T cells and by regulating NK cell activity through interactions with NK cell receptors (NKRs) (24). The human MHC molecules are called human leukocyte antigens (HLA) since their expression was first characterized on lymphocytes. A better understanding of the regulation of the *HLA* genes and the process leading to cell surface expression of HLA molecules has not only resulted in better outcome in transplantation, but has also provided essential information on how specific immune responses by T cells and NK cells arise and are regulated. The HLA molecules can be divided into two major classes, namely HLA class I and HLA class II, of which both are mapped to chromosome 6 in the genome but differ in structure, source of peptides presented and immune function (25).

The HLA class I molecules constitute the classical HLA-A, HLA-B and HLA-C and the non-classical HLA-E, HLA-F, HLA-G and HLA-H (26). They are all composed of an α-chain containing three extracellular domains that are non-covalently bound to the β_2-microglobulin (β_2m) (27). The peptides presented on HLA class I are about 8-10 amino acids long and derive from endogenous cytosolic proteins that are digested by the immunoproteasome and transported into the endoplasmatic reticulum (ER) via transporter associated proteins (TAPs). Most of the HLA class I molecules are loaded with the proper peptide in the ER via the peptide

loading complex (PLC). Stable cell surface expression relies on the association with both the β_2m and the peptide. The immunoproteasome that digest cytosolic proteins to short peptides, the peptide transportation system and the PLC are together called the antigen-presenting machinery (APM).

The HLA class II molecules include HLA-DR, HLA-DP and HLA-DQ and consist of one α-chain and one β-chain that both are anchored to the cell membrane (28). The first domain of both chains forms the peptide-binding groove, where peptides of about 13-25 amino acids derived from extracellular pathogens are presented (29, 30). The HLA class II/peptide complex is presented on bursa of Fabricius (B) cells, macrophages and dendritic cells (DCs) and is recognized by the TCR on $CD4^+$ T cells (25). HLA class II molecules are central for adaptive immunity and can also be presented on NK cells in late maturation stages.

1.1.1.2.1 Classical HLA class I molecules

The classical HLA class I molecules (HLA-A, HLA-B and HLA-C) are expressed by almost all nucleated cells. They are both polygenic (several loci in each individual) and polymorphic (several isoforms for each gene), giving them the capacity to present a large array of different peptides, which explains the high interindividual diversity in the population (31, 32). Both alleles are codominantly expressed on the cell, although the expression level varies between the three subclasses. DCs, known to engulf soluble molecules or cell debris, also express HLA class I but represent a cell type that has the unique capacity to present extracellular epitopes on HLA class I (33). This process is called cross-presentation and allows DCs to present engulfed foreign epitopes to $CD8^+$ T cells in the lymph node leading to induction of a specific immune response. Importantly, some infectious agents can interfere with the HLA class I processing and thereby avoid immune recognition (34, 35). Tumor cells can also employ similar mechanisms to escape from immune recognition (36-38).

1.1.1.2.2 Non-classical HLA class I molecules

The non-classical HLA class I molecules (HLA-E, HLA-F, HLA-G and HLA-H) have, in contrast to the classical HLA class I, a highly conserved structure with a narrow repertoire of peptides that fit to the peptide-binding groove (39, 40). Cells that express the non-classical HLA-E molecule can decrease the cytolytic activity of immune cells expressing the CD94/NKG2A receptor (41-43). HLA-E binds peptides derived from the leader sequence peptide of HLA class I molecules such as HLA-B7, HLA-B27, HLA-G (44). In addition, peptides from heat shock protein 60, expressed during cellular stress, can also bind HLA-E (45). HLA-E is over-expressed by several malignancies (46-49). HLA-G directly inhibit cytotoxicity of immune cells through interactions with the leukocyte immunoglobulin (Ig)-like receptor-B1 (LILR-B1) receptor (50). HLA-G is also over-expressed by several distinct different malignancies (51-56) and can also be expressed in a soluble form (57). It has also critical immunoregulatory properties by abrogating the activity of maternal NK cells against the HLA-G expressing trophoblasts in fetal tissue and thereby induce tolerance in the maternal-fetal interface. The HLA-H molecule, also called HFE, is widely expressed and has been shown to be involved in pathogenesis of haemochromatosis and beta-thalassemia minor, but the exact role and mechanisms in general and for the immune system in particular has not yet been clarified (58, 59). Moreover, the role and function of HLA-F is still unknown.

1.1.1.3 Immune cells and cellular immunity

The immune system is composed of many different cell types that have distinct functions and distributions in the body. The cells in the innate arm of the immune system include granulocytes,

monocytes/macrophages, DCs and NK cells, whereas T cells and B cells are cells of the adaptive immune system. The cells of the innate arm have the ability to detect and kill pathogens as well as activate the adaptive arm of immune system. As an example of the link between the innate and adaptive immune system, macrophages that encounter and engulf a pathogen in the skin start to express proinflammatory cytokines (IL-1, IL-6 and TNF-α) and chemokines (CXCL8) that recruit neutrophils. These cytokines and chemokines also activate the vascular endothelium that up-regulates integrins, CXCL8 and IL-1 leading to the recruitment of DCs, NK cells and other immune cells to the site of inflammation (9). Crosstalk between NK cells and DCs boost the functions of both cell types (60). The DCs engulf the pathogen, become activated and migrate to the draining lymph node, where they present the digested epitopes of the pathogen to T cells. Specific T cell subsets, recognizing the presented epitopes, proliferate and migrate to the site of infection where they kill the infected cells. This process also generates epitope-specific memory T cells within some days that can protect the host upon re-infection. The inflammatory reaction is terminated by the activation of biochemical programmes with lipid mediators that enable inflamed tissues to return to homeostasis. Regulatory T cells (T_{reg}) suppress the action of immune cells by secreting inhibitory cytokines (IL-10 and TGF-β) and induce apoptosis or exert direct cytotoxicity of the immune cells (61).

1.1.2 NK cell biology and its role in the immune system

NK cells are large granular lymphocytes (LGL) that constitute approximately 10% (5-15%) of the peripheral blood lymphocytes in humans (62). Most NK cells are found in the blood, liver and spleen, but they are also present in lymph nodes and have the capacity to migrate into specific tissue sites upon infection, inflammation or tumor development (63). NK cells are distinct from B cells and T cells since they develop to mature effector cells without rearranging its cell surface receptors and without the requirement of clonal expansion, which give them the capacity to directly lyse targets without prior sensitization (64-67). From an evolutionary perspective, NK cells express a broad repertoire of germ-line encoded NKRs constituting both ancient evolutionary preserved receptors as well as more recently evolved receptors (68). NK cells are involved in the rejection of virally transformed and tumor transformed cells and play an important role as regulators of immune responses by linking and modifying innate and adaptive immunity (69-71). As an example of the later, NK cells have been reported to promote tolerance to graft transplants such as pancreatic islet as well as hematopoietic stem cells during transplantation (72, 73). In addition, data also indicate that NK cells are involved in autoimmunity (74) and have a regulatory role of non-cytotoxic NK cells in the uterus during pregnancy (75).

1.1.2.1 The discovery of NK cells and the "missing-self hypothesis"

NK cells were first described in 1975 by two independent groups (Kiessling et al and Hebermann et al) as immune cells that were able to lyse target cells without prior sensitization of the host (64-67). At that time, many groups had observed an unexplainable "background" killing of tumor cells *in vitro* by peripheral blood lymphocytes. The identification of the responsible lymphocyte subset was a result of thorough and systematic investigations of tumor cell killing *in vitro* by mouse and human lymphocytes that had not experienced tumor antigens prior to the assay (76).

The "missing-self hypothesis", describing how NK cell activity is regulated, was first postulated in the thesis of Klas Kärre in 1981 and was later published in 1985 (77, 78). Further studies in murine models revealed the major role for MHC class I in the protection of target cells from NK cell-mediated killing (79, 80). Some years after the "missing-self hypothesis" was

postulated, Chambers et al conducted experiments masking cell surface structures on rat NK cells by monoclonal antibodies (mAbs), which resulted in the identification of the first structure on the NK cell surface that negatively regulated NK cell activity (81). However, Karlhofer et al. were the first to identify the inhibitory receptor Ly49 (expressed by murine NK cells) that specifically recognized MHC class I antigens and thereby inhibited NK cell activity (82). The Ly49 receptor family was later localized to chromosome 6 in a region that today is known as the Natural killer genes complex (NKC) (83). The human equivalent to the murine MHC class I binding receptors are the killer cell Ig-like receptors (KIRs) that were first described in the beginning of the 1990ies by Moretta and colleagues (84-88). In humans, the KIR locus constitutes a family of polymorphic genes that map to a region on chromosome 19q13.4 called the leukocyte receptor complex (LRC). The discovery of inhibitory NKRs such as the KIRs has together with the more recent identification of activating NKRs verified the role for both activation and inhibitory signals in the regulation of NK cell activity as was originally predicted in the "missing-self hypothesis" (80).

1.1.2.2 NK cell receptors and signaling pathways regulating NK cell activity

The earliest insights into the molecular specificity of NK cells (79, 80) have later been complemented with additional studies that verified the need for positive stimulation to induce target killing (24, 89, 90). It is now known that the NK cell activity is regulated by the integration of inhibitory and activating signals from MHC class I-restricted inhibitory receptors and a wide array of activating NKRs (24, 91, 92). Specific combinations of NKRs expressed on a given NK cell lead to distinct NK cell subsets with a certain degree of target selectivity. The recent advances in the understanding of intracellular signaling have also given us deeper insights into receptor synergies that are involved in the control of NK cell activity. This section aims to introduce the NKRs, their specificity and their intracellular signaling pathways that regulate the NK cell activity.

1.1.2.2.1 Inhibitory NK cell receptors and their ligands

The NK cell activity is under strict control of signals from inhibitory receptors (93) that most often bind classical and/or non-classical MHC class I molecules (24, 92). These molecules are normally expressed on most healthy cells in the body, but may be lost upon viral or malignant transformation and during tumor evolution (34-38). In humans, KIR and CD94/NKG2A play major roles as HLA class I-specific inhibitory NKRs recognizing groups of HLA-A, -B, and –C alleles and HLA-E molecules, respectively (24, 92). In contrast to most of the activating NKRs and the inhibitory CD94/NKG2A/B receptors, individuals differ in the number and type of KIRs expressed. This is partly explained by the identification of two major and divergent KIR haplotypes among the human population, which are composed of combinations of both activating and inhibitory KIRs. The inhibitory and activating KIRs share the same structural features of their extracellular domain (2D or 3D reflecting the number of Ig-like domains), but have different cytoplasmic tails with either a long (L) or a short (S) tail mediating inhibition and activation, respectively (94). Non-functional KIR pseudogenes (*P*) have also been identified. The A haplotype harbors at least eight KIRs of which six are inhibitory (3DL3, 2DL4, 3DL2, 3DL1, 2DL1, 2DL2/3), one is activating (2DS4) and one is a *KIR pseudogene* (3DP1) (95). In contrast, the B haplotypes constitute up to fourteen KIRs, of which many are activating, with at least one additional gene not represented in the A haplotype (94, 96, 97). The set of KIR genes that represent the B haplotype most often include KIR3DL3, 2DL2, 3DP1, 2DL4, 3DS1, 2DL5, 2DS5, 2DS1, 2DS2, and 3DL2 (98). The variegated expression pattern of KIR on NK cells may also be explained by the fact that specific KIR gene products are expressed randomly in distinct subsets of NK cells (99, 100). Despite a seemingly random expression pattern, most functionally

mature NK cells express at least one inhibitory receptor (i.e., KIR and/or CD94/NKG2A) that is specific for a self-MHC class I ligand. The clonal distribution of KIRs results in a system allowing NK cells to detect cells lacking expression of single MHC class I alleles (101). In addition to KIRs and CD94/NKG2A, the LILR-B1 receptor (102), binding to a variety of HLA-class I molecules, including HLA-G, and virally-derived UL18 molecules, and the KLRG1 receptor (103), binding to cadherins on epithelial and neural cells (104), may also contribute to inhibition of NK cell activity. In contrast to the KIRs that recognize polymorphic epitopes within the α1 and α2 domains of the HLA-class I heavy chain, the binding site for LILR-B1 has been mapped to the α3 domain and β_2m (105-107), which is consistent with the broad-binding specificity of LILR-B1 since α3 domain is relatively conserved among HLA-class I molecules. Importantly, under normal conditions, inhibition signals dominate over activation signals in NK cells (108). However in some situations, the activation signals may override the inhibitory signals as demonstrated for NKG2D-mediated killing of some MHC class I expressing tumor cell lines in mice (109, 110).

1.1.2.2.2 Activating NK cell receptors and their ligands

NK cells express the FcRγIIIR (CD16) receptor that induce antibody-dependent cellular cytotoxicity (ADCC) upon binding to the constant region (Fc) of IgG (111-113). They also express several other activation receptors which contribute to "natural cytotoxicity" (89).

The natural cytotoxicity receptors (NCRs), NKp30, NKp46 and NKp44 represent an important group of activating human NK cell receptors. Two of these, NKp30 and NKp46, are constitutively expressed on all peripheral blood NK cells, whereas NKp44 is induced on IL-2-activated NK cells (90). The role of these receptors in NK cell-mediated target killing has been demonstrated by blockade of the receptor with anti-NCR mAbs (114-117). Indirect evidence for NCR ligand expression on several tumor types is provided by the use of soluble NCR fusion proteins (118). However, despite considerable efforts to identify cellular ligands for the NCRs, only two candidate ligands binding to NKp30 have been described so far, i.e., the human leukocyte antigen-B associated transcript 3 (BAT3) and the B7-H6 (119, 120). In addition, data also suggest that hemagglutinin (HA) is a viral ligand for the NKp44 and NKp46 receptors (121, 122).

The activating NK cell receptor NKG2D is particularly well characterized (123). It is constitutively expressed on all NK cells and recognizes the stress-inducible molecules major histocompatibility complex class I-related chain (MIC)A and MICB as well as the UL16-binding proteins (ULBPs) expressed by human cells (124, 125). The NKG2D receptor has been shown to be involved in the rejection of both virally infected and tumor cells (123, 126, 127). In addition, data indicate that NKG2D may be involved in autoimmunity (128).

The DNAM-1 receptor was first describes on T cells (129). However, DNAM-1 is also constitutively expressed on all NK cells as well as on a subset of B cells and monocytes. The function of DNAM-1 is dependent on the physical association with lymphocyte-associated antigen-1 (LFA-1; CD18/CD11a) (130). Patients with leukocyte adhesion deficiency syndrome (LAD), lacking LFA-1, have defective DNAM-1 despite intact expression levels (130). However recent data indicate that cross-linking DNAM-1 with agonistic mAb can enhance the function of LAD-derived NK cells (131). Two ligands, CD155 (PVR) and CD112 (Nectin-2), have been identified for DNAM-1 (132). CD155 appears to have a predominant role in inducing DNAM-1-dependent activation. The DNAM-1 receptor may also cooperate synergistically with NCR and NKG2D to trigger NK cell mediated cytotoxicity (133) and has been reported to be important in the protection from tumor cell development (134).

The 2B4 (CD244) receptor is expressed on the majority of human NK cells. It binds to CD48, which is commonly expressed by most hematopoietic cells (135). Interactions between

2B4 and its ligand results in induction of proximal activating signals but the magnitude of the signal is not sufficient to induce effective NK cell activation alone (89, 133).

In addition to these receptors, many other receptors, including CD2 (LFA-2), NTBA, NKp80 and CD59, have been shown to be involved in activation (89). Several of these may have important co-activating or co-stimulatory functions in NK cell activation (89, 133).

Table 1. Specificity and signaling of human NK cell receptors*

Receptor	Signaling	Cellular ligand	Function
$Fc\gamma RIIIa$ (CD16)	Activation	IgG	Elimination of antibody coated cells (ADCC)
NKp30 (CD337)	Co-activation	B7-H6	NK cell – myeloid cell cross-talk
NKp44 (CD336)	Activation	?	?
NKp46 (CD335)	Co-activation	?	Surveillance of mitotic cells
KIR (CD158a, b, etc.)	Activation	HLA class I	?
CD94/NKG2C (CD159c)	Activation	HLA-E	?
NKG2D (CD314)	Co-activation	ULBP, MICA, MICB	Surveillance of tumor cells and genotoxic stress
NKp80	?	AICL	NK cell – myeloid cell cross-talk
DNAM-1 (CD226)	Co-activation	CD112, CD155	Surveillance of tissue integrity
2B4 (CD244)	Co-activation	CD48	Interaction with hematopoetic cells
CRACC (CD319)	?	CRACC (CD319)	Interaction with hematopoetic cells
CD2	Co-activation	CD58	Interaction with hematopoetic and endothelial cells
KIR2DL4 (CD158d)	?	HLA-G (soluble)	Trophoblast-induced vascular remodelling?
LFA-1 (CD11a/CD18)	Granule polarization	ICAM	Recruitment and activation during inflammation, efficient cytotoxicity
KIR (CD158)	Inhibition	HLA class I alleles	Assess loss of MHC class I alleles
LIR1, LILR1 (CD85j)	Inhibition	HLA class I	Assess loss of MHC class I expression
CD94/NKG2A (CD159a)	Inhibition	HLA-E	Gauge MHC class I expression
KLRG1	Inhibition	E-cadherin	Assess loss of tissue integrity
NKR-P1 (CD161)	Inhibition	LLT1	?
LAIR-1 (CD305)	Inhibition	Collagen	Control activation in extracellular matrix
Siglec-7 (CD328)	Inhibition	Sialic acid	?
Siglec-9 (CD329)	Inhibition	Sialic acid	?
IRp60 (CD300a)	Inhibition	?	?

*Adapted from Bryceson et al. Immunological Reviews 2006 (89)

1.1.2.2.3 Adhesion receptors

The adhesion receptors belong to different receptor families including the integrin, immunoglobulin, selectin, and cadherin family. The far most studied adhesion receptor expressed by NK cells is the integrin LFA-1 that besides adhesion also has many other functions. As an example, LFA-1 is critical for proper killing of NK cell targets by regulating the polarization of the cytolytic granules toward the target cell upon interaction with (Inter-cellular adhesion molecule 1) ICAM-1 (136). LFA-1 has also the capacity to induce NK cell activation when interacting with target cells expressing ICAM-1 (136). Blockade of the LFA-1 receptor results in impaired NK cell cytotoxicity mediated by ADCC (112, 113). Patients lacking the LFA-1 receptor due to mutations of CD18 (LAD syndrome type 1) experience severe infections and

display impaired NK cell function (137). The expression and affinity of LFA-1 can be increased by cytokine stimulation (IL-2 and IL-15) and by local chemokine stimulation (CX_3CL1) in the immunological synapse (136, 138-141). In addition, co-receptors such as 2B4, CD2, CD44, and CD16 can also increase the adhesive properties of LFA-1 (136, 141, 142).

