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Immunological Recognition and Tumor Escape Mechanisms of Ovarian Carcinoma

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"Fly the sky, like a Hawk!"

- Håkan Norell

To the past,
for the future

Abstract

Ovarian carcinoma is a leading cause of cancer mortality and there is an urgent need for new and better therapeutic modalities that result in improved long-term outcome for this severe malignancy. One promising treatment modality for patients suffering from ovarian carcinoma is immunotherapy. Several different immunotherapeutic approaches targeting ovarian carcinoma, including infusion of monoclonal antibodies, adoptive transfer of T cells and administration of tumor vaccines, are currently being tested in the clinic. To date, the clinical efficacy of these treatments has been limited, partially because of the development of tumor cell escape and induction of immune suppression. In order to improve the success rate of future immunotherapy protocols it will be important to gain a further understanding of the molecular mechanisms underlying the escape of ovarian carcinoma from the immune system. This thesis deals with several mechanisms that all lead to lack of immune recognition, and describes one possibility of exploiting this new knowledge in the design of alternative treatment strategies.

The first part of my thesis demonstrates that metastatic ovarian carcinoma often exhibit heterogeneous human leukocyte antigen –A2 (HLA-A2) expression. The down-regulation may reflect a specific and progressive loss of this HLA allele. One underlying molecular mechanism was found to be haplotype loss, associated with the presence of HLA-A2-restricted HER-2/neu specific T-cell immunity. Based on these findings, this study suggested that antitumoral cytotoxic T lymphocytes (CTL) favor tumor escape variants lacking HLA-A2 expression, which is required for most T-cell-based antitumoral immunotherapeutic strategies. Moreover, our studies also showed that short-term tumor cell lines from patients with advanced ovarian carcinoma (OVACs) were protected from lysis by peptide- and allospecific CD8$^+$ T cells upon interferon- (IFN-γ) treatment. This paradoxical phenomenon was dependent on enhanced inhibitory signaling via CD94/NKG2A receptors expressed on the CTL. IFN-γ treatment of OVACs induced HLA-G expression, and data suggested that the underlying mechanism of protection was increased surface expression of HLA-E molecules, binding a peptide derived from the leader sequence of HLA-G. This study reveals that IFN-γ modulation may shift the balance of triggering and inhibitory signals leading to tumor escape from CTL-mediated immunity.

Natural killer (NK) cell function in cancer patients is often impaired and we describe how down-modulation of the expression of multiple activating receptors on NK cells may be one underlying mechanism. We found that in particular DNAM-1, which is one of the activating receptors, was severely down-modulated in tumor associated NK cells as compared to NK cells in autologous peripheral blood and blood from healthy donors. NK cells in the tumor environment also had lower expression of CD16 and the co-stimulatory receptor 2B4 while exhibiting moderately increased levels of both NKG2D and the natural cytotoxicity receptor NKp46. There was also a significant over-representation of regulatory CD56^{bright} NK cells in ascites. These complex perturbations resulted in an impaired functional capacity of tumor associated NK cells as demonstrated by their poor ability to recognize K562 cells. These results provide mechanistic insights into the failure of innate immunity to control progression of ovarian carcinoma.

Loss or reduction of HLA class I expression should render tumor cells susceptible to NK cell lysis in agreement with the missing-self hypothesis. Indeed, freshly isolated ovarian carcinoma cells triggered degranulation of resting allogeneic NK cells. This lead to detectable levels of granzyme B and caspase-6 activation in the tumor cells and induction of significant tumor cell lysis. Ovarian carcinoma cells exhibited ubiquitous expression of the poliovirus receptor (PVR, a ligand for DNAM-1) and sparse/heterogeneous expression of NKG2D ligands. Blocking experiments suggested that DNAM-1 engagement was critical for the direct NK cell recognition of ovarian carcinoma, while NKG2D and natural cytotoxicity receptor signaling resulted in a complementary contribution to tumor cell recognition. These results demonstrate that resting NK cells readily recognize and kill freshly isolated human tumor cells, and identify ovarian carcinoma as a potential target for adoptive NK cell-based therapy.

In conclusion, this work provides new insights into the mechanisms of tumor escape of ovarian carcinoma and describes successful targeting of this tumor type by resting allogeneic NK cells.

List of Original Papers

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

I.   **Håkan Norell**, Mattias Carlsten, Tomas Ohlum, Karl-Johan Malmberg, Giuseppe Masucci, Kjell Schedvins, Wolfgang Altermann, Diana Handke, Derek Atkins, Barbara Seliger, and Rolf Kiessling.
     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 Research, 2006, 66, 6387-94.

II. Karl-Johan Malmberg, Victor Levitsky, **Håkan Norell**, Cristina Teixeira de Matos, Mattias Carlsten, Kjell Schedvins, Hodjattallah Rabbani, Alessandro Moretta, Kalle Söderström, Jelena Levitskaya and Rolf Kiessling.
     IFN-γ protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism
     The Journal of Clinical Investigation, 2002, 110, 1515-23.

III. **Håkan Norell**, Mattias Carlsten, Kjell Schedvins, Hans-Gustaf Ljunggren, Rolf Kiessling and Karl-Johan Malmberg.
     Altered Receptor Repertoire and Impaired Function of Tumor Associated NK Cells in Ovarian Carcinoma
     Manuscript to be submitted

IV. Mattias Carlsten, Niklas Björkström, **Håkan Norell**, Yenan Bryceson, Thorbald van Hall, Bettina Bauman, Mikael Hanson, Kjell Schedvins, Rolf Kiessling, Hans-Gustaf Ljunggren and Karl-Johan Malmberg.
     Direct Recognition of Ovarian Carcinoma by Resting Human Natural Killer cells
     Submitted

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen presenting cells
APM	Antigen processing machinery
$\beta_2 m$	β_2-microglobulin
BMT	Bone marrow transplantation
CD	Cluster of differentiation
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
DNAM-1	DNAX-accessory molecule-1
EBV	Epstein-Barr virus
ER	Endoplasmic reticulum
ERAP1	Endoplasmic reticulum aminopeptidase 1
GM-CSF	Granulocyte macrophage – colony stimulating factor
Gsp	HLA-G leader sequence derived peptide
HLA	Human leukocyte antigen
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL-1, -2, etc	Interleukin-1, -2, etc
ILT	Immunoglobulin-like transcripts
ITAM	Immunoreceptor tyrosine-based activating motifs
ITIM	Immunoreceptor tyrosine-based inhibitory motifs
KARAP	Killer cell-activating receptor-associated polypeptide
KIR	Killer cell immunoglobulin-like receptors
LAK	Lymphokine activated killer
LILRB1	Leukocyte immunoglobulin-like receptor B1
LIR	Leukocyte immunoglobulin-like receptors
LOH	Loss of heterozygosity
mAb	Monoclonal antibodies
MART-1	Melanoma antigen recognized by T cells
MHC	Major histocompatibility complex
MICA/B	MHC class I-related chain A/B
NCR	Natural cytotoxicity receptors
NK	Natural killer
NKR	NK cell receptors
OC	Ovarian carcinoma
OVAC	Short-term ovarian carcinoma cell line
PBL	Peripheral blood lymphocytes
PVR	Poliovirus receptor
RAG-2	Recombination activating gene-2
STAT-1	Signal transducer and activator of transcription-1
TAA	Tumor associated antigens
TAL	Tumor associated lymphocytes
TA-NK	Tumor associated natural killer cells
TAP	Transporter associated with antigen processing
TcR	T cell receptors
TGF-β	Transforming growth factor-β
$T_H 1$	T helper cell type 1
TIL	Tumor infiltrating lymphocytes
TLR	Toll-like receptor
TNF	Tumor necrosis factor
ULBP	UL16 binding protein

Contents

PREFACE

Over the years I have spent at Karolinska Institutet many of my friends that are not involved in cancer research have often asked me the same specific question when we come to talk about my research: "Have you solved the riddle of cancer yet?!"

Today we know a lot about why and how normal cells transform into malignant cells and in several aspects "the riddle of cancer" has been solved. The rules that govern this process are defined and seem to be quite universal. Tumor cells spontaneously elicit antitumor immune responses. These may occasionally clear the disease, but they often seem to merely prolong the battle that too many cancer patients finally loose. To prevail, malignant cells are dependent on the ability to resist both nonimmune and immune mechanisms that try to prevent their illegitimate proliferation. Thus, the ability to escape eradication is an inherent property of tumor cells, acquired during neoplastic transformation.

Nevertheless, approaches utilizing cellular immunity as well as strategies based on antibodies have recently proved themselves successful in clinical trials. There is today ample evidence for an impressive ability of immune responses to clear malignant diseases. These findings are encouraging and hold promises that novel immunotherapy-based treatments will reach clinical practice in the near future. For future therapies to be even more successful these must implement strategies to avoid and counteract problems associated with immune evasion by tumors. Mechanisms of immune escape and immune suppression may promote tumor progression and should therefore be targeted in clinical protocols.

The work described in this thesis deals with the two major research areas of tumor immunology: Immunological recognition of tumors and tumor escape from antitumor immunity. Our work provides new insights into the mechanisms of tumor escape of ovarian carcinoma and also describes successful targeting of this tumor type by NK cells.

THE HUMAN IMMUNE SYSTEM

Human beings have always been under constant attack by invading bacteria, viruses and parasites that threaten our survival (2). As a consequence, the immune system has evolved to protect us from pathogens causing infections (3). Immunological responses can be based on a variety of potent biological effector-mechanisms some of which result in effective immunity (4). Unfortunately, our natural defenses under certain circumstances may also attack the body's own cells and cause adverse reactions leading to autoimmune disorders such as multiple sclerosis, rheumatoid arthritis and diabetes (5, 6). Hence, a successful immune system should effectively eradicate everything that is infectious "nonself" but at the same time spare everything that is noninfectious "self", apparently a delicate process.

It is not my intention to give even a brief introduction to all the different types of cells of the immune system and the complex biology behind the interactions between them. Instead, I will focus on a few key players of immune responses against tumors and some basic concepts of antitumor immunity that are of particular importance for the work described in this thesis.

The major histocompatibility complex

The major histocompatibility complex (MHC) encompases a set of genes which were identified for their key roles in the rejection of transplants (7, 8). The human MHC is known as the human leukocyte antigen (HLA) and map to chromosome 6. They are membrane-bound molecules with an extracellular peptide binding motif. Their function is to present peptide antigens to T cell receptors (TcR) and interact with NK cell receptors (NKR) expressed on some types of immune cells (8-10). HLA molecules are divided in two classes: I and II, they differ in their structures, functions and expression patterns. Within each class a further subdivision into classical and nonclassical HLA is useful, as these two types of molecules seem to have somewhat different functions in the immune system (11).

