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Neuroblastoma as a target for effector mechanisms of the immune system

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Doctoral thesis
Neuroblastoma as a target for effector mechanisms of the immune system

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To my family, Stefan and mormor

Tandem finitum est

ABSTRACT

Neuroblastoma (NB) is the most common solid extracranial tumor in children. Despite intensive multimodal treatment, the prognosis for high-risk NB patients remains poor and therefore new treatment modalities are needed. The main aim of my studies was to assess NB as a target for cytotoxic T lymphocyte (CTL)-mediated immunotherapy. CTLs induce target cell death through two major pathways, namely the cytotoxic granule exocytosis- and the death receptor-mediated pathway. While an efficient T cell-mediated lysis through the release of perforin/granzyme-containing cytotoxic granules requires the expression of the relevant HLA class I complexes as well as adhesion molecules on the target cells, killing through the death receptor pathway involves the binding of the ligands to death receptors in the tumor cell membrane, and requires an intact intracellular molecular machinery that conveys the signal to execute tumor cell death. However, a number of features including low or absent surface HLA class I as well as a defective expression of caspase-8, may hamper the development of immunotherapeutic modalities for NB. Nevertheless, strategies to enhance the immunogenicity of NB cells could render them into good targets for CTL-mediated immunotherapy. Alternatively, NB cells can be targeted by means of bystander activated CTLs in an HLA-independent fashion. Both these approaches have been investigated in the studies presented in this thesis.

We demonstrated that retinoids, differentiating agents currently in use as a treatment modality for high-risk NB, act as enhancers of HLA class I antigen processing and presentation as well as increase the susceptibility of NB to CTL-mediated effector mechanisms. We found that retinoids induced the expression of proteolytic and regulatory subunits of the immunoproteasome, increased the half-life of HLA class I complexes, and enhanced the sensitivity of NB cells to both HLA class I-restricted peptide-specific and HLA class I non-restricted lysis by CTLs. Our data suggest that the application of retinoids and CTL-based immunotherapy may be an effective combination for the treatment of NB (Paper I). Though numerous studies have demonstrated the ability of IFNγ to induce components of the antigen processing machinery (APM) in NB, the impact on subsequent tumor recognition by CTLs has not been investigated in detail. Therefore, we investigated the IFNγ-induced effects on selected components of the APM in NB cells as well as on CTL recognition. Although IFNγ-treatment efficiently induced surface HLA class I on NB cells, this effect did not translate into an efficient increase in HLA-restricted killing by CTLs. We suggest two possible molecular explanations for this phenomenon; (i) an IFNγ-induced expression of the inhibitory HLA-E on NB cells, and/or (ii) a decreased susceptibility of IFNγ-treated tumor cells to the cytolytic granule content of CTLs (Paper III).

To investigate whether CTLs may eliminate NB cells in an HLA-independent manner without the involvement of perforin/granzyme-mediated cytotoxicity, we used NB-non-specific CTLs activated on third party targets as well as soluble factors released from these CTLs. We found that both caspase-dependent and-independent cell death were induced in NB cells in a bystander manner, and, in addition, TNFα released from activated CTLs was defined as an important effector molecule (Paper II). Besides inducing NB cell death, soluble factors released by activated CTLs modulated the levels of cell surface molecules implicated in HLA-restricted and HLA-independent NB cell recognition, induced the expression of caspase-8, as well as increased the susceptibility of NB cells to death receptor-mediated killing. Importantly, soluble factors released by activated CTLs skewed the surface immune phenotype of both long term cultured and primary NB cells (Paper IV).

Altogether, the results presented in this thesis suggest that recruiting activated CTLs into the proximity of the tumor may have a therapeutic effect in patients with NB. CTLs may simultaneously activate different death pathways and override the “silent” immune phenotype of NB cells. We also suggest that CTL-mediated therapy could be combined with retinoid treatment of NB.

LIST OF PUBLICATIONS

The thesis is based on the following papers, which in the text will be referred to by their roman numerals (I-IV).