1.1.2.2.4 Regulation of receptor expression

The regulation of receptor expression on NK cells is characterized for some receptors (summarized in Figure 4 and Table 3), whereas the regulation of other NKRs is less well understood today. It is known that cytokines can modulate the expression of NKRs, including the NKG2D, DNAM-1 and NCRs (14, 117, 143, 144). As an example, IL-2 has been shown to increase the expression of NKG2D (145) and stimulate NK cells to express NKp44 (146). In contrast, TGF-β may down-regulate NKG2D and IL-21 may down-regulate NKG2D and NKp44 (145, 147-151). NK cells stimulated with IL-12 were recently shown to up-regulate the inhibitory CD94/NKG2A receptor (152).

The NKR expression may also be modulated by interactions with their cognate ligands, as exemplified by trogocytosis, where the NKR is ripped of from the NK cell surface or internalized after receptor-ligand interaction (Figure 4). The involvement of receptor-ligand interactions has been demonstrated for the expression of the NKG2D, CD96 and DNAM-1 (153-157). Shedding of ligands, such as the NKG2D-ligands, can also induce loss of the cognate receptor (158). Ligation of the lower hinge region of IgG antibodies to the CD16 receptor does not only induce NK cell degranulation, but also loss of expression of the receptor due to internalization (159). It has also been reported that the loss of the signal transducing molecules FcεRIγ and CD3ζ in tumor-associated lymphocytes of cancer patients reduced the expression of CD16 and depressed the proliferative response to CD16 stimulation (160). Thus, NKR expression may be dynamic and could be altered by several mechanisms such as cytokines, soluble ligands or through direct contact with targets expressing ligands for NKRs.

1.1.2.2.5 Intracellular receptor signaling

Many receptors expressed on lymphocytes of both the innate or adaptive immune system are linked to common signaling transducing units. DNAX adaptor protein (DAP)10 and DAP12 are two central subunits that are involved in NK cell activation (161, 162). DAP10, for instance involved in activation of NK cells encountering target cells expressing ligands for the NKG2D receptor, mediate activation via tyrosine phosphorylation of YINM sequences on the short cytoplasmatic domain (163). Phosphoryaltion of these motifs allows binding of phosphatidylinosoitol-3 kinase (PI3K) and GrB-2Vav1-son of sevenless 1 (SOS1) leading to NK cell activation via activation of transcription factors. DAP12 contain immunoreceptor tyrosine-based activation motifs (ITAMs) that upon phosphorylation recruits and activate spleen tyrosine kinase (Syk) and ξ–associated protein (Zap)70 leading to activation of NK cells through the Shc-Grb2-Sos-Ras-Raf-MEK-ERK pathway (164, 165). Many activating NKRs, including the activating KIRs and the HLA-E binding activation receptor CD94/NKG2C, signals via ITAMs. DAP12 and and the adaptor molecule FcεRIγ each have a single ITAM, which is in contrast to the adaptor molecule CD3ζ that has three ITAMs (166). The two latter can be associated to the CD16 receptor (167-169).

Upon interaction with a target, inhibitory signals most often override activation signals. The blockade of activation signals occurs at a very early step, before full effector to target adhesion is obtained (170) and before release of intracellular Ca^{2+} (171). Many of the inhibitory NKRs, such as inhibitory KIRs, CD94/NKG2A and LILR-B1, signals through inhibitory motifs called immunoreceptor tyrosine-based inhibition motif (ITIM) or ITIM-like

sequences. Selective recruitment of the tyrosine phosphatases Src homology (SH)-containing tyrosine phosphatase-1 and 2 (SHP-1 and SHP-2) to ITIMs inhibit NK cell activation by dephosphorylation of intracellular signaling molecules associated to activating NKRs such as Vav, Lck and Zap70 and thereby mediate early blockade of activation signals (172, 173). The exact pathway and action of inhibitory receptors varies depending on the adaptor molecules downstream of the receptor (108). Some ITIM-based receptors may even be SHP-independent and instead signal through Csk (108). Early inhibitory signaling also abrogates the recruitment of important components of the immunological synapse (IS) (108). Emerging data suggest that NK cells that receive strong inhibitory signals through MHC class I binding receptors also acquire a more potent killing capacity, whereas NK cells without MHC class I binding receptors are hyporesponsive (174-176). This process is termed education (or licensing) and will been discussed later.

The death receptors, including Fas, TNF receptor and TNF-related apoptosis-inducing ligand (TRAIL) receptor, expressed by target cells, can also signal by SHP-1 and SHP-2 through ITIM-like (YxxL) motifs in their cytoplasic tail (177). The exact consequences of signaling through the death receptors seem to vary between normal and tumor transformed cells (178, 179).

Taken together, although relatively much is know about the control of NK cell activity, there is still a need for further studies to delineate how the signaling pathways intersect and how they synergize in the intricate regulation of NK cell target cell killing.

1.1.2.2.6 Synergy among NK cell receptors

NK cells need activation signals that reach a certain threshold to induce degranulation (133, 180). Although recent data from studies on inside-out signals for LFA-1 have provided more detailed information about the minimal requirement for NK cell activation (180), the precise molecular mechanisms and the exact checkpoints for the intersection of activation and inhibition signals are still not clear. In resting (non-cytokine-stimulated) NK cells, signals from single activation receptors do not provide enough stimulation to induce degranulation. Instead, a pair wise ligation of two activation receptors simultaneously may together reach the threshold for NK cell activation leading to the release of cytotoxic granules (136). The receptors engaged simultaneously have to be stimulated by their respective ligands expressed on the very same target cell and not by two different but adjacent cells (180). Importantly, there seem to be a hierarchy between the NKRs, where some can induce Ca^{2+}-flux, but not degranulation. The combined engagement of 2B4, NKG2D and LFA-1 has been defined as minimal requirement for natural cytotoxicity leading to lysis of the target cell by resting NK cells (180). The exact mechanism for this synergy remains unclear and future studies are needed to clarify if it is controlled by signals from different receptors in sequential steps or a sum of activation signals that eventually converge downstream (91, 181). Importantly, the CD16 receptor represents an exception since it can induce degranulation by resting NK cells alone.

1.1.2.3 The immunological synapse between the NK cell and its target

All lymphocytes have the ability to form transient conjugates with other cells (182). Conjugate formation between an NK cell and its target is a highly dynamic process and a prerequisite for the NK cell to exert its function. The formation of the IS occurs through a series of sequential steps from the first contact via adhesion receptors to the release of perforin and granzyme containing granules (181). In summary, the formation of the IS starts with an initial adhesion inducing Ca^{2+} flux that result in an even tighter adhesion by increased affinity and avidity of the LFA-1 (89, 182). Next, the NK cell reorganizes its microtubule (MTOC; microtubule organizing centre) followed by reorientation and translocation of the granules toward the target (182). At this point,

the NK cell motility is decreased due to the reorganization of its cytoskeleton (183). When the NK cell has polarized to the target cell the granules dock and fuse with the cell membrane leading to release of perforin and granzymes inside the target and subsequent killing. Hence, the outcome of an NK cell that interact with a transformed cell is not only regulated by a balance of activation and inhibition signals from cell surface receptors, but importantly also by adhesion receptors and signals leading to prolonged intercellular contact as well as polarization toward the target cell leading to more efficient target killing. When the target cell is killed, the NK cell regains its motility and can attack new target cells in the surrounding, a phenomenon called sequential killing (184).

1.1.2.4 Effector mechanisms

NK cells exert their functions by two major pathways, namely direct cytotoxicity and by the release of cytokines and chemokines. The notion that the human $CD56^{dim}$ NK cell subset is more cytotoxic than the perforin-low but immunomodulatory $CD56^{bright}$ NK cell subset (185-188) has recently been revised. In fact, data indicate that specific target cell ligands can dictate $CD56^{dim}$ NK cells to be more prominent cytokine and chemokine producers than $CD56^{bright}$ NK cells (189).

1.1.2.4.1 Cytokine secretion

NK cells produce a variety of cytokines including macrophage inflammatory protein (MIP)-1α and β, interferon-γ (IFN-γ), tumor-necrosis factor-α (TNF-α), granulocyte macrophage colony stimulating factor (GM-SCF) (190, 191). Recent data support the notion that the type of cytokines released upon interaction with a specific target is dictated by the degree of stimulation (189). MIP-1α and β (also known as CCL3 and CCL4) induce an inflammatory response by stimulating granulocytes causing acute neutrophilic inflammation. They also induce the synthesis and release of other pro-inflammatory cytokines from fibroblasts and macrophages. MIP-1α and β are released already at low degrees of stimulation (189). IFN-γ is commonly released by NK cells and have many functions, such as increasing HLA class I expression and halts tumor growth via effects on p53 (192-195). TNF-α is a pleiotropic cytokine that has been shown to mediate extensive cellular responses, including proliferation, differentiation and apoptosis, depending on the cell type and the microenvironment (196, 197). GM-CSF stimulates the differentiation of granulocytes, macrophages and MDSCs from stem cells (198). Please see Table 4 in the result section for further information about some of these cytokines.

1.1.2.4.2 Perforin/Granzyme pathway and death receptors

NK cells can directly kill target cells by releasing their granule loaded with perforin and granzymes. The exact mechanism for target penetration is not known, but perforin is believed to perforate the cell membrane of the target cell helping additional components in the granulae to enter (199). When inside the target cell, granzymes induce apoptosis by activation of the caspase system in the intrinsic pathway (200). Moreover, target killing can also be induced through interactions between death receptors expressed on the target cells and its corresponding ligand expressed by NK cells. These systems are known as Fas-Fas ligand (201) and TRAIL-TRAIL ligand (202) and induce apoptosis via activation of caspase-8 and caspase-9 in the extrinsic pathway (199).

1.1.2.5 Development and distribution of NK cells

The NK cell development has been studied for a long time and although several different models have been suggested, accumulating data support the notion that different NK cell subsets have a

common progenitor in the bone marrow and that they acquire receptors and obtain full effector function during a maturation process referred to as education.

1.1.2.5.1 NK cell development - From stem cell to mature NK cell

The bone marrow is believed to be the primary site for NK cell development at steady state, although thymus, lymph nodes, spleen and liver have been suggested as alternative sites of NK cell development (203-206). Early studies have demonstrated that recombination-activating-gene knock-out (RAG $^{-/-}$) mice as well as thymus-deficient mice, lacking both B cells and T cells, express normal and fully mature NK cells (207-210), which suggests that NK cells developed via a unique pathway, disparate from both B cells and T cells. In addition, and in contrast to B cells and T cells, the early studies also indicated that NK cells lacked a lineage unique transcription factor (211). This led to speculations that NK cells evolved as a "default pathway" when the lymphoid lineage was not directed to B cells or T cells (211). Since then, various transcription factors such as Ets-1, GATA-3, PU.1, Mef, T-bet, Irf-2 and Id2 have been suggested to control NK cell development, but none of them have been shown to be unique for NK cells since lack of either of these factors is associated with deficiencies in other lineages too (212-218). However, recently published data demonstrate a central role for the transcription factor E4BP4 in the specific development of NK cells from a common lymphoid progenitor (219). This transcription factor is detectable in NK cell progenitors, and up-regulated in immature and mature NK cells and acts by inducing Id2 that is known to be critical for NK cell homeostasis (217, 219). In addition, mice lacking E4BP4 develop normally with a normal hematopoietic system including B and T cells, but lack NK cells, verifying the critical role for E4BP4 in NK cell development (219). Data from Gascoyne et al further demonstrate that the E4BP4$^{-/-}$ mice lack the ability to lyse HLA class I-deficient tumor targets, while CD8^{+} T cells still possessed full killing capacity.

The formation of NK cell precursors (NKPs) (CD34$^{(+)}$CD38^{+}CD45RA^{+} CD117^{+}CD127^{+}CD62L^{+}CD7^{+}) from CD34^{+} multipotent haematopoietic stem cells (HSC) in the bone marrow is likely to be regulated by stromal cell interactions, lymphokine stimulation and notch signaling (206, 220-222). The acquisition of CD122 (IL-2Rβ) on NKP facilitates IL-15-dependent NK cell development (223, 224). NK cell development and homeostasis have been shown to rely on IL-15, since IL-15$^{-/-}$ and IL-15R$^{-/-}$ knock-out mice both lack peripheral NK cells and cytotoxicity against HLA class I-deficient tumor targets (225, 226). Moreover, IL-15 stimulation has also been closely linked to the up-regulation of E4BP4 expression during NK cell development, suggesting one possible mechanism of action for IL-15 (219, 227). However, the presence of a unique NK cell subset in the spleen of both IL15- and IL15R-deficient mice has been reported (226, 228) and indicates that IL-15 is important but not essential for the NK cell development. These rare IL-15-independent NK cells have the capacity to respond to viral infections and may represent a distinct NK cell subset (229). Recently published *in vitro* data suggest that NK cells differentiate and acquire their functional capacities early during development by stimulation with IL-15, whereas the continuous homeostasis depends more on stimulation by IL-2 (230). This is probably explained by the sequential and altered expression of the different cytokine receptors, where the high-affinity IL-2 receptor, in contrast to the IL-15 receptor, is acquired after NK cell differentiation. New data also demonstrate a role for IL-15 complexed to the IL-15α receptor (IL-15 trans-presentation) on stromal cells along with soluble IL-2 in the proliferation and differentiation of human CD56^{bright} to CD56^{dim} NK cells (231). Although IL-15 and IL-2 are critical for the development, proliferation, effector function acquisition and the survival of NK cells, the contribution from other γ_c cytokines such as IL-7 and IL-21 should not be underestimated (232-235). The homeostatic effect of γ_c cytokines, protecting mature NK cells from apoptosis, is probably mediated through maintenance of the

antiapoptotic factor bcl-2 and a maintained activity of the two transcription factors, IRF-2 and T-bet (212, 213, 236). In contrast to the γ_c cytokines, the anti-proliferative cytokine TGF-β halt the development and function of NK cells (148, 237, 238) (Table 4).

As for B cells, but not T cells, the main part of the NK cell maturation process is considered to occur in the bone marrow, although emerging data suggest that other sites may be required for the final maturation (239). In addition, early studies in mice also demonstrate that immature NK cells could be seeded from the bone marrow to peripheral tissue sites where the maturation process takes place *in situ* (240). Hence, the exact compartment and the proper environmental requirements for NK cell maturation still remain partly unclear. Several distinct steps of NK cell maturation, defined by the expression of CD34, CD117 (c-kit), CD94 and CD56 in humans and by CD11b (Mac-1) and CD27 in mice, have been suggested (239, 241-243). The dynamic alterations of the NKR repertoire are believed to be orchestrated in a sequential fashion by various transcription factors (239). From initially expressing CD117 and CD127 (IL-7Rα), the NKPs develop into immature NK cells by acquiring the expression of CD161 and the integrin CD11b/CD18 (206). The immature $CD161^{+}CD11b^{+}$ NK cells also start to express the 2B4 receptor at an early stage (244). Although not fully cytotoxic at this stage, NK cells also acquire the TRAIL that can induce apoptosis in targets upon interaction with the TRAIL receptor on target cells (245). NK cells are considered to reach a more mature stage when acquiring the CD94/NKG2A and NKp46 receptors as well as the Ly49 receptors (mice) and KIRs (humans), which are important receptors regulating the NK cell cytotoxicity (206). It is not yet clear what defines an end-stage NK cells, but at least mature murine NK cells display a reduced turnover rate and proliferative capacity in response to IL-15 along with a poorer homeostatic expansion potential when they acquire the inhibitory KLRG1 (MAFA1) receptor (246). In fact, in humans, the $CD56^{bright}$ NK cell subset are KLRG1-negative and display a great proliferative capacity, whereas about 80% of the $CD56^{dim}$ NK subset, that has shorter telomeres and are considered to originate from the $CD56^{bright}$ NK cell subset, express the KLRG1 receptor (247, 248). Hence, KLRG1 expression may also define late-stage human NK cells, although additional discrete stages may be identified based on the density of the CD94/NKG2A or presence of CD69, CD57 CD86 or HLA class II on the $CD56^{dim}$ NK subset (206, 249).

Recently, several independent groups have identified a previously unknown NK cell or NK cell-like lineage in the gut (reviewed in ref (250)). They are RORγt expressing lymphoid tissue inducer (LTi) cells that may express NKRs such as NKp46 (250). However, it should be stated that these cells have not been verified to be conventional NK cells. The function of the NK-like cells found in the gut is still unknown. One may speculate that they can interact with the gut epithelia expressing stress-inducible molecules during an infection, which result in the release of IL-17, IL-22 and IFN-γ (250).

In conclusion, most NK cells arise from a common lymphoid progenitor via a specific developmental pathway that is regulated by the transcription factor E4BP4 acting via induction of Id2 expression. IL-15, with contribution from other γ_c cytokines such as IL-2, IL-7 and IL-21, is central for the development, maturation and homeostasis of NK cells by controlling the expression of E4BP4. The initial phase of NK cell development is likely to occur in the bone marrow, although data suggest that maturation may occur at other tissue sites too. Recent data also indicate that there might be an additional NK cell lineage arising from LTi cells. However, it is still unclear whether the uterine (u)NK cells originate from the same progenitor as peripheral blood and other organ-residing NK cells since they display a totally different phenotype. Hence, details regarding the precise localization, cellular interactions and regulation by intracellular and extracellular factors during NK cell development are not fully clear today, but will be an important task for future studies.

1.1.2.6 Education and tuning of NK cell functionality

New insights into the regulation of the NKR repertoire (251, 252) and the need for functional maturation through interactions with self-HLA class I molecules (174-176, 253, 254) have contributed to a better understanding of how NK cells gain their functional responsiveness and preserve their tolerance (255, 256). Although NK cells express high levels of perforin and granzyme they still need to undergo a functional maturation process to attain full killing and cytokine producing capacities (256). This process, referred to as education, is considered to be regulated by interactions between inhibitory NKR (KIRs and CD94/NKG2A) and their cognate HLA class I ligands (256). The level of excess stimuli from inhibitory KIR ligand interactions over signals from activating NK cell receptors dictates the threshold of activation in a given NK cell (257). However, this threshold is likely not set permanently, but may be continuously tuned (257, 258). This process has been designated the rheostat model, where the NK cell responsiveness is dynamically tuned through repetitive interactions with surrounding cells and possibly other factors in the microenvironment (259). Hence, tolerance of NK cells may be induced in certain environments protecting the host from unwanted NK cell responses such as autoimmunity, whereas this may be reversed in other environments. As previously discussed, the cytotoxic potency of a specific NK cell is determined by the strength of the inhibitory signals provided upon interaction with the corresponding HLA class I molecules (258). Importantly, NK cells with either activating KIRs in individuals expressing the corresponding ligand or inhibitory KIRs in individuals that do not express the corresponding HLA class I molecule are hyporesponsive (260). In contrast NK cells expressing inhibitory KIRs in individuals with the corresponding HLA ligand are fully functional (260). Recent data further highlights the role for activating KIRs in tuning of NK cell responsiveness, since NK cells expressing KIR2DS1 reduced the responsiveness of NK cells co-expressing CD94/NKG2A or KIR2DL3 (261). However, NK cells co-expressing KIR2DS1 and KIR2DL1, both binding to HLA group C2, were still functional (261). All these situations are summarized in Figure 1. The inhibitory CD94/NKG2A receptor is, besides inhibitory KIRs, also involved in NK cell education and tolerance. Hence, NK cells expressing only the CD94/NKG2A receptor are fully functional (262-264), indicating that this receptor also conveys NK cell education, presumably through interactions with HLA-E during NK cell development. In addition, recent data indicate that cytokine stimulation can induce KIR expression on a restricted fraction of KIR-negative NK cells and make the NK cells that started to express self-KIRs functional competent killers (265). The authors of this paper speculated that the education, in the absence of other cells, was either caused by *cis* interactions with HLA class I molecules expressed on the same NK cell or through *trans* interactions with self-HLA class I molecules expressed by surrounding NK cells (265). Together these data suggest that NK cell education might not only be an early event during NK cell development, but could also occur continuously in the periphery and especially during immune responses.