HLA class I molecules

HLA class I molecules consist of an α-chain containing three extracellular domains that are non-covalently linked to, and stabilized by, a β_2-microglobulin (β_2m) chain (12). The first and second domains of the α-chain form a groove in which peptides of around nine amino acids bind (13, 14). Peptides presented by HLA class I molecules are mainly of endogenous origin and they are the products of the antigen processing machinery (APM) (15, 16).

Classical HLA class Ia molecules

Classical HLA class Ia molecules are expressed quite uniformly by almost all nucleated cells in the body. The HLA class Ia genes are polygenic, i.e. there are several loci in each individual. There are three different sets of classical HLA class Ia molecules, HLA-A, -B and -C. Their functions are similar in that they all present peptides to $CD8^+$ T cells and interact directly with NKR expressed by natural killer (NK) cells and subsets of T cells(9, 10). The TcR binds the HLA/peptide complex diagonally across the peptide binding groove (17). Thus, it makes contact with residues of the α-chain as well as the peptide (17, 18). TcR binding is therefore critically dependent on both the target cell itself, which express a certain set of HLA alleles,

and the antigens presented in these molecules. The CD8 receptor may bind a conserved region of the third domain of the α-chain. HLA-A molecules seem to have a preferential importance for TcR signaling while HLA-C antigens seem more important for NKR interactions (19, 20). Each locus encoding classical HLA class Ia molecules is also polymorphic, i.e. one of many related but unique alleles that are represented in the population is present and expressed in any given individual (21, 22). The combined effect of this polygenic and polymorphic system is that numerous different antigens may be presented in each individual, and there is a tremendous variety in the peptides that may be presented in the population.

Nonclassical HLA class Ib molecules

The nonclassical HLA class Ib molecules HLA-E, -F, -G and -H (also known as HFE) are part of the HLA class I gene family but are different from the classical HLA class Ia because they are relatively nonpolymorphic (11). Furthermore, HLA-G binds a relatively limited set of peptides as compared to classical HLA class Ia molecules, but its peptide repertoire is still broad compared to the very restricted number of peptides bound by HLA-E molecules (23). Nonclassical HLA class Ib molecules have restricted and sporadic patterns of expression with low levels of surface expression and/or limited tissue distribution. They have unique antigen presentation functions and play important specialized regulatory roles in immune responses (11). Since a prerequisite for surface expression of HLA-E is binding of peptides derived from the leader sequences of certain other HLA class I molecules, HLA-E expression reflects the total amount of HLA class I molecules in the cell (24, 25). In this way, effector cells may control the integrity of the expression of the polymorphic HLA class I molecules by monitoring the expression of a single nonpolymorphic HLA class Ib molecule. HLA-E directly regulates the activity of both natural killer cells and cytotoxic T lymphocytes (CTL) due to its interaction with CD94/NKG2 receptors (26, 27). HLA-G may play a role in controlling the maternal cell-mediated immune responses against the fetus. This nonclassical molecule is normally expressed only by placental extravillous trophoblasts (28). Since trophoblasts lack expression of classical HLA-A and –B molecules, expression of HLA-G has been suggested as an underlying mechanism by which maternal NK cells are inhibited (29). In line with this hypothesis, it was reported a decade ago that HLA-G regulated NK cells through CD94/NKG2 receptors (30-32). These findings have later been shown to rather reflect that HLA-G expression results in intracellular availability of leader sequences, which enable surface expression of HLA-E molecules that ligate with the CD94/NKG2 receptors (25, 33, 34). Nevertheless, HLA-G expression may be involved in some of the many different mechanisms that collectively lead to successful protection of the fetus from maternal immunity. The two inhibitory receptors Ig-like transcript-2 (ILT-2), also referred to as leukocyte Ig-like receptor-1 (LIR-1) or leukocyte immunoglobulin-like receptor B1 (LILRB1), and ILT-4 are expressed by lymphocytes and monocytes respectively and strongly, but non-exclusively, bind to HLA-G. Thus, these interactions may be important for the inhibition of decidual lymphocytes and macrophages at the maternal/fetal interface. In addition, decidual NK cells express high levels of CD94/NKG2 molecules and the binding of peptides derived from leader sequences of HLA-G to HLA-E molecules thus provide a separate mode of inhibition that act in parallel (25, 35). In line with a situation where the allogeneic fetus is perceived as foreign by the maternal immune system, insufficient inhibition of decidual NK cells may favor spontaneous abortion (36). Recent research reveals that human reproductive success is influenced by the expression of HLA-C ligands on fetal trophoblast cells and KIR on uterine NK cells (37). Paradoxically, effective inhibition of maternal NK cells does however negatively influence the

outcome of pregnancy. This is due to preeclampsia, a disorder leading to perturbation of maternal circulation and poor fetal growth. It is a major cause of death for pregnant women and their young (38). In an evolutionary perspective, some mortality from preeclampsia may be seen as the price paid for increasing the brain size (39). Formation of the placenta involves cooperation between maternal NK cells and fetal trophoblast cells that remodel the blood supply. The underlying mechanism for preeclampsia seems to be that absence of activating KIR and presence of HLA-C alleles that preferentially interact with inhibitory receptors make NK cells overly inhibited and incapable of helping trophoblasts achieving this (37, 40). Thus, a successful pregnancy seems to be dependent on delicately regulated NK cell activities.

The dependency of leader sequences from other HLA class I molecules for HLA-E molecules to be expressed on the cell surface can also explain why CD94/NKG2 receptors have been reported earlier to bind a variety of HLA molecules. In all cases, the assumed interactions between classical HLA class Ia molecules and CD94/NKG2 heterodimers are ascribed to the leader sequences provided by HLA class Ia molecules up-regulating HLA-E surface expression (25). Interestingly, the limited sequence polymorphism of peptides that may exist, while still conferring stabilization and surface expression of HLA-E, substantially influence the ability of the HLA-E/peptide complexes to bind to CD94/NKG2 (41-44). The availability of β_2-microglobulin (β_2m) chains also influences HLA-E expression (34, 45). HLA-E may also activate T cells through direct binding of their TcR (46-48). Viral antigens from e.g. human cytomegalovirus (CMV) may hence be presented by HLA-E, and in a similar way as classical HLA class Ia molecules allow CTL recognition of infected cells (49). However, as HLA-E only has the ability to bind a limited repertoire of peptides, derived mainly from the leader sequences of HLA class I alleles, it is unlikely to play a broad role in the T cell defense against pathogens. TcR mediated CTL recognition of peptides presented by HLA-E might however still be important in specific cases. These interactions allow recognition of cells that may escape both CTL and NK cells lysis due to interactions with CD94/NKG2A, and provide T cells with an alternative restriction element on target cells with down-regulated expression of classical HLA class Ia molecules (49). As aberrant HLA class I expression is frequent in tumors it is interesting to note that a recent report showed that CTL recognition of cancer cells with impaired MHC class I antigen presentation in mice indeed may be restricted by the mouse homologe of HLA-E (50). Hence, the ability to regulate T and NK cell activity is a common feature of both classical and nonclassical HLA class I molecules. However, the mechanisms for doing so are partly different. In addition, some nonclassical class Ib molecules (HLA-H) have non-immunological functions whereas the function of others (HLA-F) still is elusive.

HLA class II molecules

HLA class II molecules consist of one α-chain and one β-chain and each chain contains two extracellular domains. The first domain of each chain together forms a groove in which peptides of about 13-25 amino acids in length bind (51, 52). Mainly peptides derived from extracellular proteins e.g. from bacteria and some parasites are loaded onto HLA class II molecules. Classical HLA class II molecules are also polygenic and polymorphic. Four different classical HLA class II molecules are expressed in each individual, but only HLA DR-, DQ- and DP-molecules are have a known function i.e. to present peptides to CD4$^+$ T cells. The expression of HLA class II molecules is normally restricted to certain cell types i.e. antigen presenting cells (APC). Hence, only B cells, macrophages, thymic epithelial cells and dendritic cells (DC) express HLA class II molecules constitutively, but class II expression can be

induced in many cell types by interferon-   IFN-γ) treatment. One usually refers to HLA class Ib molecules when talking about "nonclassical HLA molecules" but some of these are in fact HLA class II molecules. Of these, HLA-DM is known to promote peptide loading of classical HLA class II molecules in the endosomal/lysosomal system. HLA-DM is abundant in most antigen presenting cells while HLA-DO expression is restricted to B cells. HLA-DO associates with HLA-DM and thereby modifies its ability to exchange peptides (53).

Innate immunity

Physical barriers make up the first line of defense against microorganisms that threaten the host. Many pathogens will fail to enter our bodies because they die in the hostile physiological conditions maintained around the skin and mucosal membranes. Hence, our immune system is only exposed to a fraction of the infectious agents that attack us. Upon successful infection, a pathogen is attacked by the immune system within minutes. To be able to respond instantly, innate immunity uses pre-existing soluble factors (e.g. the complement system) and rapidly inducible cellular agents to combat pathogens (54). Neutrophils and macrophages are phagocytic cells that engulf and eliminate infectious agents when their cell surface receptors bind lipopolysaccharide, mannose or complement fragments found on the microorganisms (55). In addition, these immune cells produce and secrete inflammatory mediators such as interleukin-1 (IL-1), IL-6, IL-8 and tumor necrosis factor-α (TNF-α) that efficiently recruit more phagocytes and other immune cells to the site of infection.

The innate immune system evolved long before adaptive immunity and there are similar defense strategies in plants, invertebrates, and vertebrates (56, 57). Toll-like receptor (TLR) like proteins are present in all of these organisms, and there are some similarities between the complement systems in invertebrates and vertebrates (58). A unique and very important attribute of innate immunity is its ability to respond instantly at the very first encounter with new pathogens. Most components of the innate immune system do not change in quantity or quality due to previous exposure to a microorganism. The ability to respond to any particular pathogen is therefore stable over time, regardless of which pathogens have been previously encountered. The innate response usually prevents establishment of infections. Even if innate immunity is incapable of clearing the pathogen it plays a critical role in controlling the infection during the first days or week until the adaptive immune response is engaged. Moreover, components of the innate immune system, especially dendritic cells (DC), play crucial roles in both initiation and direction of adaptive immune responses (59). In addition both the complement cascade and certain innate cells are important effectors of the humoral immune response. Hence, even when adaptive immune responses have developed and B cells produce large amounts of antibodies that coat the microorganisms, innate immunity is needed to eradicate antigen-antibody complexes. Antibodies may activate certain components of the complement system that initiate the enzymatic cascade resulting in formation of membrane attack complexes that eradicate the pathogens. Alternatively, the Fc portion of IgG antibodies on e.g. opsonised pathogens can be bound by the FcγRIII receptor (CD16) and activate innate cellular immunity. Human NK cells, mononuclear phagocytes and neutrophils all express CD16, and engagement leads to antibody-dependent cellular cytotoxicity (ADCC). The importance of this mechanism for antibody efficacy *in vivo* is exemplified by an association between polymorphisms in the FcγRIII receptor and the ability to clinically respond to

antibodies (60). Hence, there is a mutual dependence of the innate and the adaptive immune systems.

NK cells

Natural killer (NK) cells constitute about 10% of the lymphocytes in peripheral blood and can in humans be defined as CD3⁻CD56⁺ cells. These cells are also found in the spleen but rarely in lymph nodes, thymus and bone marrow (61). Natural killer cells play important and non-redundant roles in the immune system. Apart from being involved in ADCC, their ability to produce IFN-γ is crucial for enhancing the activity of phagocytic cells, especially in the early phase of infection before T cells respond. NK cells are therefore important for defense against certain bacteria and parasites (62, 63). NK cell function is enhanced by several cytokines including IL-2, IL-12, TNF-α and type I interferons, which are produced by e.g. plasmacytoid dendritic cells in response to viral infection (64). In contrast, transforming growth factor-β (TGF-β) produced by e.g CD4⁺CD25⁺ regulatory T cells inhibits NK cell function (65, 66). Activation of NK cells may also occur independently of pathogens or antibodies. In fact, these cells were first identified by their unique ability to spontaneously kill certain tumor cells (61). NK cells were also found to mediate this spontaneous cytotoxicity, without prior sensitization, in response to normal cells infected with certain viruses (67-69). NK cells have been found to be of importance for the defense against cytomegalovirus, Epstein-Barr virus (EBV) and herpes simplex virus (68, 70, 71). The reason for the selective killing of these different targets lies in the basis for NK cell target recognition. The first insights into how NK cell activity is regulated were provided by the "missing-self" hypothesis (72, 73). It suggests that NK cells attack target cells that lack normal levels of cell surface expression of certain "self" MHC class I molecules. Molecular proof of these predictions was provided when inhibitory receptors were identified (74). Since then, a vast variety of both activating and inhibitory receptors that regulate NK cell activity have been identified, some of these will be discussed in greater detail below (10). The signals delivered by these partly overlapping receptors determines NK cell function.

There are two functionally distinct subsets of human NK cells (Figure 1). These can be identified phenotypically by their different cell surface density of CD56 (75). CD56^{dim} NK cells are highly cytotoxic and mediate direct killing of targets by exocytosis of large amounts of perforin and granzymes. They express a specific set of receptors including high levels of CD16 and killer cell Ig-like receptors (KIR). CD56^{bright} NK cells are thought to be more regulatory in their function due to their responsiveness to exogeneous cytokines and capacity to produce IFN-γ, TNF-α and β, IL-10 and GM-CSF (76-78). CD56^{bright} NK cells constitute approximately 10% of the total NK cell population in peripheral blood. This subset has been shown to be over-represented at some locations such as in lymph nodes. In the decidual tissue of pregnant women there is marked increase of NK cells that exhibit bright expression of CD56. However, these cells express KIR and CD94/NKG2C and thus seem to comprise a different specialized type of NK cell subset that may play a role in protection of the fetus during early pregnancy (79, 80). CD56^{bright} NK cells have also been reported to be over-represented in certain pathological conditions. They accumulate within inflammatory lesions in a variety of diseases and amplify the inflammatory response (81). The enrichment of CD56^{bright} NK cells in the synovial fluid is therefore potentially important in the pathogenesis of rheumatoid arthritis (82).
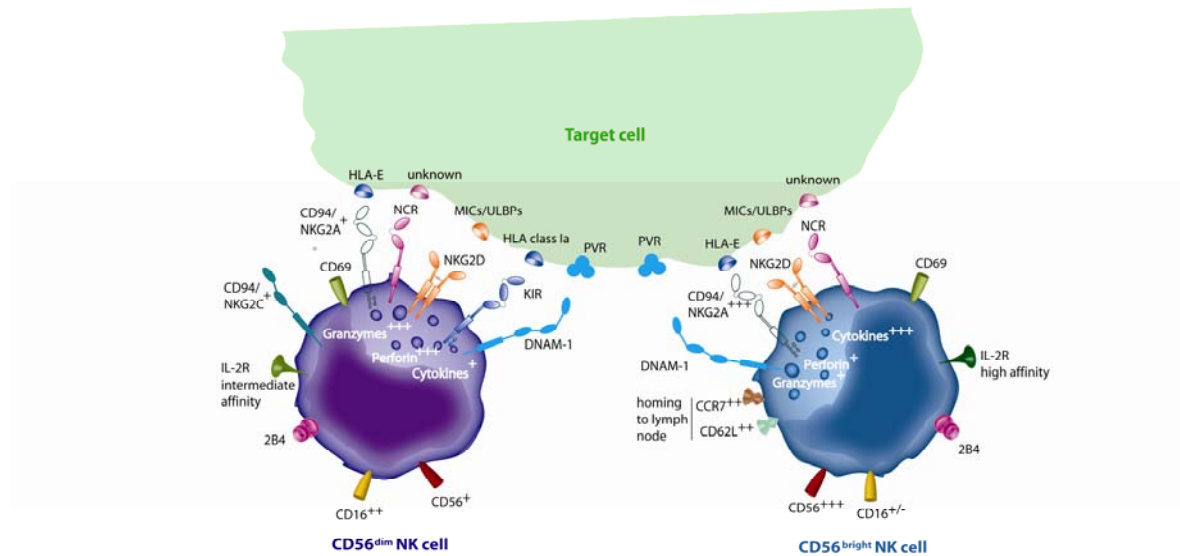
Figure 1. Schematic picture of CD56^{dim} and CD56^{bright} NK cell subsets with different receptor repertoires and immune functions. Adapted from Romero A. I. (1).

The mechanism behind the increased frequency of CD56^{bright} NK cells may be preferential proliferation of this subset in response to IL-2, as IL-2 receptors of higher affinity are constitutively expressed at increased levels by the CD56^{bright} NK subset (83, 84). Preferential apoptosis of CD56^{dim} NK cells, described in peripheral blood of cancer patients and in patients suffering from tuberculosis, could be another reason for the enrichment of CD56^{bright} NK cells (85, 86). A possible underlying mechanism for these observations could be the higher sensitivity of CD56^{dim} NK cells to oxidative stress that we recently have observed (manuscript in preparation).

Recent studies suggest that NK cells play an important role in T cell priming and thus shape the adaptive immune responses. As previously mentioned, NK cells are usually excluded from lymph nodes. However, they are rapidly recruited to lymph nodes during activation of an immune response. Specifically, NK cell migration is induced in antigen-stimulated draining lymph nodes (87). The interactions between NK cells and DC that occurs result in both NK cell activation and DC maturation (88). This process may be promoted by signaling through TLRs on innate immune cells. Interestingly, activated NK cells may acquire the ability to kill autologous DC that fails to mature properly(88). Further cross-talk between NK cells, DC and T cells initiate and sustain immune responses against pathogens and tumors (89). In this process, NK cells are an early and important source of IFN-γ, and thus necessary for T helper cell type 1 (T$_H$1) polarization (87). Considering the essential role that IFN-γ seems to play in tumor immune surveillance, the crosstalk between NK cells, DC and T cells may determine whether a tumor is rejected or not. The frequency of tumor formation in IFN-γ-receptor or STAT-1 deficient mice is similar to that of RAG-2 deficient mice, which have NK cells but lack T- and B cells (90). This argues against a major role for NK cells in IFN-γ-mediated immune surveillance. However, NK cells may contribute to tumor rejection mainly by producing IFN-γ that induces maturation of DC, which then prime T cells. Although NK cells are present in RAG-2 deficient mice, their IFN-γ production cannot confer protection based on this mechanism. In conclusion, it seems like the crosstalk between NK cells and DC may have great impact on the quality and strength of subsequent adaptive immune responses.

Adaptive immunity

Although most pathogens are rapidly cleared by innate immune responses adaptive immunity is crucial for our survival. Adaptive immune responses, in the form of humoral immunity mediated by antibodies produced by B lymphocytes and cellular immune responses that rely on different types of T lymphocytes, is essential for clearing infections (91). Each cell in the naïve adaptive immune system expresses a distinct antigen receptor produced by somatic gene rearrangements (92). Since these are partly random, all cells need to be "educated" to ensure that they have the ability to recognize a specific antigen and that they are not reactive to the host's own normal cells. A natural consequence of this arrangement is that several unique adaptive immune responses are raised for each individual type of pathogen. These features of adaptive immunity together with a continuous selection of the "fittest" cells are the basis for the extraordinary high specificity that is one of its hallmarks. The extensive training of lymphocytes is also a prerequisite for immunological memory, another important characteristic of the adaptive immune system (91). Immunological memory allows the immune system to respond instantly and vigorously to a particular antigen, a property to be retained for many years. A successful adaptive immune system must therefore allow the host to respond to a multitude of new foreign pathogens while accommodating numerous large memory pools specific for previously encountered antigens in a finite immune system.

HLA class I antigen presentation

The process known as antigen processing and presentation is complex and involves many discrete steps that all depend on different enzymes and chaperon proteins (93). Mutations that lead to impaired function or lack of expression of any of these proteins may prevent efficient presentation of specific antigens or the entire normal repertoire of intracellular peptides. Many endogenously produced cytosolic proteins are eventually targeted for degradation by the proteasome via the process of ubiquitination. Defective ribosomal products, which result from errors during transcription or translation and thus are representative of all intracellular proteins, are another main source of polypeptides degraded by the proteasome. Importantly, this also applies to proteins encoded by virus and intracellular bacteria. IFN-γ induces expression of the immunoproteasome, an altered proteolytic complex that produces peptides of lengths generally more suitable for HLA class I binding. Some potentially antigenic peptides are produced directly by the proteolytic activity of the proteasome, but presented peptides are often trimmed by peptidases in the cytosol or in the endoplasmic reticulum (ER) e.g. ER aminopeptidase 1 (ERAP1) (94). In fact, a vast majority of all peptides produced by the proteasome are destroyed via degradation by peptidases in the cytosol. Howewer, ERAP1 preferentially trims peptides longer than those that may be bound by HLA class I molecules, and thereby helps to provide more peptides that are presented to CD8^{+} T cells on the cell surface (95). IFN-γ strongly up-regulates the expression of ERAP1 and other peptidases involved in the post proteasomal trimming of antigenic epitopes, hence this is another way in which IFN-γ potentiates HLA class I antigen presentation (96). Peptide transport into the lumen of the ER is highly selective and depends on two proteins called transporter associated with antigen processing (TAP) 1 and 2 (97, 98). In the ER high affinity peptides bind to HLA/β_2m heterodimers and the HLA/peptide complexes leave the ER and are transported

via the Golgi complex before arriving at the cell membrane (99). The continuous presentation of endogenous peptides ensures that T cells may detect and eradicate cells that are infected.