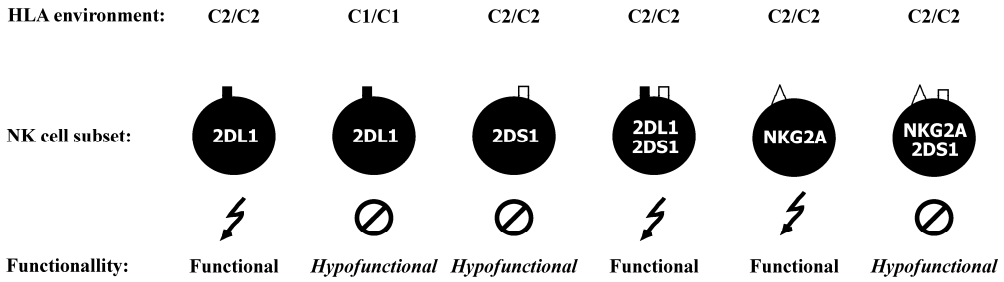

Figure 1. The impact of KIR on the education process in various HLA environments. KIR2DL1 expressing NK cells are functional through education via HLA-C2, whereas NK cells expressing the corresponding activating KIR (KIR2DS1) are hypofunctional. When both are expressed on the same NK cell, this cell become functional since the KIR2DL1 has higher affinity to the HLA-C2 than the KIR2DS1. However, KIR2DL1 single positive NK cells are hypofunctional in individuals homozygous for HLA-C1. NK cells that are either KIR2DS1 single positive or co-expressing KIR2DS1 and CD94/NKG2A are both hyporesponsive in individuals homozygous for HLA-C2.

1.1.2.6.1 NK cell distribution and trafficking

The distribution of NK cells differs slightly in mice and humans, which may be associated with partly differential expression of chemokine receptors. NK cells are most abundant in the spleen, but are also found in the lung, blood, thymus and liver whereas somewhat less NK cells are observed in lymph nodes (63, 266). NK cells are rare or totally absent in some tissues such as the digestive tract (except for RORγt^{+} LTi), muscle, brain, and skin of both species under normal conditions (239). During the maturation process, NK cells alter the expression of chemotactic receptors leading to homing to peripheral tissue. As an example, upon development, human $CD56^{bright}$ NK cells acquire the expression of CCR7 and thereby home toward the ligands CCL19 and/or CCL21 in lymph nodes (185, 266). Recently published data indicate that $CD56^{dim}$ NK cells can re-express CCR7 under some specific circumstances such as after interaction with DCs and targets cells or during IL-18 stimulation (267, 268). However, CCR7 is not expressed on mouse NK cells, which may explain the low frequencies of NK cells in mouse lymph nodes (63). The most central receptor homing NK cells to secondary lymphoid organs is CD62L (L-selectin). However, the regulation of NK cell migration is very complex. As example, upon inflammation, NK cells have to migrate through high endothelial venules (HEVs) via adhesion to integrins (ICAMs) to get to the site of inflammation, a process that is regulated by a complex concert of chemotactic compounds such as leukotrienes and C5a as well as chemokines stimulating the CCR2, CCR5, CX3CR1, CXCR3 and ChemR23 receptors (18, 63). Another complicated and still unclear process regards the trafficking of uNK cells. Data indicate that selected subsets of blood NK cells are specifically recruited to the endothelium of the decidua basalis of the uterus, a process that seem to partly be regulated by ovarian hormones with a peak of NK cells in the uterus during a 3-day window of ovulation (75). Integrins and L-selectin have been given roles in the recruitment, but further studies are needed to delineate the origin and homing of uNK cells (75).

In conclusion, NK cell trafficking is delicately regulated by chemokines that make specific NK cell subsets home to specific tissue sites. However, the receptors and mediators

controlling the trafficking are still largely unknown and represent an important field that will need further attention to delineate how NK cell migration is controlled.

1.2 IMMUNE CELLS IN THE TUMOR MICROENVIRONMENT

1.2.1 The interplay between immune cells, cytokines and ROS in the tumor microenvironment

The tumor microenvironment does not only consist of tumor cells but also extracellular matrix, stroma cells, blood vessels and immune cells. T cells are usually the most frequent tumor infiltrating lymphocyte (TIL) in the tumor microenvironment. The infiltrating T cells are often of memory (CD45RO-expressing) phenotype and may be specific for tumor-associated antigens (TAA) (269). Regulatory T cells (T_{reg}; $CD3^+CD4^+CD25^{high}Foxp3^+$) are enriched in the tumor microenvironment and constitute 5-15% of all $CD4^+$ T cells (270-272). These cells suppress immune responses from T cells and NK cells by the release of both IL-10 and transforming growth factor β (TGF-β) (273). In fact, high frequency of T_{reg} cells (274) and high levels of TGF-β both correlate with poor prognosis in many cancers (275-278). NK cells are less frequent than T cells in the tumor microenvironment (279). However, NK cells are involve in the formation of the tumor milieu since they can produce IFN-γ in response to IL-12 from tumor-associated macrophages (TAMs). TAMs react to the IFN-γ by producing more IL-12, but also IL-10, prostaglandins and reactive oxygen species (ROS) (280) (Table 4). Granulocytes and MDSCs also produce ROS and are as T_{reg} relatively resistant to oxidative stress (280, 281). The myeloid-derived suppressor cell (MDSC), recruited from the bone marrow by IL-10, VEGF and GM-CSF (282), also inhibit immune cell functions, such as DC maturation, while supporting tumor cell growth by releasing the enzyme arginase-1 (Arg-1) which synergizes with inducible nitric oxide synthetase (iNOS) to produce superoxide and nitric oxide (NO) (283). The consequences of MDSC-mediated inhibited DC maturation involve reduced capacity of cross-presentation and thereby compromised initiation a tumor-specific T cell response through interactions in the draining lymph node. Some immune cells and soluble factors found in the tumor microenvironment are depicted in Figure 2.

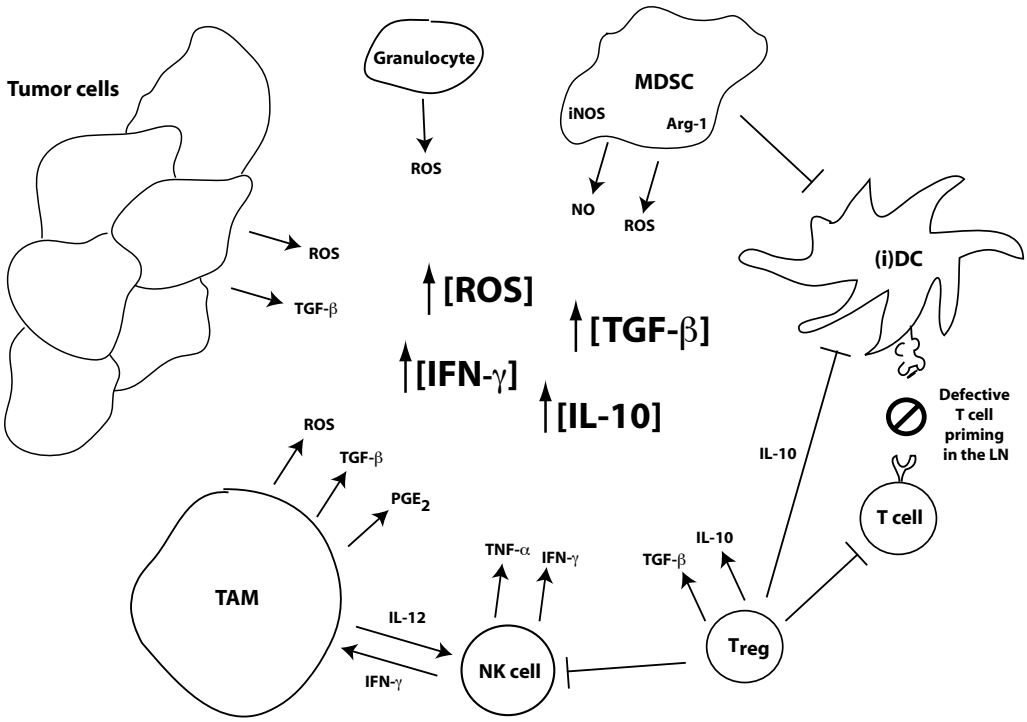

Figure 2. Examples of the interplay between cells and soluble factors in the tumor microenvironment. TAM; tumor-associated macrophages, MDSC; myeloid-derived suppressor cell, (i)DC; (immature) dendritic cell LN; lymph node.

1.2.2 Oxidative stress in the tumor microenvironment

Cells are equipped with detoxifying antioxidative systems built up from cystein- or selenocystein-containing proteins such as redox enzymes and scavanger proteins (284). In addition, free thiols groups (sulfhydryl groups; -SH) inside the cell and on the cell surface protect from the effects of ROS by being reduced to disulphide groups (S-S). These intracellular systems help the cell to keep an adequate redox balance and protect the cell from undergoing cell death. Moreover, the cell surface is also redox active and alterations in the redox state of protein thiols on cell surface receptors as well as their ligands have been shown to be important for multiple cellular processes (285, 286). There are several enzymatic antioxidant systems, including superoxide dismutases (SODs), catalases, as well as the glutathione and thioredoxin systems, inside a human cell. The two latter redox systems consist of glutathione (GSH) and thioredoxin (Trx) that are electron receivers (reductants) in enzyme-catalysed reactions with glutathione peroxidase and thioredoxin reductase (TrxR), respectively. These two systems (glutathione and thioredoxin system) work independently to neutralize oxidative agents, but are also central in the *de novo* synthesis ribonucleotides.

High levels of ROS are often observed in the tumor microenvironment (280). Immune cells are responsible for ROS producers, but ROS could also be derived from energy metabolites produced by the electron transfer reaction in the mitochondria as a consequence of rapid cellular metabolization in the tumor cells (287). ROS are highly reactive molecules that

affect all cells in the tumor microenvironment, including both the immune cells and the tumor cells, by inducing DNA damage (strandbreaks and mutations). DNA damage activates the ataxia telangietasia mutated (ATM) and ATM- and Rad3-related (ATR) protein kinase signaling pathway, which among other things may lead to up-regulation of the NKG2D receptor ligands MIC and ULBP and the DNAM-1 ligand (288-291). Oxidative stress may inhibit cellular growth since it induces massive activation of redox systems to protect the cells from damage and thereby temporarily inhibit the production of new ribonucleotides needed for the mitosis (292). Oxidative stress can also induce cellular arrest by interfering with intracellular signaling pathways, such as the MAPK pathway (293-296). It can also alter the set of proteins produced by the cell without affecting RNA transcription (292). Another consequence of oxidative stress was demonstrated in bladder cancer cell lines where ROS reversed TRAIL resistance by decreasing the threshold for death receptor-mediated apoptosis (297).

Tumor cells seem to have a perturbed sensitivity to oxidative stress compared to normal cells. This may be explained by decreased activity of the redox enzyme systems in tumor cells, as demonstrated for hepatoma cells compared to normal hepatocytes (298). Moreover, selenite, a highly reactive compound that induces oxidative stress, exerts its effect at lower concentrations in tumor cells than in normal cells (299, 300), which may be explained by altered uptake of selenite in tumor cells compared to normal cells (301).

Hence, oxidative stress may directly kill tumor cells or render them susceptible to immune cells. However, the oxidative stress my also have negative effects on non-tumor cells in the tumor microenvironment, including immune cells. Studies report dysfunction or apoptosis of subsets of T cells and NK cells due to oxidative stress. For instance, oxidative stress has been shown to down-regulate NKRs on NK cells (302-304). Taken together, oxidative stress may be a double-edged sword in the tumor microenvironment. Strategies to protect immune cells from oxidative stress in the tumor microenvironment are being developed and will be discussed in the result section.

1.3 IMMUNOLOGICAL RECOGNITION OF CANCER

1.3.1 Tumor immune surveillance and cancer immunoediting

The tumor immune surveillance theory, formulated in the 1950ies, has been heavily criticized due to reports showing no difference in the frequency of tumors in nude mice versus normal mice (305, 306), although virus-associated tumors represented an exception (307, 308). Most tumors that developed in immunosuppressed human hosts, such as transplant and AIDS patients, have also shown to be virus-associated tumors such as non-Hodgkin's lymphoma and Kaposi's sarcoma (caused by EBV and human herpes virus 8, respectively) (309-311). In contrast, arguments for the immune surveillance theory are based on data showing that immune suppressed humans display increased formation of non-viral induced cancer and that low NK cell activity in familiar breast cancer patients as well as in their clinically asymptomatic relatives links immune deficiency to increased cancer formation (312, 313). Another argument supporting the immune surveillance theory is the fact that immunocompetent cancer patients raise immune responses against the tumor cells, as exemplified by the detection of tumor specific antibodies in cancer patients (314). Data also demonstrate an increased survival in patients with high frequency of infiltrating lymphocytes in their tumors, which suggest a role for the immune system in the protection from tumor development. Moreover, studies on immunodeficient mice (lacking either the RAG, IFN-γR or perforin genes) have also added support for tumor immune surveillance (315, 316). In this respect, recently published data from two independent groups has

demonstrated central roles for the activating immune receptors NKG2D and DNAM-1 in controlling the tumor formation in murine models (127, 134).

Regardless of whether the immune system has a central role in controlling tumor formation, it is well documented that the immune system in cancer patients is impaired. Studies evaluating DTH responses to assess the function of antigen-specific T cell responses revealed decreased immune reactivity in cancer patients (317). Data also suggest that the absolute number of circulating T cells are lower in cancer patients (318) and in line with these experiments, a general decreased cytotoxic capacity by immune cells has been demonstrated in cancer patients (319, 320). Finally, immune cells isolated from tumors most often display reduced functionality (321).

During the last decade, Dunn and colleagues have published several reports on immunoediting of cancer (312, 322). They claim that anti-tumor immune responses caused by tumor immune surveillance modify the tumor cells *per se* and thereby edit the tumor cell repertoire. This theory is based on findings in mouse models showing that tumors formed in mice with intact immune system are less immunogenic due to the development of immune escape variants whereas tumors that are formed in immunodeficient mice are more immunogenic (316, 322). This process is called immunoediting and is divided into three phases that include an initial elimination phase, followed by an equilibrium phase and a final stage where the tumor cells escape immune recognition. This process, summarized as the three E's of immunoediting, has been suggested to be a 7th hallmark of cancer (312), in addition to limitless replicative potential, self-sufficiency in growth signals, insensitivity to anti-growth signals, the capacity to evade apoptosis, sustained antiangiogenisis, tissue invasion and formation of metastasis (323, 324).

1.3.2 Immune escape mechanisms by cancer cells

Neoplastic cells continuously produce new subclones due to spontaneous mutations that give them growth advantages in the tumor microenvironment and help them to evade from immunological recognition. Cancer cells can evade immune recognition by release of a broad range of biological effector molecules that impair the immune system (321, 325) or by down-regulation of MHC class I (326).

The genomic instability of cancer cells resulting in new mutations and new subclones of cancer cells is a central component of tumor immune escape. Cancer cells can acquire resistance to the proapoptotic actions of IFN-γ or increased protection from apoptosis through induction of Bcl-2 and cFLIP, leading to increased survival (192). Spontaneous mutations due to genomic instability may also result in reduced cell surface expression of HLA class I. Deletion of HLA class I genes is observed in up to 90% of human cancer cells (327, 328). Reduced or abolished HLA class I expression due to mutations of the antigen-presenting machinery (APM), including the TAP proteins, tapasin or components of the immunoproteasome, are also frequently (up to 80%) observed in cancer (38). In addition, loss of β_2-microglobulin (β_2m) results in instability of the HLA complex and reduced cell surface expression. Low levels of HLA class I can also be caused by proteolytic shedding (329). Another immune evasion strategy involves down-regulation or shedding of ligands for activating NKRs such as NKG2D (MIC/A) (158, 326). Finally, co-stimulatory molecules may also be down-regulated by cancer cells leading to impaired induction or anergy of T cell responses in the tumor microenvironment (330).

Cancer cells can also up-regulate specific molecules that inhibit immune cell functions. Examples of this mechanism are the up-regulation of HLA-E and HLA-G that abrogate immunological tumor rejection by interacting with CD94/NKG2A and LILR-B1, respectively (47, 51-56, 331). Another example is the inducible co-stimulator ligand (ICOSL)

that dampens immunological responses through interaction with the inhibitory CTL-associated antigen-4 (CTLA-4) receptor expressed on T cells (332). Moreover, tumor cells may also acquire the properties to mediate counterattack to the immune cells via up-regulation of the Fas receptor that induces killing of immune cells expressing the Fas ligand. This phenomenon is called Fas-counterattack (333).

Cancer cell can also suppress the immune system by releasing cytokines. As previously discussed, the cytokines TGF-β and IL-10 are both central in immune suppression in the tumor microenvironment (Table 4). As previously mentioned, GM-CSF is indirectly suppressive because it promotes recruitment and expansion of TAMs and MDSCs (282, 334, 335). Molecules in the TNF family such as Fas ligand, TRAIL and TNF may be released and induce apoptosis in leukocytes upon binding to TNF family receptors (336). In fact, cancer cells can also release microvesicles expressing Fas ligand (337). Small molecules including prostaglandin E_2, H_2O_2 and histamine inhibit the function of immune cells by increasing cAMP inside the effector cells, whereas inducible nitric oxide synthase (iNOS) affects Fas-mediated apoptosis (338). Furthermore, enzymes such as arginase I and indoleamine 2,3-dioxygenase (IDO) impair and suppress T cells responses by decrease the CD3ζ-chain and affecting the thryptophan metabolism, respectively (339, 340). A decrease in the CD3ζ-chain expression is associated with reduced immune responses to antigens by T cells and NK cells in cancer patients (160, 341-343).