T cell responses

Animal experiments strongly suggest that both CD4$^+$ and CD8$^+$ T cells are needed to successfully eliminate most pathogens and tumors. Adaptive immune responses are initiated by professional antigen presenting cells (APC) (100). In the periphery, antigens may be expressed endogenously due to infection, or be captured locally by APC, commonly dendritic cells. Danger signals in the environment lead to activation of these APC and probably decide if humoral and/or cellular immune responses eventually will be initiated. Activated APC migrate to lymph nodes and processed antigens are presented to T lymphocytes (101). Antigenic peptides bound to HLA class I and II molecules prime and activate naïve antigen specific CD8$^+$ and CD4$^+$ T cells respectively when presented in the context of co-stimulation e.g. B7.1 molecules, which are typically found on mature DC (102). Activated CD4$^+$ T cells have importance for virtually all adaptive immune responses. They are needed to initiate and sustain humoral immunity, but also provide important co-stimulation that enhances numbers and functions of CTL. Antigen specific CD8$^+$ T cells develop into activated effector cells with the ability to migrate into tissues (103). These cytotoxic T lymphocytes finally lyse infected or malignant cells in peripheral tissues (104).

Modulation of lymphocyte activity by human NK cell receptors

NK cell function depends on a balance between activating and inhibitory signals. There are wide arrays of both activating and inhibitory surface receptors on NK cells that may enhance or decrease the cytolytic activity and ability to produce cytokines. Since NK cell receptors (NKR) deliver potent activating or inhibitory signals, modulated expression of these receptors on lymphocytes or their ligands on target cells may render these susceptible or resistant to cell-mediated lysis (73, 105, 106).

T cell activation is mainly dependent on recognition of HLA/peptide complexes by the T cell receptor. However, it is now clear that subsets of T cells express and are regulated by NK cell receptors (107). There are several theories to explain why T cells express inhibitory receptors. They may fine-tune the T cell responses by raising the threshold for TcR triggering (108); thereby serving as a mechanism for peripheral tolerance (109). It has also been described that KIR expressing T cells are more resistant to activation induced cell death and thus that KIR molecules are involved in the development of long term memory T cells (110).

Inhibition

Until recently, it has been widely accepted that each NK cell expresses at least one self-HLA–specific inhibitory receptor. This is also the generally accepted explanation for NK-cell self-tolerance (111). However, recent publications indicate that a subpopulation of NK cells may lack inhibitory receptors specific for self-HLA class I molecules. These NK cells were proposed to instead attain self-tolerance by a mechanism of induced hyporesponsiveness (112).

In addition to NK cells, T cells with a memory/activated phenotype commonly express NKR (109, 113). Several studies have described how these receptors may inhibit antigen-specific effector functions in human CTL (26, 114). Classical HLA class I molecules expressed on melanoma cells were able to interact with KIR present on CTL and thereby protect the melanoma cells from lysis (114, 115). The lytic activity of tumor infiltrating CD8^{+}T cells against autologous renal tumor cells was dramatically and specifically increased in the presence of anti-KIR monoclonal antibodies (116). Furthermore, introduction of KIR3DL1 molecules into HLA-A2-restricted gp100-specific CTL resulted in inhibition of lysis of gp100[+] melanomas co-expressing HLA-A2 and HLA-Bw4 allotypes (115). Thus, signaling via inhibitory receptors may override a positive signal provided via the recognition of HLA/peptide complexes by the TcR, and protect targets that would otherwise have been killed by CTL.

Inhibitory signals are mainly provided by receptors that upon recognition of their respective HLA class I ligand block the activity of the cells (Table 1). Two major classes of inhibitory receptors have been identified. These two classes comprise receptors belonging to the immunoglobulin (Ig)-like superfamily; the killer cell Ig-like receptors (KIR) and the leukocyte Ig-like receptors (LIR), also referred to as Ig-like transcripts (ILT). The second class comprises heterodimers formed by the C-type lectin-like molecules CD94 and NKG2A or its splice variant NKG2B. The ligands for the KIR are the classical HLA-class I molecules HLA-A, -B and -C. ILT recognizes several classical HLA-class I alleles but also the nonclassical class I molecule HLA-G (117). As mentioned, the ligand for CD94/NKG2 hetrodimers are HLA-E molecules.

Receptor	Ligand
KIR2DL1 (CD158a)	HLA-C group 2
KIR2DL2/3 (CD158b)	HLA-C group 1
KIR3DL1	HLA-B alleles (Bw4)
KIR3DL2	HLA-A alleles (A3/A11)
LIR-1/ILT2 (CD85j)	HLA class I
CD94/NKG2A	HLA-E

Table 1. Inhibitory receptors expressed by resting NK cells and their corresponding HLA class I ligands are listed.

Activation

Activating signals may be delivered by a wide array of receptors including activating NK cell receptors that are capable of triggering resting NK cells (Table 2)(10). Induction of cytotoxicity can be mediated by natural cytotoxicity receptors (NCR; NKp30, NKp44, NKp46), NKG2D, DNAX-accessory molecule-1 (DNAM-1; CD226) and CD94/NKG2C. In addition, a large number of co-stimulatory receptors and adhesion molecules such as e.g. 2B4, CD2, CD7, CD161, NTB-A, CRACC, CD59, NKp80, CD62L and LFA-1 fine tune and direct NK cell activity.

Receptor	Ligand
CD16 (FcgRIIIA)	IgG
KIR2DS1-2	HLA-C (low affinity)
KIR2DS3-6	?
KIR3DS1	?
DNAM-1	PVR, Nectin-2
NKG2D	ULBPs, MICA, MICB
2B4	CD48
NKp30	?
NKp46	?
CD94/NKG2C	HLA-E

Table 2. Activating receptors expressed by resting NK cells and their corresponding ligands are listed.

Although KIR and CD94/NKG2 receptors are mainly known for their ability to deliver potent inhibitory signals, certain variants of these receptors instead trigger NK cell activation. KIR may have long inhibitory or short activating signaling domains. CD94 can form heterodimers with a number of different functionally distinct isoforms of NKG2 molecules (118). Activating signals are delivered by CD94 heterodimers containing NKG2C or NKG2E and its splicing form NKG2H while CD94 receptors formed with NKG2A and its splice variant NKG2B deliver potent inhibitory signals (117). The functional consequences of ligation of HLA-E by the CD94/NKG2 heterodimers therefore depend on whether this receptor contains an activating (NKG2C, NKG2E/H) or inhibitory (NKG2A/B) isoform. The ligand-binding domains of the activating forms of these receptors are virtually identical to the corresponding inhibitory receptors. The membrane bound proportions are however very different. Inhibitory receptors generally have trans-membrane sections ending with long cytoplasmic tails containing immunoreceptor tyrosine-based inhibitory motifs (ITIM). Upon ligand binding the ITIM become phosphorylated, leading to activation of phosphatases that block protein tyrosine kinases responsible for the intracellular activating signals (119). Activating receptors usually have short cytoplasmic tails without signaling motifs, instead their trans-membrane domain contains a positively charged amino acid that associates with negatively charged adaptor proteins. DAP12/Killer cell-activating receptor-associated polypeptide (KARAP) are an important examples of these signaling molecules that non-covalently link to the activating receptors (120). The adaptor proteins often contain intracellular immunoreceptor tyrosine-based activating motifs (ITAM) that get phosphorylated upon engagement of the activating receptors. This leads to recruitment and activation of tyrosine kinases and thereby causes activation of lymphocytes.

Signaling through the activating receptors NCR, NKG2D and DNAM-1 have been shown to be particulary important for NK cell recognition of human tumors (121-124). The three activating receptors NKp46, NKp30 and NKp44 are collectively known as natural cytotoxicity receptors and are all exclusively expressed by NK cells. Engagement with their as of yet unknown ligands results in strong activation of the cytolytic activity, and they play a crucial role in the recognition of many target cells (125). NK cells and most CD8$^+$ and $\gamma\delta^+$ T cells express the triggering receptor NKG2D (126). Several NKG2D-specific ligands have been characterized and are expressed on malignant cells from a variety of origins. These ligands include the stress-inducible molecules MHC class I-related chain A and B (MICA and MICB) and the UL16 binding protein (ULBP) molecules. NKG2D has been demonstrated to play a major role in the induction of cytotoxicity against various types of tumors *in vitro* as well as *in*

vivo. Recent studies have identified the receptor - ligand interaction between DNAM-1 and poliovirus receptor (PVR; CD155) to be important for the recognition and lysis of freshly isolated cells from solid tumors by allogeneic IL-2 activated NK cells (122). Monoclonal antibody-mediated masking of DNAM-1 abrogated a substantial part of the recognition of neuroblastoma cells by NK cells and the remaining cytolytic activities were NCR and/or NKG2D dependent.

Engagement of specific combinations of the above mentioned receptors govern not only the degree of activity but also dictates qualitatively distinct events, such as target cell adhesion, perforin polarization and NK cell degranulation that will occur as a result of these engagements (127, 128).

CANCER

Cancer remains a major public health problem globally. With about 1.4 million new cancer cases and over half a million deaths in USA alone every year cancer is the leading cause of death for those younger than 85 years. Huge resources have been dedicated to research and development of cancer treatments. Encouragingly, together with prevention by anti-smoking information this have led to that the total number of cancer deaths now has stabilized (129).

In the clinic and at the molecular level, cancer is by necessity seen as a whole group of disorders rather than one disease. There are more than 100 distinct types of cancer, and subtypes of tumors can be found within specific organs (130). However, the rules that govern the transformation of normal human cells into malignant cancers are universal and valid regardless of their origin. Today, cancer is no longer the mystery it used to be and in my view "the riddle of cancer" has largely been solved. Six essential alterations in cell physiology are both necessary and sufficient for the growth of cancer: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death i.e. apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. There are anticancer defense mechanisms against each of these physiologic changes that have been successfully breached by metastasizing cancer cells (130).

Ovarian carcinoma

Ovarian carcinoma is a leading cause of cancer mortality. Early diagnosis of ovarian carcinoma is well known to be associated with better clinical outcome, but unfortunately this type of tumor is usually diagnosed at a late stage and is therefore known as "the silent killer" (131). Both proteomic and genomic analyses could be useful tools to detect the disease at an early stage. Screening revealed that increased levels of the tumour marker CA125 in asymptomatic postmenopausal women were predictive of an increased risk of developing gynecologic cancer (132). Surgical bulk reduction followed by chemotherapy is the current standard of care for these patients and this treatment is often effective initially (133). However, relapse is common and in late stage disease successful therapy is rare and the overall survival is poor (129). Hence, there is an urgent need for new and better therapeutic modalities that result in improved long-term outcomes for this severe malignancy. The containment to the intraperitoneal cavity is often beneficial for the evaluation of new experimental treatments modalities for ovarian carcinoma. For example, intraperitoneal distribution of chemotherapy increases the efficacy of this type of treatment (134). Small molecular weight inhibitors, monoclonal antibodies (mAb), antisense based approaches and gene therapy strategies that target e.g. cell cycle regulators, growth factor receptors, signal transduction pathways and angiogenic mechanisms are all currently being evaluated in clinical trials (135). Immunotherapy is another promising strategy for the development of improved treatments of patients suffering from ovarian cancer. Several different immunotherapeutic approaches targeting ovarian carcinoma, including infusion of monoclonal antibodies, adoptive transfer of T cells and administration of tumor vaccines, are currently being tested in the clinic (136, 137).

A common denominator of all the studies presented in this thesis is the use of metastatic ovarian carcinoma as the tumor type studied. Tumor cells and tumor associated lymphocytes (TAL) from patients suffering from ovarian carcinoma is accessible as single cell suspensions

in ascites fluid that often accumulate in the peritoneal cavity in advanced stages of the disease. For studies II, III and IV the ascites and autologous peripheral blood were collected during primary surgery of patients with suspected ovarian carcinoma and diagnosis was subsequently determined by histological examination of the tumor specimens. None of these patients had received previous treatment with radio- or chemotherapy.

TUMOR IMMUNOLOGY

Both humoral and cellular spontaneous tumor-specific responses have been detected in many cancer patients (138-142). Thus cancer may spontaneously elicit antitumor adaptive immune responses. These may occasionally clear the disease but it seems like at least some tumor cells often escape both natural and induced immune recognition. Successful elimination of tumor cells or their escape from antitumor immunity, are the only two possible outcomes of the continuous battle ongoing in each cancer patient. Below, our work is described in the context of the current knowledge.

Immunotherapy against cancer

Increased understanding about the nature of adaptive immune responses and their interactions with innate immunity is important for the development of new treatments for disorders spanning from allergies to autoimmune diseases. It also allows us to develop strategies that improve the outcome of transplantation and enhance the efficacy of vaccines. Recent advances in the field of immunology, including the understanding of the molecular mechanisms involved in raising T cell immunity, also make immunotherapy a promising strategy for the development of improved treatments of patients suffering from cancer. Since most tumor associated antigens (TAA) defined so far are derived from self-proteins it is obvious that raising strong immunological antitumor responses will be extremely difficult. Moreover, such immunity may potentially lead to autoimmune reactions. Nevertheless, approaches utilizing cellular immunity as well as strategies based on antibodies have proved themselves successful in clinical trials in recent years. Administration of autologous adoptively transferred tumor reactive T cell populations derived from tumor infiltrating lymphocytes (TIL) may result in substantial tumor regression and completely cure a substantial proportion of the patients (143). The continuous identification of new tumor associated antigens provide new potential targets for immunotherapy and enable more accurate monitoring of the immunological outcome of immunotherapeutic approaches. These findings are encouraging and hopefully various improved immunotherapy-based treatments will be seen in the clinic in the near future.

Which tumor associated antigens should be targeted?

Since tumor cells originate from the host's own tissues most antigens expressed are normal self-antigens. These cannot be targeted by the immune system beacouse during the induction of tolerance in the thymus and later in the periphery lymphocytes are educated to not recognize "self". However, the alterations in cell physiology that all cancer cells must acquire to grow and metastasize are associated with expression of specific proteins that are more or less absent in normal cells and hence are at least predominantly expressed by the tumor cells (130). The antigens that originate from these proteins are known as tumor associated antigens (TAA). These may be presented to T cells by HLA class Ia molecules expressed on the surface of tumor cells. An ideal TAA is a non-self protein expressed exclusively in tumor cells and that is recognized by the cells of the adaptive immune system. These are probably available in all cancers but they are hard to characterize, as they are unique to each tumor. However, ambitious strategies to find and exploit these in immunotherapy treatments are under development (144). Meanwhile, theoretically less useful but defined TAA are evaluated in the clinic. There are four distinct categories of human tumor antigens, a listing of the known TAA

and the T cell epitopes defined from these can be found in databases available at www.cancerimmunity.org.

Strictly tumor-specific antigens may arise by a variety of mechanisms. Mutations, frame shifts, antisense transcripts, fusion proteins caused by translocation or altered posttranslational modifications may all give rise to altered proteins that are unique to the tumor cells and often new to the host and hence may provide "de novo" antigens (144). Often these are hard to characterize but the an interesting example is that unique immunoglobulin idiotype expressed by each B-cell lymphoma is produced by somatic mutations. This identifies a type of tumor-specific antigen that has been successfully targeted in clinical trials (145, 146). Further development of the techniques used for genome-wide expression analysis of individual patients will accelerate the use of patient-oriented, individualized tumor-specific TAA as targets of therapeutic immunization in the future (147).

The most numerous TAA are those expressed also in some normal tissues (148, 149). Germline (or cancer-testis) antigens are expressed in many tumors but not in normal tissue, with the exception of placental trophoblasts and testes. Differentiation antigens are expressed in the normal tissue of origin of the malignancy. Hence, some melanoma-associated antigens are also expressed in normal melanocytes and carcinoembryonic antigens are also expressed in embryonic tissues and in normal colon epithelium except from in most gut carcinoma.

Another set of TAA are overexpressed self-antigens, which are expressed in a wide variety of normal tissues but at so low levels that they are usually not recognized by the immune system. Since these are weakly expressed sometimes in a wide variety of normal tissues and even low level of expression may allow immune recognition, potential autoimmune reactivity is always a concern.

A separate type of TAA arises from viral infections associated with cancer, e.g. Epstein-Barr virus and human papilloma virus. These viral antigens may be considered TAA or even tumor-specific in cancer patients. Many of the peptides encoded by viral genes are highly immunogenic and therefore have great potential as targets for immunotherapy, and the risk of inducing autoimmune reactions are minimal. The use of TAA that are also expressed in some other tissues as targets for cancer immunotherapy is hazardous because of the risk of inducing autoimmune reactivity. Because neither placental trophoblasts nor testicular germ cells express HLA class Ia molecules, expression of germline antigens should not result in expression of antigenic peptides and hence no autoimmunity. The use of differentiation antigens may result in autoimmunity towards the corresponding normal tissue. Thus, targeting of melanoma-associated antigens sometimes gives rise to the appearance of vitiligo (143). It is more difficult to predict the safety of effective targeting of overexpressed antigens.

Different strategies of immunotherapy

There are many conceptually different immunotherapeutic strategies. Some treatments have, during the past few years, made their way into the clinic and are currently part of the standard treatment for certain malignancies. Several others are tested in ongoing clinical trials. Finally, a huge number of immunotherapy-based treatments are now evaluated in pre-clinical studies around the world. Passive immunotherapy relies on *ex vivo* produced or manipulated agents or cells that attack tumor cells when administered *in vivo* to patients (150). In contrast, active immunotherapeutic strategies aim to evoke immune responses *in vivo* that will mediate the antitumor effect. The paradigm of specific immunotherapy is monoclonal antibody-based therapy while cytokine distribution is an example of a non-specific approach. In Figure 2 the characteristics of different strategies for immunotherapy of cancer are summarized. Most efforts are devoted to stimulating specific cellular immune responses, especially CTL. A vast number of TAA have been defined and validated as immunological targets recently and many new antitumor immunotherapies based on these are underway. Despite this great progress in the field of tumor immunology, clinical tumor regressions in response to immunotherapeutic treatments are still rare.

Vaccines

Most immunotherapy-based clinical trials for cancer patients have focused on the use of cancer vaccines. Hundreds of studies based on active immunizations of patients with established solid tumors have been performed. In conclusion, thousands of patients have been treated with tumor vaccines but the objective response rates reported is in the order of a few percent (151). The lack of clinical effectiveness of currently available cancer vaccines emphasizes the need for profound modifications of this approach. Strategies for simultaneous generation of tumor specific CD4$^+$ T cells, pre-treatment of patients by removal of regulatory T cells, the use of improved adjuvants that may activate innate immunity and the administration of homeostatic cytokines are possible modifications to consider when formulating future cancer vaccine approaches.
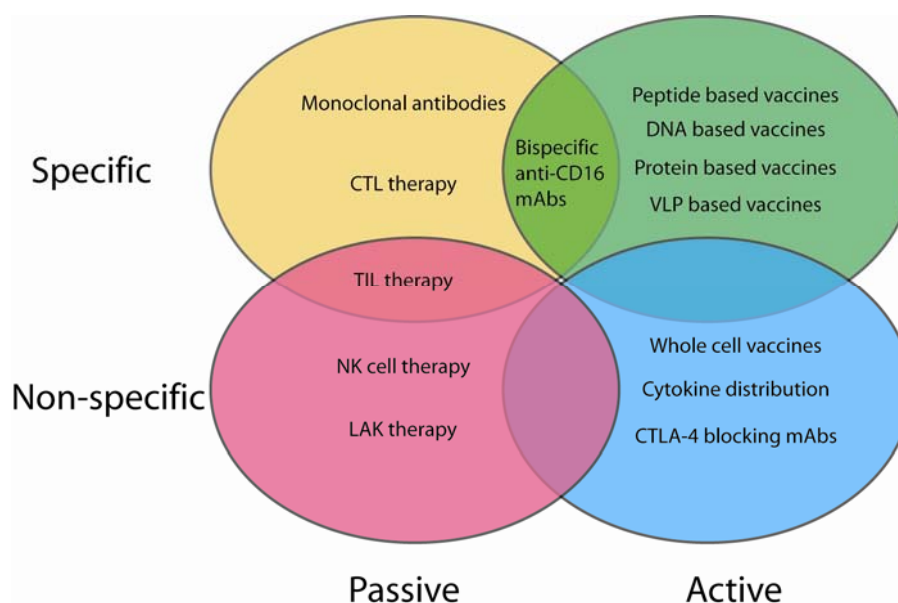

Figure 2. Different strategies for immunotherapy of cancer.

Adoptive cell therapy

Adoptive transfer of autologous tumor reactive cells is a strategy shown to be able to mediate cancer regression in preclinical and clinical models (143, 152). Recently, adoptive transfer of genetically engineered cells caused objective regression of metastatic melanoma lesions (153). Transduced cells may have great therapeutic potential and this type of treatment could be a way for adoptive transfer therapies to move into large-scale use in the clinic.