Taken together, cancer cells can alter their cell surface proteins or suppress the immune system to avoid immune recognition. Cancer cells can also dampen the immune system by modifying the receptor repertoire on the immune cells. This will be discussed later with a particular focus on NK cell receptors.

1.3.3 NK cell-mediated killing of cancer and the rational for immunotherapy

1.3.3.1 NK cell-mediated killing of tumor cell lines and the role in transplantation

Evidence for NK cell-mediated killing of tumor cells comes from several different experimental settings. Tumor cell killing by NK cells was first shown *in vitro* (64-67). Studies in murine models have also added support for the involvement of NK cells in the rejection of inoculated tumors *in vivo* (79). Two groups recently reported an increased risk of tumor development in mice lacking either the activating receptors NKG2D or DNAM-1 that is expressed on all NK cells (134, 344). For instance, DNAM-1-deficient mice developed significantly more DNAM-1 ligand-expressing fibrosarcoma and papilloma tumors compared to wild-type mice in response to the chemical carcinogens methylcholanthrene (MCA) and 7,12-dimethylbenz[a]anthracene (DMBA) (134).

A role for NK cell targeting of human tumors *in vivo* has been suggested in settings of allogeneic stem cell transplantation (SCT), in particular haploidentical SCT against acute myeloid leukemias (AML) (72, 345). Further support for NK cell targeting of human tumors have also emerged from studies involving adoptive transfer of NK cells to cancer patients (346). Moreover, NK cell infiltration in some solid tumors (347) and the correlation between increased cancer risk and low NK cell-mediated cytotoxicity (348) has also been taken as indirect evidence for a role of NK cells in the defense against cancer.

Hence, some studies have demonstrated a role for NK cells in immunotherapy and several studies have demonstrated NK cell-mediated killing tumor cell lines *in vitro* and *in vivo* (80, 349). However, many differences between primary tumor cells and tumor cell lines are associated with altered proliferation rates and disrupted tissue organization (350, 351), which may lead to false information regarding the molecular specificity of NK cells. To better understand the requirements for NK cell recognition of human tumors in the affected patient, and

prerequisites for successful human NK cell-based immunotherapy, studies have been initiated to assess the ability of human NK cells to target freshly isolated human tumor cells.

1.3.3.2 Receptor specificity for NK cell-mediated recognition of fresh human tumor cells

The evidence for NK cell-mediated killing of freshly isolated human tumor cells reported in the literature is based on a limited number of studies, some rather old (352-356) and some more recent (357-365). Recent reports have also delineated the molecular specificity involved in recognition of freshly isolated cancer cells (Table 3). The studies are based on either allogeneic NK cells or autologous NK cells and are summarized in Table 2. Some studies are hampered by technical difficulties, including monitoring the specific lysis of fresh tumor cells within heterogeneous patient-derived cell populations (see **Associated paper A**).

NK cell-mediated lysis of primary acute lymphoblastic leukemia (ALL) blasts has been observed with autologous NK cells expanded *in vitro* (357). One recently published study focusing on NK cell killing of primary AML blasts used NK cell lines with single KIR specificities for HLA class I allotypes. This study nicely demonstrates NK cell recognition of freshly isolated primary AML blasts and indicates a beneficial role for KIR ligand mismatching (more discussed later) (358). Tumor cell killing was predominantly observed in monoblastic cells expressing NKG2D ligands, whereas myeloblastic cells lacking corresponding ligands were resistant to lysis. Induction of cell surface NKG2D ligands by treatment with the histone deacetylase (HDAC) inhibitor, valproic acid, rendered cells more sensitive to NK cell-mediated lysis (358). This study pointed at the possibility of using alloreactive HLA class I-mismatched NK cells in combination with pharmacologic induction of NKG2D in clinical evaluations as a novel approach to immunotherapy for AML.

NK cell-mediated killing of freshly isolated multiple myeloma cells has also been demonstrated using either allogeneic or autologous NK cells (359-362). However, the interaction governing NK cell-mediated killing varies among the studies. One study, using allogeneic NK cells, revealed a prominent role for the DNAM-1 receptor, as demonstrated by antibody masking of activating NKRs (360). Another study, using antibody blockade of autologous NK cells indicated that several activating receptors might contribute to lysis of multiple myeloma cells in this setting (362). The recognition *in vitro* of patient-derived multiple myeloma by autologous NK cells, as demonstrated with either IL-2 stimulated or long term expanded autologous NK cells (361, 362), led to speculation that this tumor might be targeted *in vivo by* immunotherapeutic strategies involving autologous NK cells. It should be noted that multiple myeloma frequently display reduced levels of HLA class I on the cell surface which may explain the effectiveness of autologous NK cell preparation in this setting. Indeed, NK cell killing correlated inversely with the level of HLA-class I on the myeloma cells (361).

Freshly isolated neuroblastoma cells, obtained from bone marrow samples from patients with metastasizing diseases, represent one solid tumor characterized with respect to NK cell susceptibility (363). Killing of freshly isolated neuroblastoma cells was shown to involve NKp30 and NKp46. A significant heterogeneity in susceptibility to lysis was found among neuroblastomas derived from different patients. Interestingly, susceptibility to lysis directly correlated with the surface expression of the DNAM-1 ligand CD155. CD155 expressing neuroblastoma cells were efficiently killed by NK cells, and mAb masking of either DNAM-1 on the NK cells or CD155 on the tumor cells resulted in strong inhibition of tumor cell lysis. These findings indicate that assessment of CD155 levels on cell surfaces may represent a criterion for predicting the susceptibility of neuroblastomas to NK cell-mediated killing.

Observations of NK cell-mediated killing of a limited number of freshly isolated human tumor cells of various histotypes, including gastric, ovarian, colon and renal cell cancers have also been made in a recently published study (365). Although the observations are based on few experiments, the results indicate an enhanced killing in the KIR ligand mismatched setting, which highlights the possibilities of using alloreactive NK cells against solid tumors.

All the studies mentioned above have added information about the molecular specificity for NK cell recognition of fresh human tumor cells and form the rationale for NK cell-based immunotherapy. Hence, there are several activating NK cell receptors involved in the recognition of fresh human tumors, some are more central for certain tumor types, whereas several of them cooperate in the recognition of other tumor types. Finally, these studies have demonstrated a critical role for KIR ligand mismatching in settings based on allogeneic NK cells.

Table 2. Summary of studies on NK cell recognition of freshly isolated human tumor cells*‡

Tumor type	NK cell source	NK cell preparation	NK cell stimulation	Patients included	Reference
Acute lymphatic leukemia	Autologous	Polyclonal	Expanded for 10-12 days on feeders and activated with IL-2, IL-12 and IL-15	7	(357)
Acute myeloid leukemia	Allogeneic	Clonal	Expanded for 14-21 days on feeders in IL-2 medium	10	(358)
Multiple myeloma	Allogeneic	Polyclonal	Unstimulated	9	(359)
	Allogeneic	Polyclonal	Cultured for 5-7 days in IL-2 medium	4	(360)
	Autologous	Polyclonal	Unstimulated and IL-2 activated for 2 days	6	(361)
	Autologous	Polyclonal	Expanded on feeders for 20 days, in IL-2 medium with OKT-3	7	(362)
Neuroblastoma	Allogeneic	Polyclonal	Expanded on feeders in IL-2 medium with PHA	8	(363)
Ovarian carcinoma	Allogeneic	Polyclonal	Unstimulated and IL-2 activated over-night	6	**Paper II**
	Allogeneic	Clonal	Expanded on feeders for 14 days in IL-2 medium with PHA	1	(365)
Colon	Allogeneic	Clonal	Expanded on feeders for 14 days in IL-2 medium with PHA	3	(365)
Renal	Allogeneic	Clonal	Expanded on feeders for 14 days in IL-2 medium with PHA	4	(365)
Gastric	Allogeneic	Clonal	Expanded on feeders for 14 days in IL-2 medium with PHA	1	(365)

* The table is modified from **Associated paper A**.
‡ The table is not intended to provide a complete survey of all studies on NK cell-mediated recognition of fresh human tumor targets. A particular focus has been on more recently published studies.

1.4 IMMUNOTHERAPY AGAINST CANCER

During the last decades, high hopes have been set on the development of new therapies against cancer using the immune system. However, the results from studies in both animal models and in clinical trials of various immunotherapeutic approaches have not yet been as successful as first anticipated. In fact, only a few strategies, such as mAbs and BCG, may be used in clinical practice today (366, 367). This section will discuss the different strategies of immunotherapy, with a particular focus on NK cell-based immunotherapy.

1.4.1 Strategies for immunotherapy against cancer

In the beginning of the cancer immunotherapy era, much effort focused on the development of tumor vaccines. Unfortunately, the results from several tumor vaccine trials on over 1000 patients, using either DNA vaccines, peptides vaccines, proteins or whole cell lysates vaccines delivered with adjuvants and/or loaded on DCs, have been disappointing with only approximately 3-4% objective response in the cancer patients (368, 369). In contrast to vaccination against viral epitopes preventing the development of virally induced tumors, such as HPV-induced cervical cancer (370, 371), vaccination against non-viral induced tumors is much more difficult. One major obstacle with tumor vaccine strategies is that the antigens presented by the tumor cells most often are self-antigens which makes it difficult to mount strong specific

immune responses against the tumor cells without affecting normal cells. In fact, autoimmunity has been observed in several cancer vaccine trials (369). As an example, antibody blockade of CTLA-4 on T cells, the inhibitory counterpart to CD28, that aimed to increase the efficacy of the vaccines (372) by abrogating inhibitory signals to the T cells, was associated with increased autoimmunity (373). A large number of human tumor antigens (TAAs) have been characterized and cloned during the last 25 years, many of which now are now being tested in clinical trials (374). Most of these antigens were originally isolated from malignant melanomas utilizing patient derived T cells as tools for their isolation, but the majority of these antigens are also expressed on other types of tumors (375). Due to the high genetic instability, the antigens presented by the tumor cells may rapidly change and the raised immunological pressure may negatively select subclones of tumor cells which are less immunogenic (376). One way of preventing such escape is to use multi-epitope-based vaccination strategies (377). However, apart from these matters, there are still many unanswered questions to solve before the increased efficacy of cancer vaccines could be expected. As an example, further investigations need to clarify what type of vaccine, delivery route and adjuvant setting that should used for a selected types of malignancy at a certain stage of the disease.

During the 1980th and 1990th several groups tried to boost the immune system of cancer patients by the administration of recombinant (r)IL-2 (378, 379) alone or in combination with adoptive cell therapy (ACT) based on lymphokine-activated killer (LAK) cells (380-382), which are autologous peripheral mononuclear cells that are exogenously stimulate *in vitro* for 5 days to induce potent killer cells. The anti-tumor cytotoxicity was identified to be mainly mediated by activated NK cells, however, the clinical outcomes in the trials were modest with moderate anti-tumor responses *in vivo* (381, 383, 384). The best effects were generally observed in patients receiving high dose rIL-2 (e.g. 1x10^5 IU/kg three times per day) and that were treated with 4 mg dexametasone intravenously four times per day to suppress the toxic side effects of rIL-2 (e.g. dyspnea, confusion, fever, and increasing serum creatinine and bilirubin levels) (385, 386).

T cell-based cellular therapy with expanded tumor specific CD8$^+$ CTLs, from naturally occurring tumor-infiltrating lymphocytes (TILs), represents another attractive immunotherapeutic strategy. However, the initial trials with TILs only showed marginal improved survival compared to LAK therapy (387, 388). With advances in tumor immunology and identification of the critical role for preconditioning, new strategies focusing on adoptive transfer of TILs following lymphodepletion, have shown objective responses in over 50% of patients with metastatic melanoma as measured by the Response Evaluation Criteria in Solid Tumors (RECIST) criteria (389-391). Unfortunately, recurrent disease with tumor escape variants has eventually been observed in several of these patients (392). T cell-based immunotherapy has also been associated with severe adverse effects including vitiligo, ureitis, retinitis and autoimmune destruction of the epithelial of the bile duct (41, 389, 393). Another source of tumor-reactive lymphocytes is the tumor draining lymph node, the so-called sentinel node (394). Preliminary results from ongoing clinical trials with adoptive transfer of lymphocytes isolated and expanded from the sentinel nodes are promising, but need to be further evaluated (395). Adoptive transfer of T cells, transduced with gene-engineered tumor specific T cell receptors (TCRs) using viral vectors, is another attractive approach that so far has only been tested in few patients (396) and merits further attention (397, 398). The use of chimeric activating receptors (CARs; receptors gene-modified to have a strong intracellular activation adaptor) such as T bodies that are cell surface bound antibody-like receptors targeting tumor antigens in an HLA class I-independent manner giving co-stimulation of endogenous TCR signaling upon encountering of TAAs presented by HLA class I is another example of gene modified immune

receptor-based immunotherapy.

Tumor specific mAbs represent a distinct modality of immunotherapy that has several mechanisms of action. mAbs can directly eradicate tumor cell by binding to growth receptors and thereby inhibiting cellular growth but also induce ADCC by binding to tumor cells with its antigen-binding fragment region (Fab) and crosslink its Fc region with Fc receptors on immune cells such as NK cells and monocytes. Several mAbs (Rituximab®, Herceptin®) are currently being used in clinical therapy against cancer (374). mAbs that coat tumor cells may also initiate complement-dependent cellular cytotoxicity (CDCC). Modified mAbs such as bidirectional, "magic bullets" and antibodies or fragments thereof that block inhibitory immune receptors may also be studied in the future.

Hematopoietic stem cell transplantation (HSCT) has been used for many decades as a treatment of hematological malignancies and is now an established therapy for recurrent acute leukemias. In contrast, there are less reports on the clinical effect of HSCT against solid tumors, where most studies have been conducted on renal cell carcinomas and breast cancer and a few on colon carcinoma, ovarian carcinoma and pancreatic cancer (399). It has long been known that T cells have a central role in the recognition of minor epitopes in matched HSCT (400), however, a role for NK cells in haploidentical HSCT was recently shown (72). The study by Ruggeri and colleagues, demonstrated a beneficial role for KIR ligand mismatching in the graft-versus-host (GvH) direction in patients with AML (72). These results have generated much interest for the role of NK cells in cancer immunotherapy, which is currently being investigated in several settings, including SCT and NK cell-based donor lymphocyte infusion (DLI). NK cell-based immunotherapy will be discussed more in detail in the next section.

In conclusion, during the last decades most tumor immunotherapy strategies have shown modest results. Only a few strategies have been successful and hold promises for future therapeutic interventions. Antibody-based immunotherapies against lymphoma and breast cancer as well as some cellular immunotherapies involving T cells or NK cells have showed good clinical results, but there is still a need for further improvements. Preparative regiments, selection of susceptible patient groups and diseases, the use of combinatorial treatments, enhancement and increased specificity of tumor targeting among others are examples of factors that may improve tumor immunotherapy. Other aspects and possible future obstacles in the advances of tumor immunotherapy involve the handling of the expenses and logistics of a highly patient-customized therapy. The modern immunotherapy era with rapidly increasing knowledge of the molecular specificity behind immune-mediated tumor targeting will hopefully improve the outcome of immunotherapies.

1.4.2 NK cell-based immunotherapy of cancer

As previously discussed, autologous NK cells have been used in the context of LAK therapy during the 1980th, with or without synchronous administration with rIL-2. The poor results were probably attributed to hampered anti-tumor reactivity *in vivo* of the suppressed patient-derived NK cells (14, 401-403), along with short *in vivo* persistence and incomplete pre-conditioning of the patients. The rational for NK cell-based cancer immunotherapy today is still based on the fact that NK cells are rapid and potent tumor cell killers without a need for prior sensitization. In addition, deeper knowledge in basic NK cell biology and tumor reactivity as well as new insights into the prerequisites for NK cell-mediated tumor rejection *in vivo* have contributed to a better understanding that may advance NK cell-based cancer immunotherapy. The prerequisite and premises for NK cell-based immunotherapy and the results obtained to date will be discussed in this section.

1.4.2.1 Context and setting of NK cell-based strategies

As mentioned above, Ruggeri and colleagues recently showed that NK cells were involved in tumor rejection in haploidentical HSCT (72). The results from their clinical trial revealed low GvH disease (GvHD), good engraftment and an increased overall survival in haploidentical HSCT against AML (72). A detailed stratified analysis further revealed that AML patients (n=34) undergoing KIR ligand mismatched haploidentical HSCT in the GvH direction showed an increased overall survival with a probability of relapse within 5 years that was 0% compared to 75% in matched transplants (n=58). The AML patients receiving alloreactive NK cells had a 60% survival compared to only 5% in the other group. Since then, several studies have investigated the role for NK cells in immunotherapy in general, and the potential beneficial effect of KIR ligand mismatching in particular (404-410). Some of these studies have reported no or even negative effects of genetically predicted NK cell alloreactivity due to KIR ligand mismatching (407-410). Several possible mechanisms, such as different preparative regimens of the graft and the patients and different post-transplantation immune suppressive regimens, may explain the discrepancies between the studies. Another parameter to consider is the high frequency of immature NK cells early following SCT (251, 262, 411-413). In fact, one study has recently shown that the cytotoxicity of KIR ligand mismatched but CD94/NKG2A expressing NK cells, initially reconstituting after transplantation, displayed low lysis of primary AML cells, which affected the outcome of haploidentical SCT (412). The low lysis was abrogated *in vitro* following blockade of the CD94/NKG2A and HLA-E interaction, which underlines the notion that the CD94/NKG2A receptor must be considered in therapies against HLA-E expressing tumor cells (412). Hence, NK cells have shown potential in the contexts of HSCT and in adoptive transfer, but further efforts are needed to delineate the conditions and settings to improve NK cell-based cancer immunotherapy.

1.4.2.2 Patients and susceptibility to NK cell-based immunotherapy

1.4.2.2.1 Patient selection and prediction of susceptibility to NK cells by studies on fresh tumor targets

Strict selection criteria for the patient cohort are critical and should be considered to improve the outcome of NK cell-based immunotherapy. Several criteria may be applied, however, some factors seem to be more important than others. The physical status of the patient is pivotal since the pre-conditioning *per se* most often has serious (sometime lethal) adverse effects and the immune suppression following immunotherapy increases the susceptibility to infections. The type of cancer and stage of disease are two critical matters to consider prior to therapy, since some cancers are NK cell resistant and be cause patients with bulky disease and advanced stage cancers often have a worse outcome of immunotherapy (414).