T cells

There are a number of distinct types of T cells that may be used for adoptive cell transfer therapy. In cancer patients, at least three different sources of T cells have been identified and evaluated for their antitumor effect *in vitro* and/or *in vivo*. Tumor infiltrating lymphocytes (TIL) have been studied extensively over the last 20 years. Different protocols have been evaluated and little by little the important circumstances necessary and sufficient for generating dramatic objective clinical responses have been revealed (143, 154-157). With the response rate of 50% that is currently seen for patients suffering from metastatic melanoma, this is one of the most successful clinical protocols for the treatment of epithelial tumors. Interestingly, successful treatment is dependent on the inclusion of a lympho-depleting chemotherapy regimen. The cytostatic treatment enables the antitumoral CTL to undergo *in vivo* expansion, which seems necessary for long-term persistence. Importantly, the persistence of individual CD8$^+$ T cell clonotypes in peripheral blood lymphocytes (PBL) showed a significant correlation with objective tumor regression. Antigen-specific CD8$^+$ T cells from metastatic melanoma patients can be readily generated from TIL cultures or by *ex vivo* stimulation of PBL with autologous dendritic cells pulsed with peptides from TAA (154, 158). Such TAA reactive T cells were cloned and CTL clones demonstrating specific lysis of antigen-positive tumor targets *in vitro* were expanded to huge numbers. These antigen-specific CD8$^+$ T cell clones were then adoptively transferred back to patients and preferentially localized to the tumor but did generally not persist for more than a few weeks *in vivo*. They mediated some antigen-specific tumor recognition but neither major clinical tumor regression nor serious toxicity was observed (154, 158) Recent studies have identified tumour reactive lymphocytes from sentinel nodes as potentially better effector cells than the autologous tumour infiltrating lymphocytes that are commonly used in current clinical trials (159). Future studies will show if the use of lymphocytes from lymph nodes rather than the tumors will improve the efficacy of adoptive cell transfer therapies even further.

NK cells (IV)

Natural killer cells are well known for their ability to kill a wide variety of tumors. They therefore hold great promise for the use of immunotherapy against cancer. However, major clinical benefits for patients suffering from malignancies by manipulation of NK cells have up until recently not been achieved. However, promising results that indicate that NK cells can be important in the therapy of cancer are now emerging. Pre-clinical experiments in mice injected intravenously (i.v.) with tumor cells have shown that they seem to be particularly efficient in eliminating blood borne tumor cells (160, 161). Clinical data strongly suggests that NK cells play a critical role in preventing leukemia relapse after allogeneic bone marrow transplantation (BMT) (162). Also for uveal melanoma there are observations compatible with an efficient NK cell lysis of blood borne tumor cells. Low expression of HLA class I is in contrast to in other

malignancies associated with a better survival (163). Furthermore, metastases of uveal melanoma show high HLA class I expression (164). The reason may be that metastatic cells of uveal melanoma spread via the blood instead of via the lymphatic system (165). Therefore, metastasizing tumor cells of this particular malignancy are more exposed to NK cells.

Protocols preventing HLA class I-mediated inhibition of NK cell activity have recently been evaluated in clinical trials. These studies demonstrate that the NK cell-mediated donor versus recipient alloreactions that occur following HLA-haplotype-mismatched BMT are highly beneficial. Alloreactive NK cells may reduce the risk of relapse in acute myeloid leukemia, while improving engraftment and protecting against graft-vs-host disease, associated with increased survival (162). Several protocols for immunotherapy using adoptive transfer of NK cells to patients with hematopoietic and solid tumors have been performed and several more are currently being evaluated in clinical trials (166-168). Some studies involve infusion of resting or short-term IL-2 activated haploidentical NK cells (167, 168). The repertoire of these NK cells is likely to resemble a resting phenotype, but few studies have explored the interactions between such non-activated NK cells and freshly isolated human tumor cells. Hence, preclinical studies of the receptor-ligand interactions that govern recognition of tumor targets with complex NKR ligand repertoires by resting NK cells are needed. We investigated the receptor-ligand interactions that mediate activation of resting NK cells in response to freshly isolated tumor cells from ovarian cancer patients (**IV**). We evaluated NK cell recognition of ovarian carcinoma, a malignancy that may be particularly relevant for these studies as these cancer cells frequently exhibit perturbated expression of certain HLA class Ia molecules and have overall low expression of HLA class I molecules (**I** and refs. 169, 170). These attributes should potentially make them more sensitive to NK cell-mediated lysis as the interaction of certain HLA class Ia molecules, especially HLA-C, with KIR expressed by T and NK cells decreases the activity of the immune cells. Hence, loss and decreased expression of HLA class I will make the tumors suitable targets for effector cells expressing KIR. An interesting immunotherapeutic approach for ovarian carcinoma is therefore to target these tumor escape variants with NK cell therapy. We therefore performed preclinical studies of the ability of resting allogeneic NK cells to kill ovarian tumor cells isolated freshly from ascites (**IV**). Overall cytotoxicity reflects the net sum of several discrete events including NK cell activation, target cell adhesion, perforin polarization, release of cytolytic granules and susceptibility of the tumor target to the effector mechanisms. Some of these events are governed by different combinations of activating and co-receptor signals (127). Moreover, efficient triggering of effector cells may be masked by a general resistance of the tumor cells to apoptosis or a specific effector pathway (171, 172). To be able to conclude why some tumors are killed better than others it is therefore necessary to analyze each of these discrete events. We observed recognition of these tumors as manifested by degranulation by resting allogeneic NK cells and parallel induction of granzyme B activity in tumor cells (**IV**). Importantly, this led to substantial apoptosis of target cells as caspase-6 activity was induced and significant tumor cell lysis was observed (**IV**). The ability of NK cells to degranulate in response to ovarian carcinoma correlated to the extent to which the tumors were lysed and hence revealed that poor recognition of targets resulted in failure to induce NK cell degranulation. NK cells may be triggered both by increased expression of stress-associated ligands for activating receptors and lack of inhibitory signals due to impaired HLA class I expression on target cells (73, 173). NK cell killing of ovarian carcinoma cells was inversely correlated to the level of HLA class I expression on the cell surface of the tumors, presumably due to ligand interaction with inhibitory receptors on the NK cells (**IV**). Ovarian carcinoma displayed ubiquitous expression

of the DNAM-1 ligand poliovirus receptor (PVR), whereas the expression of NKG2D ligands was more variable (**IV**). In line with the NK cell receptor ligand expression profiles, recognition of ovarian carcinoma cells was mainly dependent on DNAM-1, although NKG2D signaling gave a complementary contribution against tumors that expressed any of the NKG2D ligands (**IV**). Similarly, the susceptibility of myeloid and lymphoblastic leukemia to IL-2 activated NK cells correlated with expression of DNAM-1 and NKG2D ligands, and the killing of freshly isolated neuroblastoma cells was completely blocked in the presence of anti-DNAM-1 antibodies (122, 124). Natural cytotoxicity receptors have been shown to regulate the recognition of various tumors by NK cells (121, 123). Antibody mediated masking of NCR also partially inhibited NK cell recognition of ovarian carcinoma indicating that unknown ligands for these receptors may be expressed on this type of tumor (**IV**).

Since metastatic ovarian carcinoma exhibit overall low expression of HLA class I molecules, autologous NK cells could theoretically be used to target this type of tumor (**I** and ref. 169). However, NK cells derived from these patients often display reduced function as a consequence of tumor-induced immune suppression (**III** and refs. 174, 175). Therefore, it may be more advantageous to use allogeneic NK cells from healthy donors as this circumvents that lack of recognition due to intrinsic dysfunction of the effector cells. This also admits triggering of NK cells by KIR/HLA-mismatches, i.e. situations when a patient lacks one or more of the HLA motifs for inhibitory KIR expressed on donor NK cells. A major concern with this approach is that graft-versus-host reactions theoretically could occur. However, no such adverse reaction were observed in recent clinical trials involving infusion of allogeneic NK cells to patients with malignant disease (167, 168). This implies that NK cells have a capacity to discriminate tumor cells from normal tissues. In support of this we found that NK cells lysed ovarian carcinoma cells but spared the corresponding autologous fibroblasts (**IV**). The fibroblasts expressed higher levels of HLA class I and lower levels of activating NKR ligands, possibly explaining the tumor-specific targeting by allogeneic NK cells (**IV**).

Immune escape mechanisms

Most human tumors eventually continue to grow in spite of spontaneous or therapy induced immune responses. Thus, the presence of sufficient numbers of antitumoral effector cells with adequate avidities is usually not enough to result in effective clinical responses that cure the malignant disease. There is ample evidence that this is sometimes due to the ability of a fraction of the cancer cells to continuously evade potentially effective immune recognition and destruction. The work presented in this thesis deals mainly with the immunological mechanisms exploited by individual cancer cells that through natural selection are chosen to establish new tumors on the basis of their ability to escape antitumor immunity. Several mechanisms of tumor immune escape might act simultaneously and the overall tumor-host interaction may be extremely complex (**I, II, III** and refs. 172, 176, 177). In addition, cancer also suppresses host immunity by multiple mechanisms. Collectively, these phenomena may have substantial implications for both spontaneous immunity and immunotherapy against cancer and may be one reason that systemic immunity often cannot effectively eradicate tumors (178). Tumor immune evasion can also explain the relatively poor results of most immunotherapeutic clinical trials.

Increased thresholds of apoptosis induction

Protection against illegitimate growth of somatic cells is a crucial need for all multicellular organisms. Without apoptosis and other nonimmune surveillance mechanisms that protect us against oncogene-driven malignant development we would all die of cancer early in life (179). Even though ample evidence exists that the immune system is involved in tumor elimination, the surveillance against malignancy is mainly dependent on intrinsic control mechanisms that sense DNA damage and illegitimate cell growth (172, 179). Thus, neoplastic transformation will only occur if the cell is able to resist these internal mechanisms that naturally induce apoptosis upon illegitimate excessive proliferation. As a consequence, tumor cells often exhibit increased thresholds of apoptosis induction (172, 180). Apoptosis is also the downstream consequence of cytotoxic lymphocyte recognition. Thus, mechanisms of tumor cell escape from nonimmune surveillance result in that T and NK cells will always have to deal with tumor escape variants already at their first encounter with the malignant cells.

Loss of antigen processing and presentation (I)

Defective expression and presentation of HLA class I molecules is common in a variety of human tumors including ovarian carcinoma (**I** and refs. 169, 170, 181). HLA class I antigens can be lost to various degrees and by many different molecular mechanisms (182). Total loss of HLA class I expression is often due to mutations that result in lack of expression of functional β_2-microglobulin (183, 184). Alterations in expression of one or several components of the antigen processing machinery are frequently observed in malignant lesions and can cause impairment of HLA class I surface expression (185-187). Expression can then often be restored by IFN-γ treatment (186, 188, 189). This is not possible after genomic deletions or translocations that often cause loss of heterozygosity (LOH) or expression of one or several HLA alleles (**I** and refs. 190, 191).