Tumor susceptibility to NK cells is an obvious criteria and therefore critical to assess before conducting NK cell-based immunotherapy. However, as previously discussed, the knowledge of tumor susceptibility most often comes from *in vitro* studies on cell lines or on studies based on the *in vivo* rejection of cell lines in animal models. However, these data may not always be directly extrapolated to fresh human tumors. NKR ligand phenotyping of the tumor cells may represent one way to predict the susceptibility of the tumor to NK cells. Low levels of HLA class I expression, together with expression of ligands for activating NK cell receptors, may favor NK cell-mediated tumor rejection and thereby improve the outcome of NK cell-based immunotherapy. However, a deeper understanding of the molecular specificity of the NK cell-mediated tumor recognition requires *ex vivo* studies on freshly explanted human tumor cells.

Such studies may also be used to test whether tumor cells from a given patient are sensitive to selected NK cells. It also opens up the possibility to test tumor-specificity of the NK cells.

1.4.2.2.1.1 Source and isolation of patient-derived tumor cells

Isolation of fresh human tumor cells to be used as targets for NK cells in *ex vivo* screening of susceptibility is not always a straightforward process. Several obstacles may limit the possibilities of obtaining adequate tumor material for experimental studies. Human tumor targets of hematological origin can often be isolated directly from bone marrow aspirates or from peripheral blood. Fresh human tumor cells derived from solid malignancies are normally not as easily obtainable. Material from solid tumor can be derived from fine needle aspirations, tissue biopsies, or from surgically removed tumor samples. In some cases, solid malignancies give rise to effusions, including pleural and peritoneal effusions, which may be relatively more available sources of tumor cells.

Several additional obstacles are encountered when processing fresh tumor samples. Separation of tumor cells from solid tumor masses often requires enzyme digestion or manual cutting and filtration through strainers, and/or other tumor cell separation procedures, including magnetic bead separation. Processing of fresh tumor material, including enzymatic digestion of solid tumor tissue, carries the risk of introducing changes in the ligand expression and possibly other properties of the fresh tumor cells. Another problem that may be encountered is low viability of the collected material due to necrosis within the tumor tissue. Even though viable fresh tumor samples are obtained and successfully prepared in single cell suspensions, the material may still contain a mix of normal and tumor cells. Thus, difficulties in processing tumor material may limit the available methods for studying NK cell recognition of freshly isolated tumor cells.

1.4.2.2.1.2 Assessment of tumor susceptibility to NK cells

The use of apoptosis or lysis as a final end-point is not only the best measurement but also in many cases the only choice since most tumor cells are isolated in heterogeneous cell populations. Assessment of the indirect tumor killing by measuring NK cell degranulation may give false results due to interactions with normal cells when evaluated in the context of a heterogeneous cell population. Nevertheless, FACS-based methods that assess the induction of apoptosis in tumor cells following co-incubation with NK cells are both fast and have a high accuracy. Moreover, this method could be used to delineate the NK cell susceptibility of specific subsets of tumor cells as well as assess the reactivity versus normal cells (**Associated paper I** and **Paper II**). Hence, a fast assessment of the NK cell-mediated rejection of freshly explanted tumor cells along with a phenotyping of the NKR ligands could be used to predict the *in vivo* reactivity and clinical efficacy. As shown in Table 2, data from *in vitro* experiments on fresh tumor material support the notion that several tumor types are susceptible to NK cells. The molecular specificity of the NK cell-mediated tumor killing has been delineated in most of these studies (Table 3). Additional investigations have reported impaired tumor targeting of the autologous NK cells due to perturbation of the NKR repertoire (Table 3), which indicate a role for allogeneic NK cells or strategies that abrogate the receptor down-regulation in the tumor microenvironment. Furthermore, a pre-assessment of the NK cell reactivity could also be used to test a panel of different donors and also investigate what NK cell subset that is most reactive and tumor specific along with the role for combinatorial treatments.

1.4.2.3 The role for preconditioning

Preconditioning of mice with lymphodepleting chemotherapy prior to adoptive transfer was shown to significantly improve the outcome in studies of cell-based immunotherapy conducted already 20 years ago (415). More recent studies have verified a critical role for pre-conditioning of patients in clinical trials with cell-based immunotherapy and strengthen the notion that pre-conditioning is one of the major factors for a successful outcome (346, 391, 416, 417). The rational for preconditioning in cell-based immunotherapy is based on studies that demonstrate improved survival of the transferred immune cells due to better immunological space with increased availability of cytokines and growth factors as well as a distinct reduction or total elimination of immune suppressive T_{regs} (416, 418). In addition, the direct cytotoxic effect of the preconditioning may also reduce the tumor burden. The pre-conditioning regimens used today mainly consist of chemotherapy (e.g. the purine analog fludarabine (Flu) in combination with the alkylating agent cyclophosphamide (Cy) and/or total body irradiation (TBI). Gathered data from the studies performed to date, propose that better outcome after cellular immunotherapy is seen in patients undergoing more intensive pre-conditioning resulting in high-grade lymphodepletion (419). As exemplified in a recent study, low intensity Cy/Flu pre-conditioning only induced a transient *in vivo* persistence of donor cells, whereas more intense regimens resulted in high endogenous IL-15 levels, *in vivo* expansion of donor NK cells, and induction of complete remission in some patients with AML (346). However, chemotherapy and TBI can both induce severe adverse effects and could in high dose-regimens cause prolonged neutropenia. Several ongoing studies aim to refine the pre-conditioning procedure to find the most optimal doses and combination of drugs inducing high-grade lymphodepletion with few side effects promoting the best possible outcome of cell-based immunotherapy.

1.4.2.4 Source and preparation of NK cells

Several aspects of the graft cells should be taken into consideration when conducting NK cell-based immunotherapy. It is not only important to decide the setting of the therapy (autologous versus allogeneic), it is also important to consider the source (bone marrow, cord blood or peripheral blood) and preparation (naïve versus cytokine-activated NK cells, KIR ligand matched versus mismatched NK cells and bulk NK cells versus specific NK cell subsets) of NK cells when conducting NK cell-based immunotherapies.

Anti-tumor responses have been observed in some patients undergoing autologous NK cell therapy, however, new protocols for *ex vivo* expansion of autologous NK cells prior to adoptive transfer are currently being evaluated and may improve the results of autologous NK cells in clinical therapy (420). As discussed previously, increasing number of studies using healthy donor-derived allogeneic NK cells have showed promising clinical responses. Cellular therapy with allogeneic NK cells also has the advantage of using KIR ligand mismatched alloreactive NK cells (72, 404-406). The clonal distribution of KIRs and the diversity of NK cell subsets it creates is potentially beneficial in settings of SCT and adoptive NK cell-based immunotherapy against cancer (421). However, genetic prediction of alloreactivity may need to be complemented with information about the actual size of alloreactive subset in a given donor, since the size has been shown to vary greatly (1-50%) among donors (**Associated paper C** and ref (99)).

More persistent anti-tumor cytotoxicity through prolonged NK cell survival *in vivo* may be one factor that improves the outcome of cellular therapy. Hence, the NK cells that are injected should ideally have good replicative potential. Cord blood represents a source of NK cells that may lead to enhanced expansion and good survival of NK cells *in vivo* (422). Tumor transformed NK cell lines have a high replicative potential and represents another source of NK

cells. The NK-92 cell line is the only NK cell line that has entered clinical trials (423). Most of the studies on the NK-92 cell line have been phase I clinical trials assessing the tolerance to the treatment in small number of patients. Although data from patients with renal cell carcinoma and melanoma indicate a clinical effect in some patients receiving adoptive transfer of NK-92 cells, further studies are needed for more solid conclusions. Due to their replicative potential, NK-92 cells could theoretically be easily genetically modified to express NK cell receptors or cytokines. For instance, genetically modified NK-92 cells, expressing chimeric receptors specific for Her2/neu, were recently shown to display strong and specificity lysis of primary Her2/neu-expressing tumor cells that were resistant to lysis by parental NK-92 cells *in vitro* (424). Another advantage of using a cell line is the limitless source and number of cells that could be obtain.

Taken together, the source and preparation of NK cells for NK cell therapy are likely to play central roles for increasing the efficacy of immunotherapy and merits further attention.

1.4.2.5 Combinatorial treatments

The tumor recognition by NK cells as well as the increased tumor susceptibility to NK cells can be manipulated in several aspects. NK cell-mediated recognition could be manipulated both on the effector and the target side. Enhanced NK cell effector function, tumor homing potential and improved *in vivo* survival could be manipulated to increase the efficacy of NK cell therapies. This could be achieved by selecting subsets of NK cells or treat them with cytokines prior and adoptive transfer or *in vivo*. Increased expression of activating NKRs as well as down-regulation or blockade of inhibitory NKRs may lead to increased tumor cell recognition. Manipulation of the NKR ligand repertoire represents another attractive possibility. Combining several strategies that shift the balance to activation of NK cells may enhance the efficacy of NK cell-based immunotherapy of cancer.

1.4.2.5.1 Enhancement of NK cell activity

Cytokines can be used to enhance NK cell activity and to support their proliferation and homeostatis (362, 420). Improved outcome of AML patients treated with histamine dihydrochloride and low dose IL-2 post-transplantation is considered to boost the function of NK cells (425). Another interesting strategy may be to transduce NK cells with genes coding for IL-2 or IL-15, which may elevate the levels of these important cytokines locally within the tumor microenvironment leading to increased NK cell activity, proliferation and improved homeostasis. Antibodies blocking inhibitory interactions (426) or over-expression of genetically modified activating NK cell receptors and CARs (424) may also increase target recognition and improve NK cell activity. These and other potential strategies to enhance anti-tumor responses by NK cells have recently been reviewed (421). Importantly, combinations of several of these strategies may be considered to further improve the outcome of NK cell-based therapies.

1.4.2.5.2 Sensitization of cancer cells

Strategies to render tumor cells more susceptible to NK cells may significantly improve the outcome of clinical NK cell therapies. Examples of such strategies are irradiation, all-trans-retinoic acid, arsenic trioxide, heat-shock inhibitors and HDAC inhibitors that all induce expression of stress-inducible ligands, such as MICs and CD155 (288, 291, 358, 427-431) and the proteasome inhibitor bortezomib and HDAC inhibitors that up-regulate death receptors such as the TRAIL receptor (432-436). Oxidative stress induced by drugs or by cells in the tumor microenvironment, may also alter the ligands expressed by tumor cells and render them more susceptible to NK cells. Administration of antibodies, such as the anti-Her2/neu mAb trastuzumab against breast cancer and the anti-CD20 mAb Rituximab against lymphoma, may

argument NK cell-mediated recognition through ADCC. Hence, there are several distinct strategies to further render tumor cells more susceptible to NK cells. New drugs and further insights into the regulation of the expression of NKR ligands may also add new possibilities in the future.

2 AIMS OF THE THESIS

The general aim of this thesis was to gain further insights in the molecular specificity of NK cell-mediated recognition of human tumor cells. More specifically, this thesis aimed to:

1. Gain further insights into the expression and mechanisms that affect the HLA class I expression on tumor cells *in vivo*

2. Evaluate the capacity of NK cells to kill autologous and allogeneic freshly isolated tumor cells

3. Delineate the NK cell receptor-ligand interactions involved in the NK cell-mediated recognition of freshly isolated human tumor cells

4. Explore the mechanisms by which tumor cells evade recognition by NK cells

5. Develop new strategies to render tumor cells susceptible to NK cells, with a special focus on inhibitory interactions mediated via non-classical MHC molecules

3 METHODS

The following methods have been used and are briefly summarized in this section. More details are found in the papers.

3.1 CELLS AND FLOW CYTOMETRY

Ficoll-Hypaque was used to eliminate red blood cells from blood samples, bone marrow samples and buffy coat speciments. Two magnetic bead-based negative separation techniques were used. The NK cell isolation kit (MACS) was used to isolate NK cells. The CD45 depletion kit (EasySep) was used to isolate freshly isolated ovarian carcinoma cells from peritoneal effusions. Cell lines, freshly isolated PBMCs, NK cells and carcinoma cells were all maintained in complete medium.

All flow cytometry samples were stained with mAbs on ice. Permeabilization buffer and PermWash/Fix (both BD) were used for intracellular stainings. The samples were finally washed twice and fixed in CellFix (BD) prior to acquisition on a CyAn™ ADP LX 9 color flow cytometer (DAKO Cytomation). The data was subsequently analyzed with FlowJo software (Treestar, Ashland, OR, USA). Graphs and statistical analyses were performed with GraphPad PRISM (GraphPad Software Inc., San Diego, CA, USA).

3.2 DETECTION OF NK CELL CYTOTOXICITY AND T CELL REACTIVITY

NK cell activity and cytotoxicity to targets was measured by different techniques. A non-radioactive flow cytometry-based method was used to detect NK cell-induced apoptosis as measuring by naked DNA (7-aminoactinomycin D; 7-AAD) or by caspase (Caspase-6 substrate) or granzyme B (Granzyme B substrate) activity inside the target cell. 7-AAD was added prior to acquisition, whereas the caspase or granzyme substrates were added the last 30 minutes of the assay. Importantly, flow cytometry-based assays allow assessment of NK cell specificity by the possibility to discriminating tumor cells from normal cells as discussed in **Associated paper A**. The NK cell activity was acquired by assessing NK cell degranulation (CD107a expression) and production of cytokines (TNF-α and IFN-γ) upon co-incubation with the tumor cells. The techniques for detection of NK cell cytotoxicity are summarized in **Associated paper A**.

Enzyme-linked immunospot assays (ELISpots) were performed in **paper I** to explore the CTL-mediated immune response in patients. PBMCs were stimulated with peptides and cultured for 7 days in U-bottomed 96-well tissue culture plates (TechnoPlastic Products, Trasadingen, Switzerland). IL-2 was added on day 4 and irradiated (30 Gy) autologous PBMCs pulsed with the same peptides used initially were added on day 7 and incubation for another 24 hours. The numbers of IFN-γ-secreting cells were estimated by the use of an IFN-γ-specific enzyme-linked immunospot (ELISpot) kit (Mabtech, Nacka Strand, Sweden). The number of spots was enumerated by using an automated ELISpot reader system (Autoimmun Diagnostika, Strassberg, Germany).

3.3 RNA EXTRACTION, REAL-TIME PCR AND REVERSE TRANSCRIPTASE PCR

Total RNA used for measurement of HLA-E mRNA in **paper V** was extracted with the RNeasy Mini kit (Qiagen, Stockholm, Sweden) and converted to cDNA with the cDNA reverse

transcription kit from Applied Biosystems (Foster City, CA, USA). Amplification of cDNA was performed using the TaqMan Gene Expression Master Mix and a 7500 Fast Real-Time PCR System both from Applied Biosystems. The primers and probes for HLA-E (Hs00428366_m1) and 18S rRNA (4310893E) were purchased as Pre-Developed TaqMan Gene Expression Assays (Applied Biosystems). 18S rRNA served as endogenous control to normalize the amount of sample cDNA. Relative amounts of HLA-E were calculated using the comparative threshold cycle (CT) method (437).

The mRNA expression levels of the APM components and HLA-A2 were evaluated by using set of specific primer pairs: TAP1, TAP2, β2-microglobulin and HLA-A2 as described in **paper I**. Annealing temperatures ranged from 57°C to 60°C and between 26 and 30 amplification cycles were used. The PCR products were separated by electrophoresis in 1.5% agarose gels together with molecular weight markers and visualized by ethidium bromide.

3.4 DNA EXTRACTION AND GENOTYPING OF KIRS AND HLAS

DNA for KIR and KIR ligand typing in **paper II** and **paper V** was isolated from peripheral blood cells by using the DNeasy® Blood & Tissue Kit (Qiagen). *KIR* genotyping was performed using PCR-SSP technology and *KIR* typing kit (Olerup-SSP, Stockholm, Sweden). KIR ligands were determined using the *KIR HLA* ligand kit (Olerup-SSP) for detecting the -*Bw4*, -*Cw3* and -*Cw4* motifs. For analysis of *HLA-A3/A11*, complementary *HLA* genotyping was performed with the *HLA-A* low-resolution kit (Olerup-SSP).

DNA for HLA typing was extracted from microdissected tumor tissue samples from three slides of paraffin-embedded primary tumor material as described in **paper I**. PCR-SSP kits (GenoVision, Vienna, Austria) were used in accordance with the manufacturer's instructions for the HLA-A*, HLA-B*, and HLA-DRB* low-resolution genotyping and for HLA-A*02 high-resolution genotyping. The PCR products were separated by electrophoresis in 2% agarose gel and visualized by ethidium bromide and documented with a photo printer.

3.5 DETECTION OF PROTEIN EXPRESSION AND OXIDATIVE STRESS IN WHOLE CELL LYSATES

Cells used for HLA-E protein quantification in **paper IV** was resuspended and lysed in SDS-sample buffer supplemented with dithiothreitol by repetitive heating and freezing to degrade DNA. The isolated protein fraction was separated on a NOVEX SDS-PAGE gel (Invitrogen) and transferred to a nitro-cellulose membrane. The membrane was blocked with BSA followed by over-night incubation with the mouse anti-human HLA-E mAb. After washing, the membrane was incubated with Horse Radish Peroxidase conjugated goat anti-mouse antisera (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The blot was developed with Super signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and the chemiluminecent signal was acquired with LAS 4000 (Fuji Film Life Science). The signal density was finally analyzed with image J (http://rsbweb.nih.gov/ij/).

Cells used to measure oxidative stress in **paper V** were sonicated in a Tris-HCl/EDTA buffer one ice followed by centrifugation. The protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, CA, USA) and a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA, USA) (438). The concentration of free thiols was measured through the addition of DTNB and guanidine–HCl in Tris–HCl, to a cell homogenate in a quartz cuvette. The absorbance at 412 nm was measured and the concentration was calculated using ε412 = 13.6/mM.

4 RESULTS AND DISCUSSION

The interplay between the immune system and cancer is a dynamic equilibrium. For instance, molecules expressed on the tumor cell surface are continuously sculptured by the immune system. On the contrary, tumor cells may also induce impairment of the immune system. Both these events may cause immune evasion. I will discuss the results from the papers included in this thesis in three separate sections. The first section focuses on the molecular specificity of NK cell-mediated killing of freshly isolated human tumor cells. The second section focuses on perturbations of NKR expression in the tumor microenvironment and the implications for tumor immune evasion. Finally, I will discuss how combinatorial treatments may be used to improve NK cell-mediated tumor cell killing. The NKRs, their role in the recognition of human tumor cells and mechanisms for loss of receptor expression are summarized in Table 3.

4.1 MOLECULAR SPECIFICITY OF NK CELL RECOGNITION OF TUMORS

4.1.1 Down-regulation of HLA class I on tumor cells due to immunological pressure

Low levels of HLA class I has been associated with aggressive tumor growth and poor prognosis for several cancers (439). Several well-characterized mechanisms for the loss of HLA class I on tumor cells have been described (440), where mutations affecting components of the antigen processing machinery (APM) represent one frequently observed mechanism (37, 38). In **paper I**, we analyzed the cell surface expression of HLA class I and the integrity of APM components in freshly isolated tumor cells from patients with ovarian carcinoma. The level of HLA class I was reduced on the ovarian carcinoma cells from all patients compared to autologous lymphocytes. In this study we focused our analysis on the expression of HLA-A2 in particular, since individuals with HLA-A2 are overrepresented among ovarian carcinoma patients and HLA-A2 has been linked to poor prognosis in this cancer type (441). A heterogeneous cell surface expression was observed in six out of nine HLA-$A2^+$ patients, where decreased or totally absent HLA-A2 expression was observed on a subpopulation of the tumor cells. Genetic analysis revealed a total deletion of the HLA class I allele carrying the HLA-A2 gene in one of these patients. No mRNA for HLA-A2 was be detected and INF-γ did not up-regulate HLA-A2 on the cell surface of the ovarian carcinoma cells (**paper I**). Importantly, no defects in the expression of the APM components, TAP1, TAP2, and β_2-microglobulin were detected in this patient. Although loss of heterozygosity (LOH) of HLA class I has been described previously in other cancers (442-446), **paper I** represents the first publication reporting this phenomenon in ovarian carcinoma.

Interestingly, an HLA-A2-restricted HER-2/neu specific T cell response was observed in PBMCs of the patient with the LOH. Deletions of specific HLA class I genes or alleles have previously been associated with anti-tumor T cell responses in other cancers such as malignant melanoma, lung cancer and renal cell carcinoma (447-449). Moreover, adoptive immunotherapy with TAA specific tumor infiltrating CTLs and other T cell-based immunotherapies has been shown to induce loss of HLA class I in malignant melanoma (449-451). Hence, immunological pressure by tumor specific T cells may select for tumor variants with low HLA class I expression (440). However, while escaping from T cells, the abnormal HLA class I expression may render tumor cells susceptible to NK cell cytotoxicity due to lack of inhibition via HLA class I (24). For instance, it is speculated that uveal melanoma, that form metastasis with high levels of HLA class I but display low levels in the primary tumor, are cleared by circulating NK cells while spreading through the hematological system (452). The role

for autologous NK cells was not tested in the patient with the ovarian carcinoma cells that displayed LOH.

4.1.2 Activating NK cell receptor ligands mediate tumor cell killing by NK cells

Low levels of HLA class I (**paper I** and **paper II** and ref (453)) have been associated with poor prognosis in ovarian carcinoma patients (453-455). Ovarian carcinoma is often diagnosed at a late stage with metastatic disease (approximately 67% of the cases) with a poor 5-year overall survival (NCI's SEER Cancer Statistics Review, USA). Based on the low HLA class I expression, we though that NK cells could recognize ovarian carcinoma cells. Indeed, as shown in **paper II**, ovarian carcinoma cells were susceptible to NK cells and the killing correlated with the levels of HLA class I expressed on the tumor cells. A novel FACS-based method, allowing detection of granzyme B and caspase-6 activity inside the target cell (see the Methods section and **Associated paper A**), was used to conduct these studies and was paralleled with NK cell degranulation assays. Although the expression of NKR ligands could not fully predict the NK cell susceptibility, the ubiquitous expression of CD155 and the sparse/heterogeneous expression of MICA/B and ULBP1-3 seemed critical for NK cell recognition. Indeed, masking of activating receptors on the NK cells revealed a prominent role for DNAM-1 with minor contribution from the NKG2D receptor. As expected, the NKG2D receptor had a more central role in the rare cases of tumor cells expressing higher levels of the NKG2D ligands. The expression of the NCR ligands were not assessed on the ovarian carcinoma cells since they are not yet fully defined. However, masking of the NCRs revealed a minor role for these receptors. Importantly, the role for KIR ligand mismatching was not addressed in **paper II**. However, continued studies in our laboratory showed no beneficial role for KIR ligand mismatching, regardless of the level of HLA class I (Figure 3). One may speculate that the levels of HLA class I are too low since the data in Figure 3 indicate that there is no impact of HLA class I expression even in freshly isolated ovarian carcinomas (OC37) expressing relatively high levels of HLA class I. Another possible explanation for the lack of effects of the KIR LIGAND mismatching may be that the size of the alloreactive NK cell subset was too small to have an impact on the killing in these experiments. A matched donor with 0% alloreactive NK cells is compared to a mismatched with on average 5-10% alloreactive NK cells (**Associated paper C**).

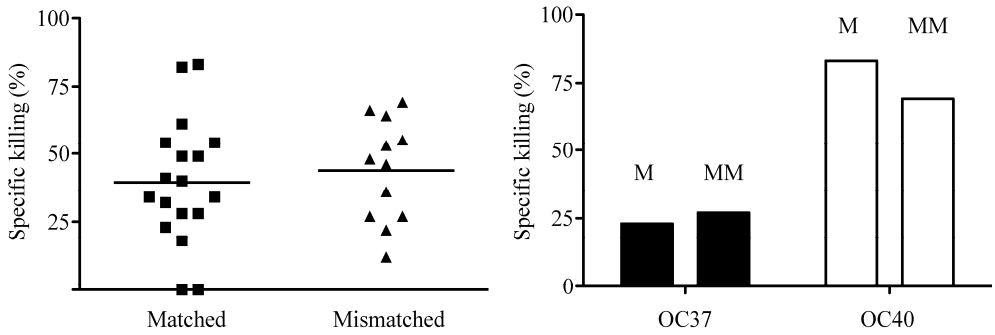

Figure 3. Killing of ovarian carcinoma (OC) cells by KIR LIGAND matched and mismatched allogeneic NK cells. *A*, Specific killing of OC cells, n=18 matched and n=12 mismatched NK cells. *B*, two representative examples of OC expressing high and low levels of HLA class I, respectively.

In **paper II** we also show a degree of tumor specificity in the NK cell-mediated recognition of ovarian carcinoma cells since fibroblasts derived from the same patient were resistant to killing. This was likely due to the fact that the fibroblasts expressed higher levels of HLA class I while having lower levels of activating NKR ligands (**paper II**). These results illustrate the advantage of using FACS-based killing methods, where apoptosis can be assessed at a single cell level and discriminate NK cell sensitive targets from resistant ones.

An increased amount of studies on the molecular specificity of NK cell-mediated recognition of freshly isolated human tumor cells have emerged during the last decade (352-365) (summarized in Table 2, Table 3 and **Associated paper A**). Direct evidence for NK cell-mediated lysis of fresh tumor cells isolated from patients with ALL (357), AML (358), MM (359-362), NB (363), Ewing sarcoma (456), gastric cancer (365), colon cancer (365), renal cell cancer (365), malignant melanoma (457) and ovarian carcinoma (**paper II** and ref (365)) have added information about the molecular specificity of human NK cells. These studies have demonstrated that NKG2D, DNAM-1 and the NCRs are involved in the recognition of fresh human tumor cells and that some receptors are more central for certain tumor types, whereas several of them cooperate in the recognition of other tumor types. Moreover, these studies do not only put forward the possibilities of using NK cells against hematological cancers, but also provide a basis for the design of new cell-based immunotherapies against solid tumors.

4.2 MECHANISMS OF IMMUNE EVASION FROM NK CELLS

4.2.1 NK cell receptor alterations in the tumor microenvironment of OC and MDS

Cancer patients often exhibit hypofunctional immune responses (319, 320) as exemplified by the poor responses to recall antigens in DTH reactions (317). However, more recent studies have dissected the function of the immune cells associated to the tumor cells within the tumor microenvironment. As previously discussed, tumor cells can evade from the immune system by altering their expression of classical and non-classical HLA class I molecules or by changing their expression of ligands for activating immune receptors. However, immune evasion can also occur due to changes of the molecular specificity of anti-tumor immune cells. Multiple factors have been shown to alter the function and specificity of NK cells in the tumor microenvironment (Table 3). In **paper III** and **paper IV**, we assessed the function of NK cells in peripheral blood and in the tumor microenvironment of patients with ovarian carcinoma and MDS.

In ovarian carcinoma patients (**paper III**), we found that DNAM-1 was severely down-regulated on tumor-associated NK cells compared to NK cells in autologous peripheral blood and from healthy donors. We also demonstrated a loss of the co-stimulating receptor 2B4 and the CD16 receptor. Since data from **paper II** demonstrated that DNAM-1 was critical for the recognition of ovarian carcinoma cells, we speculated that a loss of DNAM-1 on the tumor-associated NK cells might be linked to impaired NK cell function and poor disease control. Indeed, the function of the tumor-associated NK cells was impaired and they displayed poor recognition of autologous ovarian carcinoma cells (**paper III**). DNAM-1 has also been shown to be important for NK cell-mediated recognition of several other human tumors, including neuroblastoma, multiple myeloma, melanoma and Ewing sarcoma (360, 362, 456, 457), but loss of DNAM-1 in the tumor microenvironment has so far only been observed or studied in ovarian carcinoma and recently also in melanoma (Table 3).

In MDS, previous papers have reported reduced function of peripheral blood NK cells (458-461), but no study has assessed the functional integrity of NK cells associated to the malignant blasts in the bone marrow. In **paper IV** we assessed the function and receptor expression of bone marrow-derived NK cells of MDS patients. In this study we used peripheral

blood and bone marrow cells from a unique cohort of aged-matched healthy controls. Relative to the healthy controls, we observed a severe hypofunctionality of NK cells in MDS patients that was linked to a reduced expression of the activating NK cell receptors NKG2D and DNAM-1 whereas expression of the NCRs including NKp30 remained intact. The reduced receptor expression was more severe in the bone marrow compartment than in peripheral blood and most severe in patients with high content of bone marrow blasts. Further studies revealed a hyporesponsiveness of NK cells against K562 cells, which was more pronounced in the bone marrow-derived NK cells. In contrast, when assessing the functional consequences of the receptor loss by reverse ADCC (rADCC), where receptor specific agonistic monoclonal antibodies can be combined to co-stimulate selected NKRs, we observed an equally poor degranulation capacity in the two compartments. Hence, although NKG2D and DNAM-1 were expressed at normal levels on peripheral blood-derived NK cells, the data from the rADCC experiments suggested that down-stream signaling may also be defective in MDS patients.

Importantly, and in sharp contrast to ovarian carcinoma, MDS represents a disease of the hematopoietic system caused by genetic aberrations that may directly affect cells of the immune system. Defects in NK cell proliferation and cytotoxicity in MDS have previously been described to be independent from the expression of activating NK cell receptors (460). The poor function of NK cells in the periphery of MDS patients, despite relatively intact expression of activating NK cell receptors, observed by Kiladjian et al., was explained by the fact that a proportion of the NK cells carried the same genetic aberration as the $CD34^+$ blast cells (460). Other groups have also observed a clonal involvement of NK cells in MDS patients (462). In contrast to these results, Epling-Burnette et al. report that NK cell dysfunction correlated with a reduced expression of NK cell receptors and was primarily observed in patients with high-risk MDS (458). In congruence with our study, Epling-Burnette et al. observed a reduced expression of NKG2D. However, deviating somewhat from our study, they did not observe any alteration of the DNAM-1 expression, but instead reduced expression of NKp30 on the peripheral blood NK cells of MDS patients. Concordantly with **paper IV**, they also report that the reduced receptor expression occurred primarily in patients with high blasts counts. Hence, these findings reinforce the reported association between NK cell dysfunction and higher International Prognostic Scoring System (IPSS) scores as well as the presence of excess blasts (458). Based on the data in **paper IV** and the results from other studies, one may speculate that NK cells or subsets of NK cells in MDS may be hypofunctional due to genetic aberrations but exhibit a more prominent hypofunctionality in the bone marrow due to additional receptor alterations induced by factors in the tumor microenvironment or by interactions with tumor cells. Together with the fact that reduced NK cell receptor expression have been shown to correlate with poor survival in AML (463), these studies also indicate that reduced NK cell receptor expression may lead to impaired immune surveillance of MDS blasts in the bone marrow which may facilitate disease progression and increase the risk of transformation to AML.

Taken together, NK cells in the tumor microenvironment of patients with ovarian carcinoma and MDS are hyporesponsive compared to NK cells in the peripheral blood. DNAM-1 plays a critical role for NK cell-mediated recognition of ovarian carcinoma cells. Changes of DNAM-1 expression as well as the NKG2D expression may influence the disease progression in MDS. Although genetic aberrations cannot be excluded as an underlying mechanism for the reduced NK cell function in MDS, factors in the tumor microenvironment are likely to be involved in the modification of the activating NKR repertoire that may lead to a more severe hypofunctionality of the tumor-associated bone marrow-residing NK cells. Additional studies are needed to further delineate the underlying mechanism for receptor alterations and NK cell hypofunctionality in the tumor microenvironment in cancers, including MDS and ovarian carcinoma.

4.2.2 Mechanisms of NK cell receptor alterations in the tumor microenvironment

Several mechanisms, such as receptor-ligand interactions and soluble factors, have been shown to be involved in the perturbation of the NKR repertoire and will be discussed in this section. The receptors, their involvement in the recognition of fresh human tumor cells and mechanisms for altered receptor expression on NK cells in cancer patients are listed in Table 3. Moreover, Figure 4 summaries some mechanisms for loss of NKR in the tumor microenvironment.

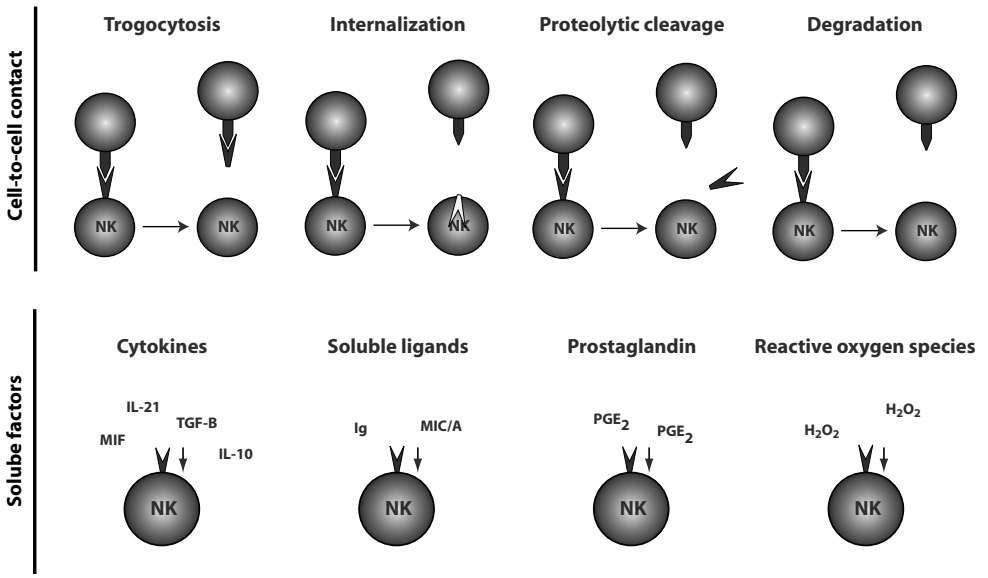

Figure 4. Overview of possible mechanisms for reduced NK cell receptor expression in the tumor microenvironment.

4.2.2.1 The role for receptor-ligand interactions

It has previously been described that the CD96 receptor is down-regulated upon engagement with its cognate ligand CD155 (155). Since ovarian carcinoma cells constitutively express CD155 (**paper II**) we speculated that similar mechanisms could be responsible for the down-modulation of DNAM-1 on tumor-associated NK cells (**paper III**). Indeed, we were able to demonstrate that NK cells lost DNAM-1 expression within hours of exposure to CD155 expressing targets. Down-modulation of DNAM-1 was dependent on physical contact with target cells expressing CD155 since no change in DNAM-1 expression was observed when transwell membranes separated effectors and targets. Interestingly, we found an inverse correlation between the expression of CD155 on ovarian carcinoma cells and the expression of DNAM-1 on autologous tumor-associated NK cells, supporting the notion that the increased levels of CD155 led to reduced DNAM-1 expression. The role for receptor-ligand interactions as mediator of loss of DNAM-1 has later been verified in melanoma (464).

Paper IV is the first report on reduced expression of DNAM-1 on NK cells in MDS and there are no available data published today on the mechanisms for receptor loss in this

disease. However, it is possible that chronic ligand exposure also mediated the reduced DNAM-1 expression observed in MDS, since the MDS blasts also express CD155 (Baumann et al. unpublished data). The expression of the NKG2D receptor was also shown to be low on the NK cells in MDS (**paper IV**). It is well documented that the NKG2D receptor can be lost due to trogocytosis or following chronic ligand exposure or by interactions with NKG2D ligand-expressing exosomes (153, 154, 157). As seen in Table 3, the loss of NKG2D expression on NK cells in cancer patients is a wide spread phenomenon and has been observed in AML, multiple myeloma, squamous cell cancer and cervical cancer. Although not addressed in **paper IV**, an involvement of receptor-ligand interactions cannot be excluded as a mediator of reduced NKG2D expression since the MDS blasts also express NKG2D ligands (Baumann et al. unpublished data).

4.2.2.2 The role for soluble factors in the tumor microenvironment

Soluble factors such as cytokines and shedded ligands can also induce down-regulation of NK cell receptors. The involvement of such factors in the down-regulation of DNAM-1 remains poorly studied in the literature. Nevertheless, the influence of soluble factors on the DNAM-1 expression in ovarian carcinoma was excluded in **paper III**, since the receptor expression was unaltered when NK cells were exposed to tumor cells but separated by transwells or to peritoneal effusions. In contrast, the impact of the various isoforms of CD155 that are known to be shed from cells expressing membrane-bound CD155 (465) or soluble molecules such as MUC16 (466) were not assessed and may play a role in regulation of DNAM-1 expression in MDS. The mechanism behind the observed down-modulation of the NKG2D receptor on NK cells in MDS patients was not addressed either. As shown in Table 3, there are several soluble mediators, including shedded MIC/A, that have been described to down-regulate the NKG2D receptor on both NK cells and T cells (144, 147-151, 153, 154, 302, 467-470). This mechanism has been observed in several cancers including malignant melanoma, colon cancer, gastrointestinal cancer and cervical cancer as well as in aggressive end-stage cancer of the breast, lung and ovarian cancer (467, 468, 470). It should be noted that shedding of MIC-A has been mostly demonstrated, whereas shedded MIC-B has not been shown to induce down-regulation of NKG2D (471). Hence, detection of soluble NKG2D ligands is not synonymous with reduced expression of NKG2D.