CTL-mediated immunity is severely compromised by the emergence of tumor variants exhibiting pertubated expression of HLA class Ia molecules. Loss of HLA class Ia expression, may be the most relevant tumor escape mechanism preventing effective T-cell–mediated immunity and constitutes a major challenge to T-cell–based immunotherapy (177). Indeed, the underlying mechanism for lack of HLA class Ia expression on tumors often seems to be immune selection mediated by tumor specific CTL, favoring the selective outgrowth of HLA class I-negative variants (**I** and refs. 192-195). These T cells may be the result of a natural response elicited by the cancer itself, but may also be the product of effective immunotherapy. Most T-cell-based antitumoral immunotherapeutic strategies currently in use require expression of HLA-A2 on target cells, since they are based on CTL with HLA-A2 as the restriction element (155, 158, 196, 197). In addition, there is an overrepresentation of the HLA-A2 allele among patients with advanced stage ovarian cancer compared with patients with less advanced disease and healthy individuals (198, 199). Hence, HLA-A2 expression on the tumor cells is of critical importance for the effectiveness of immunotherapy and naturally occurring antitumor immunity in cancer patients in general and for patients suffering from ovarian carcinoma in particular. We have described that metastatic ovarian carcinoma often exhibit heterogeneous HLA-A2 expression, with a subpopulation of tumor cells exhibiting decreased or absent HLA-A2 expression. This reflects a specific loss of this HLA allele during progression of disease and therefore has direct implications for the design of future immunotherapeutic treatments (**I**). The results suggest that a majority of the ovarian carcinoma

patients cannot benefit from most immunotherapy-based clinical trials currently performed with patients suffering from this malignancy. We also provide the first report that haplotype loss may be the underlying molecular mechanism for abberant HLA class I expression in ovarian carcinoma (**I**). Interestingly, this was associated with presence of spontaneous autologous HLA-A2-restricted HER-2/neu specific T-cell immunity and the study suggests that antitumoral CTL favor tumor escape variants lacking HLA-A2 expression. In line with the hypothesis that CTL sculpt the phenotype of tumor cells and cause a progressive loss of HLA class I expression, down-regulation was found to be more frequent in metastases than in the primary tumors (**I** and refs. 181, 200, 201). An exception to this paradigm is metastases of uveal melanoma, which show high HLA class I expression (164). Interestingly, low expression of HLA class I was also associated with a better survival (163). Metastatic cells of uveal melanoma spread via the blood instead of via the lymphatic system (165). In contrast to the lymphocytes of the adaptive immune system NK cells are abundant in the blood while quite rare in lymph nodes. Therefore, metastasizing uveal tumor cells are as compared to other types of cancer relatively more exposed to NK cells. Hence, it seems like NK cells rather than T cells are the major type of effector cell mediating immunoediting during the metastatic spread of this malignancy.

Impaired function of TAP or tapasin results in dramatically reduced HLA class I expression (202). Paradoxically, a group of CTL that specifically recognize antigen processing machinery (APM) defect cells was recently identified (50). These cells target epitopes from self-antigens that only are presented in situations where the normal peptide repertoire is unavailable. Hence, these epitopes are expressed specifically on cells with impaired TAP, tapasin or proteasome function. These cytotoxic T-lymphocytes therefore specifically target tumor immune escape variants (50).

Antigenic-loss variants

Besides HLA class Ia molecules and a functional APM, expression of the targeted tumor associated antigen is necessary for presentation to and recognition by antigen specific tumor reactive T cells. Another immune escape mechanism frequently observed following partially effective immunotherapy is that tumor cells have down-modulated or lost expression of a TAA (203-205). The best example of this phenomenon was observed after adoptive transfer of tumor-reactive antigen specific CD8^{+} T cell clones. In three of the five patients analyzed, the relapsing or residual nodules displayed selective loss of the targeted antigen (gp100 or MART-1) while expression of two other melanoma antigens remained intact (158). Many of the currently targeted tumor antigens are not derived from proteins that are essential for cell survival, and can therefore readily be lost by the tumors. Vaccines and other immunotherapeutic strategies that target antigenic molecules critical for cell viability may be more effective as tumors are forced to express these molecules for their own survival and thus can not evade recognition by mutating to antigenic-loss variants.

Enhanced negative signaling (II)

All NK cells and subsets of CTL express inhibitory NK cell receptors that may inhibit their cytolytic activity. Some of these are specific for nonclassical HLA class Ib molecules. These ligands are normally expressed at low levels and show restricted tissue distribution. Ectopic expression of these nonclassical HLA class Ib molecules, which negatively regulate NK cells
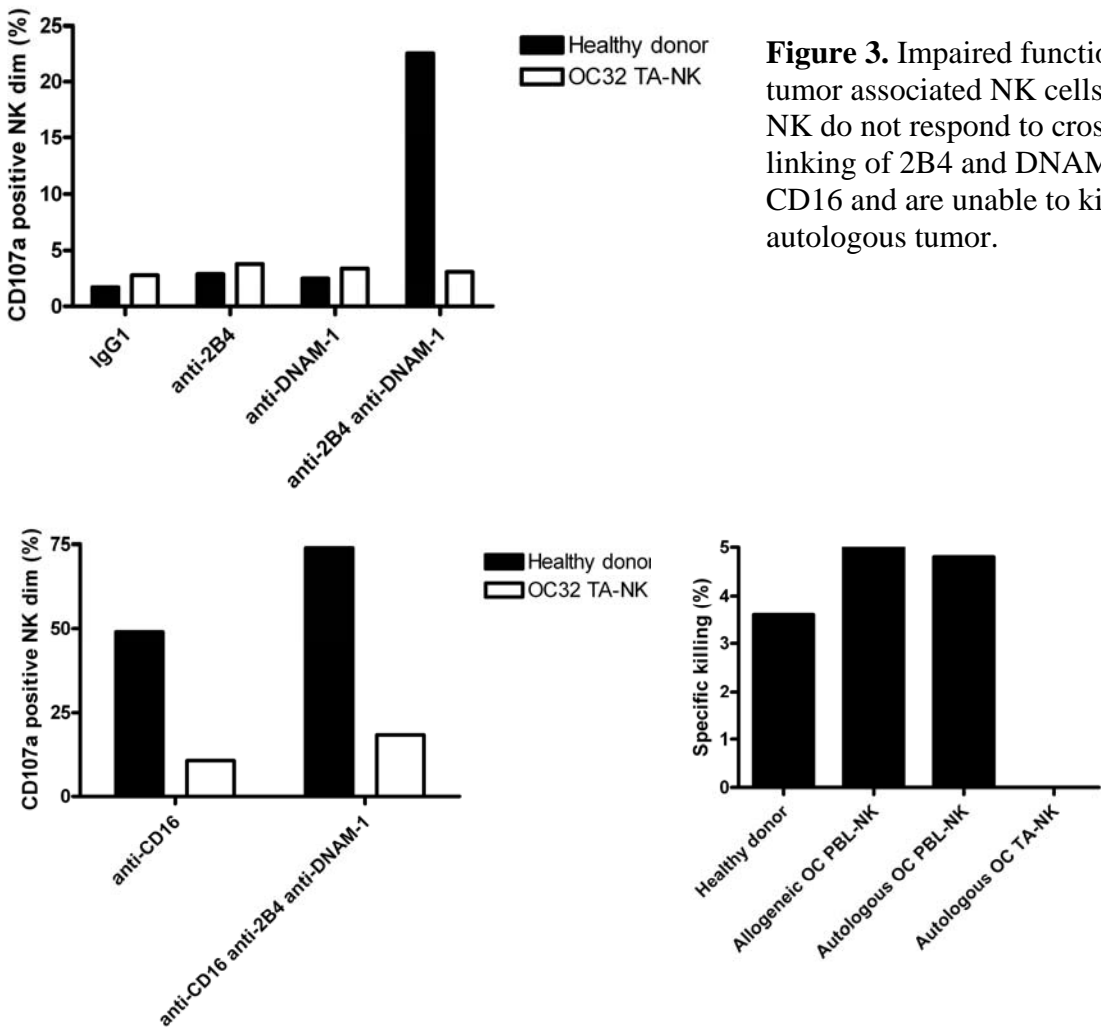
and antigen specific T cells, is yet another mechanism by which tumors evade immune recognition.

HLA-G may physiologically inhibit maternal decidual NK cells and macrophages at the maternal/fetal interface through interactions with the inhibitory receptors ILT-2 and ILT-4 respectively. Ectopic expression of HLA-G has been reported in several types of cancer (206-208). We found that ovarian carcinoma cells also frequently express this HLA class Ib molecule (**II**). As both CD56^{dim} tumor associated NK cells and CD56^{+} T lymphocytes in ascites of ovarian carcinoma patients frequently expressed ILT-2, the expression of HLA-G on tumor cells may constitute an important immune escape mechanism (**III** and unpublished observation Norell et al). HLA-G expression also enables surface expression of HLA-E by providing stabilizing peptides from leader sequences (i.e. Gsp) (**II** and refs. 25, 209). HLA-E expressed on tumor cells have been shown to inhibit the activity of both NK cells and CTL through interactions with CD94/NKG2A receptors (**II** and refs. 26, 27). However, we describe a new mechanism by which tumor cells may paradoxically escape CTL recognition (**II**). By expressing both classical and nonclassical HLA class I molecules in response to IFN-γ treatment, short-term cultured ovarian carcinoma cells could effectively inhibit cytotoxic T cell responses (**II**). The protection from lysis was dependent on enhanced inhibitory signalling via CD94/NKG2A receptors expressed on the effector cells (**II**). Thus, the increased inhibitory signaling via CD94/NKG2A was more important for CTL recognition than the up-regulation of the positive signaling to the TcR that both occurred upon IFN-γ treatment. Interestingly, the results indicate that IFN-γ-mediated intracellular expression of HLA-G lead to increased cell surface levels of HLA-E/Gsp complexes that interacted with the inhibitory receptor (**II**). Thus, immune cells may be inhibited through either ILTs or CD94/NKG2A upon HLA-G expression (**II** and ref. 10). The nature of the peptide that binds HLA-E drastically influences the ability of this complex to interact with CD94/NKG2 receptors (41, 44). The Gsp binds to the HLA-E molecule with high affinity and promotes strong signaling to the CD94/NKG2A inhibitory receptor expressed on NK cells and subsets of T cells (**II** and refs. 41, 44). In contrast, a peptide derived from the leader sequence of human heat shock protein 60 bound and up-regulated cell-surface expression of HLA-E on stressed cells but did not mediate signaling through CD94/NKG2A (42).

Importantly, we also showed that IFN-γ modulation protected ovarian carcinoma from lysis by autologus tumor associated lymphocytes (TAL). These cells frequently expressed CD94/NKG2A, mainly on CD8^{+} T cells of the terminally differentiated effector phenotype (**II** and ref. 210). Increasing evidence of the functional importance of expression of NK cell receptors on T cells is accumulating. Inhibitory CD94/NKG2A receptors may inhibit cytokine production and cytolytic activity of antigen specific T cells (26). It was also demonstrated that the inability of antiviral CD8^{+} T cells to control virus-induced oncogenesis was due to up-regulation of CD94/NKG2A receptors on the T cells (211). In addition, we now show that CD94/NKG2A receptors inhibit the recognition of ovarian carcinoma by CTL and TAL cultures (**II**). Interestingly, recent studies have shown that there was a bias towards expression of the inhibitory form of CD94/NKG2 heterodimers specifically on tumor infiltrating CD8^{+} T cells in human cervical and endometrial carcinoma patients (212, 213). These findings may have biological implications and clinical relevance. Although tumor infiltrating lymphocytes are often antigen specific CD8^{+} T cells, they are likely to be non-functional against HLA-G and HLA-E expressing tumors cells due to selective expression of inhibitory CD94/NKG2A (**II** and refs. 212-215).

Decreased activation (III)

As described above, many types of malignancies including ovarian carcinoma display reduced levels of HLA class Ia expression (**I** and refs. 169, 170, 181). Although the loss of expression of HLA class Ia antigens clearly decreases T cell activation, loss of certain of these alleles also decreases the negative signaling through KIR expressed by NK cells and T cell subsets, thus favoring immune activation. Thus, NK cells complement the T cells by eradicating cells that do not express HLA class I molecules. This unique ability of NK cells make them an attractive effector cell type to exploit in the battle against tumor escape variants that have lost or down modulated their HLA class I expression. NK cell tolerance to self is partially preserved by expression of inhibitory receptors for self HLA class I molecules. Because metastatic ovarian carcinoma cells or tumors exhibit overall low expression of HLA class I molecules, autologous NK cells could theoretically be used to target these tumor cells (**I** and ref. 169). However, NK cells derived from ovarian carcinoma patients may be impaired due to factors in the tumor microenvironment (**III** and ref. 174). Therefore lack of tumor recognition may be a consequence of intrinsic dysfunction of the NK cells. Indeed, there is substantial evidence of poor *ex vivo* activity of NK cells derived from ovarian carcinoma patients against the prototype NK cell target K562 and autologous tumors (**III** and refs. 216-218). Therefore, it may be more advantageous to use allogeneic NK cells from healthy donors, which may mediate potent recognition of these tumors (**IV**). Our findings of important regulatory roles of both activating and inhibitory NK cell receptor signals in the recognition of ovarian carcinoma prompted us to perform a comprehensive analysis of the NK cell receptor repertoire of the tumor associated

Figure 3. Impaired function of tumor associated NK cells. TA-NK do not respond to cross-linking of 2B4 and DNAM-1, CD16 and are unable to kill autologous tumor.

lymphocytes that infiltrate the peritoneal cavity in patients with ovarian carcinoma (**II, III, IV**). A potent activating NK cell receptor expressed by both NK cells and most CD8^{+} and γδ^{+} T cells is the triggering receptor NKG2D. Its ligands are up-regulated upon replicative stress following transformation and may consequently be found on cancer cells from a variety of origins (219-223). Although, NKG2D has been demonstrated to contribute significantly to recognition of cancer, some tumors may specifically impair the function of this triggering receptor (121, 123). The shedding of soluble MICA from human tumors lead to the down-modulation of NKG2D receptors on lymphocytes *in vivo* (224). The ligands for the natural cytotoxicity receptors on tumors remain unknown but signaling through NCR is obviously critical for NK cell recognition of many human tumors (121, 123). The ligands for DNAM-1 were shown to be the poliovirus receptor (PVR) and Nectin-2 (225). Signaling through DNAM-1 affected the NK cell lysis of leukemias and was critical for recognition of neuroblastoma and ovarian carcinoma (**IV** and refs. 122, 124). Down-regulation of NCR and NKG2D expression on NK cells in cancer patients severely impairs the capacity of NK cells to kill autologous tumor cells (226, 227). Thus, a perturbed receptor repertoire may provide one possible mechanism behind the poor function of NK cells in cancer patients, providing a partial explanation for the inefficient elimination of ovarian carcinoma during the immunoediting process. Our studies revealed a novel pathway by which tumors may avoid recognition by the immune system (**III**). We found multiple alterations of receptors that regulate NK cell function on tumor associated NK cells isolated directly from the tumor environment in patients with ovarian carcinoma (**III**). We demonstrated that all freshly isolated ovarian carcinoma ubiquitously express the DNAM-1 ligand PVR (**IV**). Moreover, DNAM-1 signaling was indispensable for activation and killing of ovarian carcinoma by allogeneic NK cells (**IV**). In accordance we also revealed that DNAM-1 was severely down-modulated in tumor associated NK cells compared to NK cells in autologous peripheral blood and blood from healthy donors (**III**). NK cells in the tumor environment was in an activated state displaying increased CD69 expression and additional alterations including a shift in the CD56[bright/dim] NK cell ratio and profoundly lower expression of CD16 and the co-stimulatory receptor 2B4 (**III**). In contrast, NKG2D and the natural cytotoxicity receptor NKp46 were slightly up-regulated (**III**). These complex perturbations of the receptor repertoire resulted in a reduced capacity of tumor associated NK cells to recognize K562 cells. To more precisely pin point the consequences of the reduced expression of individual NK cell receptors by TA-NK we performed re-directed lysis experiments where P815 cells were co-incubated with anti-2B4, anti-CD16, anti-DNAM-1 or combinations thereof (Figure 3). These experiments confirmed the previously described synergy between DNAM-1 and 2B4 in the triggering of resting NK cells from healthy donors (128). In contrast, TA-NK cells were not responsive to this combination of mAbs and only weakly to anti-CD16 stimulation. Moreover, preliminary data indicate that TA-NK cells are unable to kill ovarian carcinoma cells (OC32) in contrast to NK cells derived from peripheral blood. These data support the interpretation that the alteration of the NK cell receptor repertoire in TA-NK has functional consequences leading to poor targeting of autologous tumor cells.

Tumor induced immune suppression

Patients with advanced cancer often experience a systemic tumor-induced immune dysfunction as manifested by functional abnormalities of T cells and NK cells (e.g. down-regulation of TcR signal transduction, inadequate ability to secrete cytokines, poor cytolytic capacity or sensitivity

to apoptosis upon tumor encounter) (175, 228). The underlying mechanisms are often complex and numerous, including immunosuppressive substances e.g. TGF-β and IL-10 secreted by tumor cells or tumor-associated immune cells.

Moreover, a more profound immunosuppression often forms in the tumor microenvironment as compared to the peripheral blood. For example, defects in TcR signalling molecules have been found to be more pronounced in CD8^{+} T cells in TIL when compared to those in peripheral blood. Regulatory CD4^{+}CD25^{+} T cells have also been shown to migrate to and accumulate within tumors in ovarian carcinoma patients (229). Importantly, their presence within the tumor is associated with reduced survival in cancer patients. Moreover, regulatory T cells may mediate direct inhibition of CTL and NK activity *in vitro* and block TAA-specific immunity *in vivo* (65, 230).

In addition to the immune dysfunction induced by the disease itself, some of the chemotherapeutic agents used for treating malignancies also induce immune suppression. In an effort to determine immune competence we investigated the DTH reactions to recall antigens in 29 patients with advanced cancer. The majority of patients mounted normal responses, indicating immune competence. However, patients suffering from ovarian carcinoma had significantly weaker responses than both melanoma and prostate cancer patients (manuscript in preparation). A better knowledge of the mechanisms underlying the local and systemic immune suppression in these patients is critical for the development of future treatment strategies based on the immune system. We foresee that such protocols may involve non-specific adjuvants that help to restore the immune competence of the patients prior to specific immunotherapy targeting the tumor cells.

CONCLUDING REMARKS

The possibility to manipulate the immune system to recognize and kill tumor cells is the ultimate aim of the science of tumor immunology. Immunotherapy may be an attractive complement to conventional therapies, particularly in the targeting of residual disease. The overall tumor-host interaction is however extremely complex and a better understanding of the tumor-host interaction is required to design novel treatments that enhance the ability of the immune system to eradicate cancer.

The strikingly different results obtained with short- versus long-term ovarian carcinoma lines described in paper II, have had a considerable impact on our subsequent work. Since long-term *in vitro* culturing is known to alter the characteristics of tumor cells, we performed almost all experiments described in this thesis on freshly isolated cells which had not been manipulated or cultured. We have developed several flow-cytometry-based functional assays that allow monitoring of the interaction between resting lymphocytes and freshly isolated tumor targets in a setting that, as closely as possible, mimics the *in vivo* situation. I believe these efforts are important to gain further insights into the processes that regulate T and NK cell interactions with tumor cells.

The work presented in this thesis deals with the mechanisms by which tumors avoid recognition by the immune system. Several lines of evidence suggest that, during tumor progression and metastasis, the immune system plays a major role in selecting tumor cells of a less immunogenic phenotype. We describe that metastatic ovarian carcinomas exhibit heterogeneous HLA-A2 expression, reflecting a specific and progressive loss of this HLA allele. One underlying molecular mechanism was found to be haplotype loss, associated with the presence of HLA-A2-restricted HER-2/neu specific T-cell immunity. Many antitumoral immunotherapeutic strategies are severely compromised by perturbated HLA-A2 expression on target cells and my results indicate that the majority of the ovarian carcinoma patients cannot benefit from most T-cell-based treatments currently evaluated in clinical trials. The cytokine IFN-γ has been considered for immunotherapy against cancer based on its ability to increase the expression of HLA class I, thus rendering target cells more susceptible to CTL activity. However, we show that IFN-γ modulation of ovarian carcinoma cells leads to increased expression of the nonclassical HLA class Ib molecules HLA G and HLA E and enhanced signaling through inhibitory NK cell receptors expressed by CTLs. These results demonstrate that IFN-γ may shift the balance of triggering and inhibitory receptor signals leading to tumor cell resistance to CTL-mediated immunity despite restored levels of classical HLA class I molecules.

Having uncovered several immune escape mechanisms utilized by ovarian carcinoma, we set out to develop strategies that circumvent or counteract these. It is well known that NK cells are negatively regulated by HLA class I molecules and recognize targets that lose HLA class I expression. Therefore, we explored the capacity of NK cells to kill freshly isolated ovarian carcinoma cells that expressed reduced levels of HLA class I. We have demonstrated that resting allogeneic NK cells readily recognize and kill freshly isolated ovarian carcinoma cells. This was dependent on signaling through the activating NK cell receptor DNAM-1. Interestingly, the DNAM-1 receptor was significantly down-modulated on patient derived, tumor associated NK cells. The perturbations of the receptor repertoire on tumor associated

NK cells was associated with an impaired function, thus providing mechanistic insights into the failure of innate immune surveillance to control progression of ovarian carcinoma.

The results presented in this thesis clearly demonstrate that several mechanisms of tumor immune escape may act in parallel. However, some of these mechanisms may be exploited by alternative strategies of immunotherapy. The identification of HLA class I^{low} ovarian carcinoma cells as sensitive targets for resting NK cells may set the stage for pilot studies exploring the *in vivo* potential of adoptively transferred NK cells. Future work will address the possibility of restoring/enhancing activating NK cell receptor signalling by introducing chimeric DNAM-1 receptors into tumor specific T and/or NK cells. Other strategies may involve *in vivo* blockade of inhibitory NK cell receptors including KIR and NKG2A. If successful, such efforts may lead to the development of novel, more effective immunotherapy-based treatments of ovarian carcinoma.

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