Down-regulation of the NKG2D receptor can also be mediated by cytokines (472). In one study, macrophage migration inhibiting factor (MIF) was shown to mediate loss of NKG2D expression on NK cells and was assumed to cause the reduced NKG2D expression on NK cells in the tumor microenvironment of ovarian carcinoma observed in that study (144). The role for MIF or IL-21, also known to mediate down-regulation of NKG2D (144, 147, 151), has not yet been addressed in MDS. Tumor growth factor-β (TGF-β) can also mediate down-regulation of NKG2D on NK cells (148, 150, 473). For instance, increasing levels of TGF-β inversely correlated with surface expression of NKG2D on NK cells in patients with lung cancer and colorectal cancer (14). As for patients with lung cancer and colorectal cancer, most patients with MDS also display elevated levels of TGF-β (14, 474). One study demonstrated that patients with excess bone marrow blasts had higher levels of TGF-β than patients with low blast count (475). Interestingly, another study reported significantly reduced levels of TGF-β in the bone marrow of MDS patients following treatment with thalidomide (476). Based on these observations, it is tempting to speculate that the high levels of TGF-β observed in the bone marrow of MDS patients could be involved in the reduction of NKG2D expression on NK cells and that this may be mediated by thalidomide or similar analogs. In addition, gene expression profiles of bone marrow precursors in MDS have provided evidence for overactivation of the TGF-β signaling

pathway due to mutations of a downstream mediator of TGF-β receptor I kinase (TBRI) activation called smad2 (477). Mutations of smad2 resulting in constitutive TGF-β signaling without increased cytokine expression (477), may also contribute to loss of NKG2D expression on NK cells that derive from the malignant MDS clone. Restoration or inhibition of this pathway may therefore also improve the receptor repertoire and functional integrity of NK cells in MDS. In fact, one study reported that blockade of the signaling of the TGF-β receptor via inhibition of the TBRI kinase promoted normal hematopoesis in MDS patients (477). However, data from this study did not show whether this was attributed to direct effects on the malignant cells or if restored NKG2D expression and NK cell function caused the normalization of the hematopoiesis by immune-mediated rejection of the malignant clone. Further studies are warranted to gain insights into the role of TGF-β in the regulation of NKG2D and other NK cell receptors.

There may also be other factors in the tumor microenvironment that mediate alterations of the NK cell receptor repertoire. For instance, ROS have been shown to have an impact on NKp46 and NKG2D expression (302, 304). NKp46 and NKG2D receptors were down-regulated on the $CD56^{dim}$, but not the $CD56^{bright}$ NK cell subset, by ROS released from phagocytes (304). Administration of histamine, targeting H2 receptors on the phagocytes, inhibited the down-regulation of both receptors. Additional support for ROS-mediated down-regulation of the NKG2D receptor on the $CD56^{dim}$ NK cell subset comes from studies on patients with end-stage renal disease that undergo dialysis (302). The NKG2D expression on NK cells from healthy donors was decreased upon exposure to serum from the uremic patients and catalase reversed the expression. Moreover, the cell surface and mRNA expression of NKG2D were low on NK cells from these patients compared to healthy controls. Hence, these studies suggest that ROS have an impact on the NKR repertoire, which may impair NK cell function. PGE_2 is another immune suppressive factor found within the tumor microenvironment that was recently shown to mediate reduction of the NCRs (464). However, the roles of ROS and PGE_2 have not yet been addressed in either OC or MDS.

4.2.2.3 Factors regulating the expression and function of the CD16 receptor

CD16 is a unique receptor that alone, without the involvement of other activating NK cell receptors, can induce target killing by ADCC. Hence, altered cell surface expression of CD16 can dramatically change the capacity of NK cells to induce ADCC. However, the regulation of the CD16 expression and the down-stream components is not fully clear today. Data in **paper III** demonstrate that a severe loss of CD16 expression on tumor-associated NK cells led to impaired ADCC of trastuzumab-coated fresh ovarian carcinoma cells. In contrast, autologous peripheral blood NK cells, expressing normal levels of CD16, displayed proper activation against trastuzumab-coated targets. Although trastuzumab may have direct effects on some tumors (478), by restraining the continuous growth signals mediated by the tyrosine kinase pathways downstream of the Her2/Neu receptor, our data may explain the poor outcome of clinical therapy with trastuzumab against Her2/neu expressing ovarian carcinoma (479-481). The mechanism remains elusive. However, a previous study has shown that loss of the signal transducing molecules FcεRIγ and CD3ζ led to reduced cell surface expression of the CD16 receptor on tumor-associated lymphocytes in ovarian carcinoma (160). Since we observed a more severe loss of the CD16 receptor on tumor-associated NK cells compared to NK cells in autologous blood, we favor the interpretation that specific factors in the peritoneal compartment are involved in tuning of the CD16 expression and function. Chronic inflammation in general and in the tumor milieu in particular has been shown to mediate down-regulation of the signaling adaptor protein CD3ζ in both NK cells and T cells (341, 482). Thus, inflammatory cytokines may be potential mediators of the CD16 loss on tumor-associated NK cells in ovarian carcinoma patients. In fact,

we observed increased levels of inflammatory cytokines in peritoneal effusions from ovarian carcinoma patients compared to blood plasma (Figure 5). High levels of cytokines, including IFN-γ, IL-2 and IL-15, that are observed at sites of chronic inflammation (483), were associated with up-regulation of the activation marker CD69 on the tumor-associated NK cells (Carlsten et al. unpublished observation). The levels of TGF-β, known to be elevated at sites of chronic inflammation, were not measured in our material. TGF-β is a potent lymphocyte suppressor and mediates its effects via distinct molecular pathways. Although TGF-β does not reduce the expression of CD16 *per se* (14), it is known to inhibit the downstream signaling of the CD16 receptor as recently demonstrated by dampened production of IFN-γ and poor release of granzymes upon stimulation of CD16 on NK cells treated with TGF-β (484). Activation-induced internalization or protease cleavage that has been reported upon interaction between the CD16 receptor and the Fc-portion of mAbs may be additional mechanisms that contribute to reduced CD16 expression (159, 485). Finally, it is tempting to speculate that estrogen, produced by the ovaries and that was recently shown to reduce the expression of CD16 by inhibiting the transcription through signaling via the ER-α (486), may contribute to the reduced CD16 expression observed in ovarian carcinoma. As will be discussed later, the reduced CD16 expression on tumor-associated NK cells in ovarian carcinoma provides one possible explanation for the poor results from clinical trials with trastuzumab in ovarian carcinoma (479).

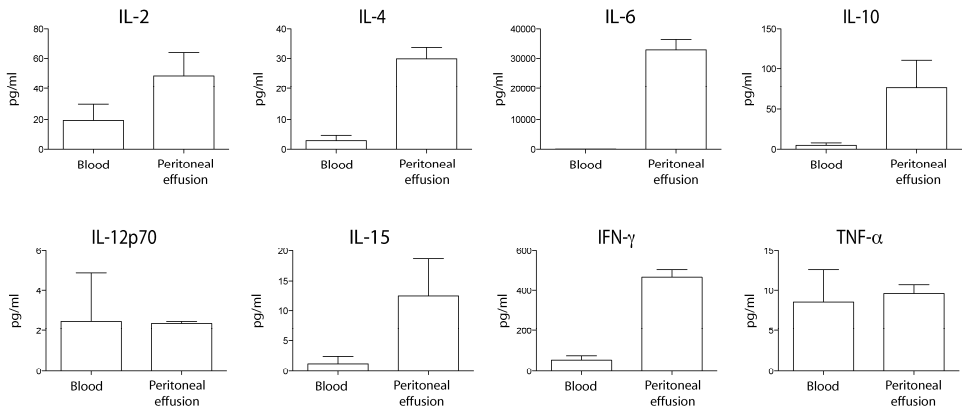

Figure 5. Increased expression of proinflammatory cytokines in peritoneal effusions from patients with ovarian carcinoma. The levels of the indicated cytokines was measured in blood plasma (Blood) and peritoneal effusions.

4.2.2.4 Studies on immune evasion by down-regulation of NK cell receptors

As shown in Table 3, there are now emerging data reporting down-regulation of activating NK cell receptors on NK cells in the tumor microenvironment. A broad repertoire of activating receptors, including the NKG2D, DNAM-1, NCRs, 2B4 and CD16 receptors, have shown perturbed expression in the tumor milieu. Some studies have also linked the loss of receptor expression to reduced function of the tumor-associated NK cells. The integrity of tumor-associated NK cells have been assessed in several distinct cancer types, including hematological (ALL, AML, MM, MDS), endodermal (Mel) and ectodermal (OC, SC, CC, HCC) cancers. Hence, receptor loss on NK cells in the tumor microenvironment is a widely spread phenomenon.

Several mediators of receptor alterations have been identified today, but the mechanisms are not fully understood and differ between the tumor types (Table 3). Receptor-ligand interactions, cytokines and reactive molecules such as ROS, NOS and PGE_2 are examples of factors that are involved in the regulation of NK cell receptor expression (Figure 4). Further studies are warranted on the mechanisms and consequences of altered NK cell receptor expression in the tumor microenvironment. Such studies will hopefully help us to better understand the interplay between the immune system and cancer and might thereby improve the current protocols of tumor immunotherapy.

Table 3. An overview of NK cell receptors that are involved in the recognition of fresh human tumor cells and factors that regulate their expression.

Receptor	Expression pattern	Signaling mechanism(s)	Ligand(s)	Tumor specificity	Tumors containing NK cells with NKR loss	Regulators of expression
2B4	All NK cells	SHP-2 and SAP (487)	CD48 (488, 489)	n.d.	OC (**paper III**, (466)) MM (490)	?
CD16	$CD56^{dim}$ NK cells	CD3ζ (167) FcεRIγ (168, 169)	IgG (491)	OC (trastuzumab) (492) L (rituximab) (493)	OC (**paper III**, (160)) MM (490)	(-) mAbs (159)
CD96	Act. NK cells	ITIM-like (494)	CD155 (155)	n.d.	n.d.	(-) Ligand (155)
DNAM-1	All NK cells	?	CD155 (132) CD112 (132)	OC (**paper II**) ALL (495) ES (456) MM (360, 361) NB (363) Mel (457)	OC (**paper III,** (466)) MDS (**paper IV**) Mel (464)	(-) Ligand (**paper III**, (464)) (-) TGF-β (150) (-) MUC16 (466) (+) hGIFT2 (496)
NKG2D	All NK cells	DAP10 (497)	MIC/A-B (124) ULBP1-4 (125)	AML (358) MDS (498) MM (362) ES (456)	AML (463) MDS (**paper IV**, (458)) OC (144, 466) MM (499) CC (468, 469) SC (469)	(-) TGF-β (147-150) (-) IL-21 (151) (-) ROS (302, 304) (-) MIF (144) (-) soluble MIC-A (467-469) (-) exosome (153, 154, 470) (+) IL-2 and IL-18 (145, 500) (+) TNF-α and IL-15 (501)
NKp30	All NK cells	CD3ζ (114)	BAT-3 (119) B7-H6 (120)	MDS (498) AML (463) Mel (457) NB (363) MM (361)	AML (463) MDS (458) CC (469) SC (469)	(-) Ligand (463) (-) TGF-β (148, 150) (-) PGE_2 (464)
NKp44	Act. NK cells	DAP12 (146)	Viral HA (122)	n.d.	OC (466)	(-) PGE_2 (464) (-) IL-21 (145) (+) IL-2 (117, 146)
NKp46	All NK cells	CD3ζ (115)	Viral HA (121)	ALL (502) AML (463) Mel (457) NB (363) MM (361)	AML (463) CC (469) SC (469) OC (466)	(-) Ligand (463) (-) ROS (303, 304) (+) IL-2 (460)

AML; acute myeloid leukemia, ALL, acute lymphatic leukemia, CC; cervical carcinoma, ES; Ewing sarcoma, HA; hemagglutinin, hGIFT2; GM-CSF/IL-2 fusion protein, L; Lymphoma, MDS; myelodysplastic syndrome, Mel; malignant melanoma, MM; multiple myeloma, NB; neuroblastoma, n.d.; not done, OC; ovarian carcinoma, SC; squamous cell carcinoma.

4.3 STRATEGIES TO IMPROVE NK CELL-MEDIATED KILLING OF TUMORS

In more recent years, new insights into the molecular specificity of NK cells have led to studies that exploit the role for NK cells in immunotherapy of human cancer. As discussed in the two previous sections, the NK cell-mediated killing of tumor cells is dictated by multiple receptor-ligand interaction that are continuously tuned by dynamic alterations of the receptor and ligand expression. As shown in **paper I** and **paper II**, metastatic ovarian carcinoma express low levels of HLA class I and are susceptible to allogeneic NK cells by interactions between DNAM-1 and CD155. However, DNAM-1/CD155 interactions may result in reduced DNAM-1 expression on

tumor-associated NK cells, which may lead to an increased risk for disease progression (**paper III**). Hence, tumor cells may evade NK cell-mediated recognition by modifying the NK cell receptor repertoire (**paper III** and **paper IV**) through physical contact or by release of immune suppressive soluble factors within the tumor microenvironment. It is important to understand the biology of the tumor microenvironment to improve the current protocols of NK cell-based immunotherapy. Future regimens may involve strategies that specifically abrogate negative regulators of NK cell activity (as exemplified in **paper V**) and factors regulating the receptor repertoire or that directly restore the expression of critical activating receptors. Such strategies will be discussed in this section.

4.3.1 Cytokine-mediated enhancement of NK cell functions and homeostasis

Enhanced activity and improved survival of NK cells *in vivo* can be obtained by administration of cytokines such as γ_c-chain cytokines and type I IFNs. For instance, systemic administration of IL-2 has been widely used in clinical trials to improve immune cell functions in the context of immunotherapy. Moreover, cocktails of cytokines have been shown to efficiently expand patient-derived NK cells *ex vivo* and potentiate their anti-tumor properties (362, 420). However, the dynamic expression of cytokines in the tumor microenvironment is a complex matter that can influence the anti-tumor response both positively and negatively.

4.3.1.1 Manipulation of the cytokine milieu to improve NK cell functions

Immunomodulatory drugs were recently shown to enhance NK cell function by inducing cytokine production *in vivo* (503). The thalidomide derivate, lenalidomide, represents one example of an immunomodulatory drug that promote survival and proliferation of both NK cells and T cells by increasing the expression of several cytokines in the tumor milieu of B cell lymphoma (504). For instance, lenalidomide stimulates DCs to increase their production of TNF-α (503) that in turn enhance NK cell-mediated ADCC. Hence, administration of cytokines *per se* or drugs that induce cytokine production in the tumor microenvironment can improve NK cell function. However, recent data have indicated that tumor cells can dampen the biological effects of cytokines in the tumor microenvironment by breaking intramolecular disulphide bridges (**Associated paper H**). This mechanism is mediated by enzymes of the protein disulphide isomerase (PDI) family that are expressed on the cell surface of some tumor types (505). PDIs may also modulate the redox state of the tumor cell-surface by altering the density of thiols, which in turn have been shown to protect the tumor cells from NK cell-mediated lysis by inhibiting conjugate formation between the effector and target (506). Hence, PDI-mediated dysregulation of disulphide-containing soluble mediators and perturbed properties of conjugate formation may both protect from anti-tumor immunity and promote tumor progression. Interestingly, the drug auranofin that is used to treat rheumatoid arthritis (507), was recently shown to abolish the PDI-mediated reduction of cytokines (**Associated paper H**) by specifically inhibiting the electron donor to PDI, thioredoxin reductase (TrxR) (508). In fact, auronofin can also inhibit PGE_2 production by macrophages (509) and thereby indirectly protect NK cells from PGE_2-mediated down-regulation of both the NCRs (Table 3) and the common γ_c cytokine receptor, which was recently shown to impair IL-15-mediated enhancement of NK cells (510). Hence, auronofin or similar drugs may be potential candidates used to improve the survival and function of NK cells by protecting cytokines from disulphide cleavage and NKR from down-regulation in tumor microenvironments of cancer patients.

Tumor cells can directly release factors that negatively influence the immune system. As previously discussed, TGF-β is one such factor that is released by tumor cells and impairs NK

cell function by down-regulating the NKG2D receptor (147-150). Several strategies to neutralize the suppressive actions of TGF-β have been suggested. Administration of anti-TGF-β antibodies to human breast cancer-bearing mice resulted in preserved NKG2D expression on T cells and prevented metastasis formation (511). TGF-β blocking antibodies have also been shown to potentiate the anti-tumor effects of tumor vaccination by reducing the formation of a suppressive tumor microenvironment (512). Moreover, IL-12 was shown to protect from TGF-β-induced down-regulation of NKG2D on T cells (513). Tamoxifen, an estrogen receptor antagonist used in breast cancer therapy, have been shown to reduce the release of TGF-β from breast cancer cells and thereby protect NK cells from down-regulation of NKG2D (514). Hence, IL-12, tamoxifen and monoclonal antibodies blocking TGF-β represents different approaches that can prevent TGF-β-mediated down-regulation of activating NK cell receptors and improve NK cell-mediated rejection of tumor cells. Taken together, several approaches could be used to modulate the immune suppressive tumor microenvironment.

4.3.1.2 Improved survival of NK cells in the tumor microenvironment

Cytokines can improve the proliferation and homeostasis of NK cells and thereby prolong NK cell-mediated anti-tumor responses (515). This was recently exemplified in a clinical trial where increased levels of endogenous IL-15 were associated with improved *in vivo* expansion of NK cells adoptive NK cell therapy against AML (346). IL-15 is known to support proliferation and homeostasis of NK cells by trans-presentation via the cell membrane-bound IL-15 receptors on cells surrounding the tumor cells (231, 516, 517). Other cytokines that improve NK cell homeostasis and survival are IL-2 and IL-7 (518).

The tumor microenvironment contains many other non-cytokine substances such as ROS that are cytotoxic to most immune cells. The cytotoxic $CD56^{dim}$ NK cell subset is particularly sensitive to oxidative stress-induced apoptosis (303, 519-522), whereas the $CD56^{bright}$ NK cell subset and regulatory T cells are less sensitive (281). The non-cytotoxic cytokine producing $CD56^{bright}$ NK cell subset is often over-represented at sites of chronic inflammation (281) such as in the tumor microenvironment (**Paper III** and **IV**). Although the exact mechanisms for an altered ratio between the two subsets at these sites are not fully clear today, one may speculate that a combination of selective recruitment of inflammation seeking $CD56^{bright}$ NK cells and apoptosis of $CD56^{dim}$ NK cells due to oxidative stress may contribute. Increased proportion of $CD56^{bright}$ NK cells and decreased number of $CD56^{dim}$ NK cells may directly lead to reduced NK cell-mediated cytotoxicity. Moreover, IFN-γ, that is produced by $CD56^{bright}$ NK cells and commonly secreted at sites of chronic inflammation, might indirectly impair NK cell-mediated tumor rejection since it can up-regulate classical as well as non-classical HLA class I molecules on tumor cells. One such example is the up-regulation of including HLA-E that inhibits NK cell responses by interactions with CD94/NKG2A (**Associated paper E**).

Treatment with antioxidants, such as vitamin-E (**Associated paper D**), and N-acetylcysteine (NAC) or induction of catalase expression represents possible strategies that may protect NK cells from apoptosis in the tumor microenvironment. In this respect, histamine dihydrochloride (HDC) was shown to protect immune cells from oxidative stress-induced apoptosis by inhibition of ROS formation in monocytes (425, 523). In fact, administration of HDC in combination with low-dose IL-2 after transplantation of patients with AML was shown to significantly improve the leukemia free survival after 3 years in a clinical phase III trial (523). Hence HDC, that improves the survival and cytotoxicity of immune cells in the context of transplantation, represents a drug that has made it from bench to bedside and was recently approved by the Food and Drug Association (Ceplene®).

As discussed above, administration of cytokines or drugs and antibodies that manipulate the cytokine milieu represent potential therapeutics that may improve the outcome of NK cell-based immunotherapy. Several other interesting approached are also currently being investigated, including drugs and antibodies that control chemokine signaling and thereby alter the cellular composition in the tumor microenvironment (524). See Table 4 for an overview of cytokines and soluble factors within the tumor microenvironment that affect NK cells and NK cell cytotoxicity.

Table 4. A brief overview of soluble factors and their effects on NK cells.*

Factor	Source	Stimuli	Receptor	Signaling mechanism(s)	Effect(s) on NK cells	Reference(s)
IFN-γ	- T cells - NKT cells - NK cells	- IL-12/IL-18 - NK cell activation - TLRs	- IFNGR1-2	- JAK-STAT pathway	- ↑ HLA presentation	(13, 525)
IFN-α/β	- All nucleated cells	- Viruses - Microorganisms	- IFNAR1-2	- JAK-STAT pathway	- ↑ HLA presentation	(13)
IL-2	- T cells - NK cells	- IL-2 autocrine - Immune responses	- IL-2R	- JAK-STAT pathway - Ras/MAPK pathway - PI3K/Akt pathway	- ↑ Proliferation - ↑ Homeostatis	(11)
IL-7	- Stromal cells (in BM & thymus)		- IL-7R	- JAK-STAT pathway - Ras/MAPK pathway - PI3K/Akt pathway	- ↑ Differentiation - ↑ Homeostasis (in early development)	(526)
IL-10	- Monocytes - CTLs - T_{reg} - B cells - Tumor cells	- Viral infections - Autoimmunity	- IL-10R	- JAK-STAT pathway	- ↓ Function	(15)
IL-12	- DCs - Macrophages - B cells	- Antigenic stimulation	- IL-12R	- JAK-STAT pathway	- ↑ Activation - ↑ Cytotoxicity - ↑ IFN-γ & TNF-α	(527)
IL-15	- Stromal cells - Mononuclear cells	- Viral infections	- IL-15R	- JAK-STAT pathway	- Differentiation - Proliferation - ↑ BCL-x(L)	(528-530)
IL-18	- Macrophages - T cells - B cells - Epithelial cells	- Inflammatory mediators	- IL-18R	- MyD88/IRAK/TRAK	- ↑ IFN-γ	(531, 532)
IL-21	- T cells ($CD4^+$) - NKT cells		- IL-21R	- JAK-STAT pathway	- Low dose ↑ proliferation - High dose ↓ proliferation	(151, 533)
TNF-α	- NK cells - Macrophages - Neutrophils	- Infections	- TNF-αR	- MAPK pathway	- ↑ Activation - ↑ Cytotoxicity	(12)
TGF-β	- DCs - Macrophages - Cancer cells - Fibroblasts	- ROS - MMP - low pH (release from LAP)	- TGF-βR	- Smad pathway - DAXX pathway	- ↓ NKRs - ↓ Activation	(147-150, 534)
MIF	- Tumor cells	- UV light	- MIFR	- Erk1/2 pathway	- ↓ NKRs	(144, 535)
ROS	- Tumor cells - MDSCs - TAMs	- Hypoxia - TNF-α		- Trx/Ref-1 - MEKK pathway - Ras/RAF pathway	- ↑ Apoptosis - ↓ NKRs	(302-304, 536, 537)
NOS	- Phagocytes	- IFN-γ - TNF-α		- NF-κB		(538)
PGE_2	- Macrophages - Tumor cells - Fibroblasts	- IL-1 - IL-6 - TNF-α	- EP1-4	- EP1: PLC ↑ Ca^{2+} - EP2: PKA ↑ cAMP - EP3: ↓ cAMP - EP4: PKA & P13K/Erk1 ↑ cAMP	- ↓ Antitumor responses - ↓ NKRs	(464, 539, 540)

IL; interleukin. TNF; tumor necrosis factor, TGF; tumor growth factor, ROS; reactive oxygen species, NOS; nitric oxygen species, PGE_2; prostaglandin-E_2. MMP; matrix metalloproteinases, LAP; Latency Associated Peptide, MyD88; myeloid differentiator 88, IRAK; IL-1R associated factor, TRAK; TNF receptor-associated factor.

* This table does not intend to summarize all aspects of each soluble factor.

4.3.2 Directed tumor killing via mAb-mediated ADCC

Administration of tumor specific monoclonal antibodies is an interesting approach. Tumor-specific mAbs may have both direct effects and induce NK cell-mediated ADCC as well as CDCC. Today, there is robust evidence for the beneficial clinical effectiveness of monoclonal mAbs in tumor immunotherapy (541). The anti-CD20 mAb rituximab and the anti-Her2/neu mAb trastuzumab against lymphoma and breast cancer, respectively, are two mAbs that are used in standard anti-cancer therapy today (541). Since metastatic ovarian carcinoma cells uniformly express the tumor antigen Her2/neu, one might expect that they could serve as targets for trastuzumab in this disease (542). However, as described in **paper III** and by others (160), the expression of the FcγIIIR receptor CD16 and its signaling molecules are reduced in patients with ovarian carcinoma, which may explain the unsuccessful results from early clinical trials with trastuzumab (480, 481). The role for bispecific antibodies, that can cross-link tumor epitopes with other activating NK cell receptors than CD16, have not yet been addressed in ovarian carcinoma. Preliminary data from our group also indicate that the CD16 expression is reduced on NK cells in the bone marrow of MDS patients, however, the functional consequences seem not as severe as for NK cells from ovarian carcinoma patients and the role for mAb therapy remain elusive. Hence, further studies and clinical trials are needed to assess whether bispecific antibodies and monoclonal antibodies may be used in MDS and ovarian carcinoma, respectively.

4.3.3 Manipulation of the NK cell receptor-ligand interactions

The intricate interplay between the NK cell receptors and the ligands expressed by the target can be manipulated to favor NK cell-mediated killing of tumor cells. There are several strategies that are currently being evaluated. This section will discuss the different strategies that could be used to improve the outcome of NK cell-based immunotherapies.

4.3.3.1 Augmented NK cell-mediated killing by abrogation of inhibitory interactions

4.3.3.1.1 KIR ligand mismatching

Since the first paper was published on the successful outcome of KIR ligand mismatching in hematopoietic stem cell transplantation against AML (72), several studies have focused on the this matter in both hematological and solid malignancies. However, not all studies examining this concept have reported as convincing results (407-410). One possible explanation for the divergent results might be that the grafts and transplantation settings varies between different studies. Another factor to consider is that the KIR ligand mismatching is based on genetic analysis of the KIR receptors and not on the cell surface expression of the KIR protein on the NK cell as well as HLA class I levels on the target cell. Although there is a genetic KIR ligand mismatch, the actual size of the alloreactive subset varies substantially among donor-recipient pairs (**Associated paper C**). Hence the KIR protein expression on the cell surface is an important factor that may affects the outcome of transplantation and should be taken into consideration when evaluating the role for NK cell alloreactivity. Moreover, additional therapeutics could potentially inhibit the negative interactions between KIRs and HLAs. Antibodies blocking KIRs have been shown to improve the anti-tumor effects of both autologous and allogeneic NK cells in mouse models (426, 543). The role for humanized anti-KIR mAbs are currently addressed in clinical trials (544). Additional studies are needed to demonstrate that this approach has a role in the context of transplantation or as a single therapy blocking KIR on autologous NK cells.

4.3.3.1.2 Avoidance of inhibition via CD94/NKG2A

The non-classical HLA class I molecule HLA-E is widely expressed in several tumor types, including AML, colon carcinoma, lymphoma, glioma, melanoma and ovarian carcinoma (545).

As for KIRs, mAb-mediated blockade of the inhibitory CD94/NKG2A receptor would be an interesting approach, but further investigations are needed before such approach could be tested in the clinic.

In **paper V** we show that the oxidative agent selenite sensitizes human HLA-E expressing tumor cells to CD94/NKG2A-positive NK cells by down-regulating HLA-E. The loss of HLA-E was caused by oxidative stress-induced abrogation of *de novo* protein synthesis at a post-transcriptional level. Selenite induces oxidative stress when metabolized to the intermediary metabolite selenide (546). However, selenite can also be enzymatically metabolized and incorporated into selenocystein (SeCys) that in itself could be further metabolized to selenide and thereby induce powerful oxidative stress (292, 547). In fact, SeCys was also shown to induce loss of HLA-E in **paper V**, whereas selenomethionine (SeMet), that has another metabolizing pathway that do not cause reactive metabolites, did not affect the HLA-E expression. Importantly, selenite has previously been administrated to patients (548), albeit not with the aim to induce loss of HLA-E on tumor cells. Since CD94/NKG2A is widely expressed on human NK cells, selenite-induced loss of HLA-E may promote anti-cancer immunity by endogenous NK cells directly as a single therapy or synergistically in the context of NK cell-based immunotherapy. The high frequency of CD94/NKG2A-positive NK cells following transplantation (251, 262, 411-413) highlights the potential usefulness of disrupting inhibitory CD94/NKG2A and HLA-E interactions. One advantage of using selenite is that its effects seem to be tumor specific, sparing normal cells, as exemplified in melanoma and AML (549, 550). Selective accumulation due to efficient uptake of extracellular reduced selenide that is facilitated by cysteine recycling through the Cystine/Glutamate antiporter and multidrug resistant proteins (MRP) has been observed in tumor cells compared to normal cells (301). Of note, this system is frequently over-expressed by drug-resistant tumor cells and also by MDSCs, indicating that selenite might selectively induce tumor cytotoxicity while eradicating the immune suppressive MDSCs (301). Intracellular ROS-formation, caused by redox cycles between selenide, thiols and oxygen, is considered to be the main mechanism behind selenite-induced cytotoxicity (546). The intracellular effects of selenite may be specifically pronounced in tumor cells due to its increased levels of thiols leading to increased redox cycling activity (551). Although it is not fully clear, it has been speculated that the expression of TrxR, that varies between different tissues (**Associated paper F** and **associated paper G**) and that can be altered during tumor transformation (546), influence the effects of selenite. Further studies are needed to define the role for TrxR and other proteins in the cellular redox system and if these could be used to predict susceptible tumor types. Hence, HLA-E expression may be preserved on normal cells while reduced on tumor cells specifically rendering them susceptible to CD94/NKG2A-positive NK cells.

In **paper V**, we demonstrate that selenite induces loss of HLA-E on tumor cells at the post-transcriptional level. There are several lines of evidence demonstrating a global reduction of the protein synthesis without affecting transcription during oxidative stress (292). Although this mechanism is likely to be involved in the reduction of HLA-E on the cell surface, other mechanisms cannot be excluded, such as increased protein degradation or misfolding due to malformation of the critical disulphide bridges in the HLA-E molecule *per se* or in the PLC (Figure 6). The selective loss of HLA-E on the tumor targets, without major perturbations of other NKR ligands, is probably due to the high turn-over rate on the cell surface caused by its relatively unstable tertiary nature (552). Since other important NKR ligands, including CD155, was unaffected by selenite exposure, one may speculate that that this drug could be used to selectively suppress HLA-E on cancers such as melanoma, ovarian carcinoma and neuroblastoma that are recognized via DNAM-1 dependent signaling (**paper II** and ref ((363, 457)).

Taken together, one mechanism of action of selenite, in addition to its direct cytotoxic effects, could be to potentiate NK cell-mediated killing of HLA-E expressing tumor cells by inducing loss of HLA-E expression.

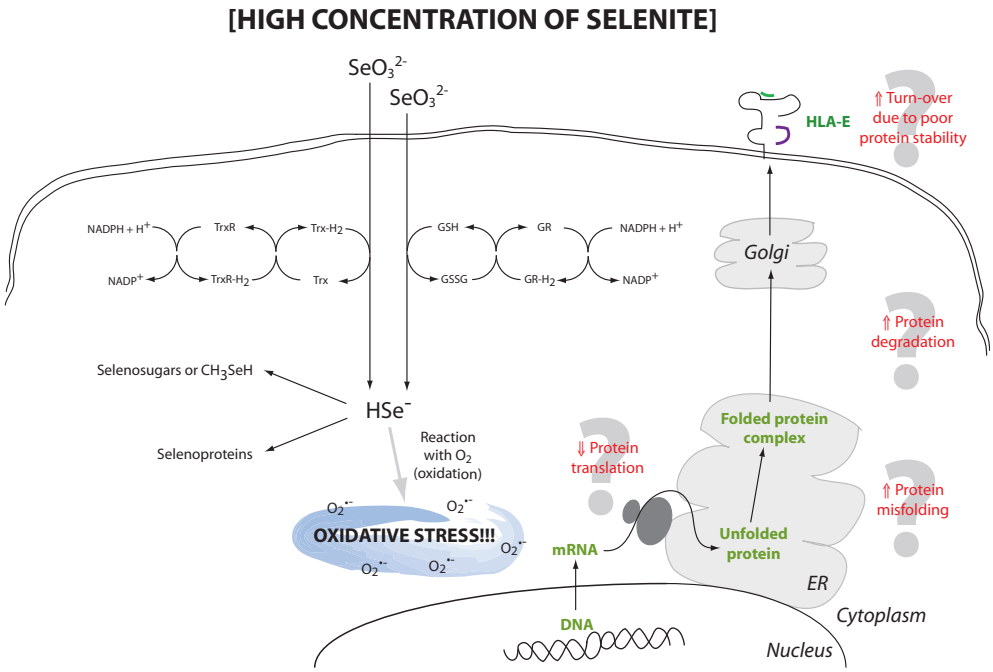

Figure 6. Schematic overview of the intracellular metabolism of selenite (based on ref (553)) and its possible effects on HLA-E protein expression. Selenite; SeO_3^{2-}, selenide; HSe^-, superoxid; O_2^-.

4.3.3.2 Improved tumor cell targeting via activating NK cell receptors

4.3.3.2.1 Increased expression of ligands to activating NK cell receptors

Although inhibition of NK cell activity may be avoided through KIR ligand mismatching or abrogation of CD94/NKG2A and HLA-E interactions, NK cells may still require activation signals to induce proper tumor rejection. Therefore, enhanced NK cell-mediated tumor killing may also be achieved by manipulating the tumor cells to express ligands for activating NK cell receptors. There are several lines of evidence that target cell susceptibility to NK cells can be enhanced by inducing a favorable NK cell receptor ligand repertoire (summarized in **Associated paper B** and ref (421)). As previously discussed, several studies have shown that DNA damage induces up-regulation of ligands to NKG2D (288, 291, 358, 427-431). One recent study nicely demonstrated augmented AML blast killing by alloreactive KIR HLA mismatched NK cells in combination with valproic acid-induced NKG2D ligand expression via activation of the ATM/ATR pathway (358). However, NK cell-mediated rejection of the AML blasts following HSCT can be abrogated by interactions between CD94/NKG2A and HLA-E (412). It is therefore tempting to speculate that additional therapies, interfering with the inhibitory CD94/NKG2A-

HLA-E interactions, may be used in combination with those that augment tumor recognition by up-regulation NKG2D ligands to improve the outcome of haploidentical stem cell transplantation against AML.

4.3.3.2.2 Enhanced tumor recognition by chimeric receptors

A concern raised by the fact that NK cell receptors can be lost upon target cell contact (**paper III**) is that sequential killing of multiple target cells may be hampered as NK cells turn hypofunctional (184). Repetitive adoptive transfer of NK cells may help to override the continuous down-regulation of NK cell receptors in the tumor microenvironment. However, the increasing knowledge of the molecular specificities and intracellular events occurring upon NK cell-mediated tumor recognition may provide new possibilities to develop more effective immunotherapeutic interventions. Better target recognition and enhanced NK cell activation may be mediated via chimeric receptors (421). For instance, chimeric NKG2D receptors have been shown to enhance tumor targeting by cytotoxic T cells (554, 555). Similar approaches based on NK cells that stably express chimeric DNAM-1 receptors and chimeric NKG2D receptors could theoretically also enable effective tumor rejection by NK cells.

5 CONCLUDING REMARKS

This thesis provides data on the molecular specificity of NK cell-mediated recognition of fresh human tumor cells and how the specificity and function of NK cells can be modulated in the tumor microenvironment. Below, I have listed the major conclusions from the present work.

- Freshly isolated human ovarian carcinoma cells were generally low in HLA class I and expressed ligands for activating NK cell receptors (**paper I** and **paper II**)
- Low levels of HLA class I due haplotype loss to was associated with the presence of tumor specific T cells (**paper I**)
- Resting allogeneic NK cells killed ovarian carcinoma cells while sparing normal cells (**paper II**)
- DNAM-1/CD155 interactions were crucial for NK cell-mediated killing of ovarian carcinoma cells, with minor contributions from NKG2D and NCRs (**paper II**)
- KIR ligand mismatching had no influence on NK cell-mediated killing of ovarian carcinoma cells (**Fig 3 in the thesis**)
- NK cells derived from the ovarian carcinoma environment expressed low levels of DNAM-1, 2B4 and CD16 (**paper II**)
- Physical interactions with CD155 induced down-regulation of DNAM-1 (**paper III**)
- Ovarian carcinoma-associated NK cells displayed a reduced tumor cell killing capacity, with a specific defect in activation via the DNAM-1 receptor (**paper III**)
- Reduced CD16 levels resulted in poor ADCC against ovarian carcinoma cells (**paper III**)
- Bone marrow-derived NK cells in MDS displayed reduced expression of DNAM-1 and NKG2D, which resulted in poor effector function (**paper IV**)
- MDS patients with ≥ 5% blasts in the bone marrow had more severe reduction of DNAM-1 and NKG2D expression (**paper IV**)
- Selenite induced a post-transcriptional loss of HLA-E expression rendering tumor cells susceptible to CD94/NKG2A-positive NK cells (**paper V**)

In summary, data presented in this thesis identify ovarian carcinoma as an interesting candidate for NK cell-based immunotherapy. Similarly, it should be exciting to explore the role for NK cells in cellular therapies for MDS with a particular focus on patients with high blast counts displaying impaired function of endogenous NK cells. Furthermore, selenite represents an interesting compound that may be used to render target cells more susceptible to NK cells. Combinatorial treatments that interfere with specific molecular pathways merit further attention and hold promises for the development of more effective NK cell-based immunotherapies.

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