I. Simona Vertuani*, **Anna De Geer***, Victor Levitsky, Per Kogner, Rolf Kiessling, and Jelena Levitskaya. Retinoids act as multistep modulators of the major histocompatibility class I presentation pathway and sensitize neuroblastomas to cytotoxic lymphocytes. *Cancer Research*, 2003, 63; 8006-13.

II. **Anna De Geer**, Rolf Kiessling, Victor Levitsky, and Jelena Levitskaya. Cytotoxic T lymphocytes induce caspase-dependent and -independent cell death in neuroblastomas in a MHC-nonrestricted fashion. *The Journal of Immunology*, 2006, 177; 7540-50.

III. **Anna De Geer**, Elian Rakhmanaliev, Diana Handke, Lena-Maria Carlson, Rolf Kiessling, Per Kogner, Barbara Seliger, and Jelena Levitskaya. Interferon gamma-induced changes either do not promote or negatively affect MHC class I-restricted T-cell recognition of neuroblastoma cells. *Submitted*.

IV. **Anna De Geer**, Lena-Maria Carlson, Per Kogner, and Jelena Levitskaya. Soluble factors released by activated cytotoxic T lymphocytes interfere with death receptor pathways in neuroblastoma. *Submitted*.

* both authors contributed equally

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LIST OF ABBREVIATIONS

ABMT	Autologous bone marrow transplantation
ADCC	Antibody-dependent cell-mediated cytotoxicity
AICD	Activation induced cell death
AIF	Apoptosis inducing factor
APC	Antigen presenting cell
APM	Antigen processing machinery
AS	Activated supernatant
ATRA	All-*trans* retinoic acid
β2m	β2-microglobulin
Bcl	B-cell lymphoma
BDNF	Brain-derived neurotrophic factor
BH	Bcl-2 homology
CAD	Caspase-activated deoxyribonuclease
Caspase	Cysteinyl aspartate specific protease
CD	Clusters of differentiation
CI-MPR	Cation-independent mannose-6 phosphate receptor
COX-2	Cyclooxygenase-2
CS	Control supernatant
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DD	Death domain
DED	Death effector domain
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
DNA-PK	DNA-dependent protein kinase
DRiPs	Defective ribosomal products
EBV	Epstein-Barr virus
EFS	Event free survival
ER	Endoplasmic reticulum
ERAP	ER aminopeptidase
FADD	Fas-associated death domain
FasL	Fas ligand
FLIP	Flice/caspase-8 inhibitory protein
GD2	Disialoganglioside 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HC	Heavy chain
HLA	Human leukocyte antigen
HMGB1	High mobility group B1
HSP	Heat shock protein
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of CAD
ICAM-1	Intercellular adhesion molecule-1
IDO	Indoleamine-2, 3-dioxygenase
IFN	Interferon
IL	Interleukin
INSS	International neuroblastoma staging system
KIR	Killer immunoglobulin-like receptor

LAK	Lymphocyte activated killer
LFA-1	Lymphocyte function-associated antigen-1
LMP2/7	Low molecular weight protein 2/7
LOH	Loss of heterozygocity
L-RAP	Leukocyte-derived arginine aminopeptidase
MECL-1	Multicatalytic endopeptidase complex-like-1
MHC	Major histocompatibility complex
MIC	MHC class I related chain
MIF	Macrophage migration inhibitory factor
MSC	Myeloid suppressor cell
M-SCF	Macrophage colony-stimulating factor
NCR	Natural cytotoxicity receptor
NB	Neuroblastoma
NGF	Nerve growth factor
NK	Natural killer
PAF	Platelet-activating factor
PARP	Poly (ADP-ribose) polymerase
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PCD	Programmed cell death
PGE2	Prostaglandin E2
PI-9	Proteinase inhbitor-9
POMP	Proteasome maturation protein
PVR	Poliovirus receptor
RA	Retinoic acid
RAG	Recombinase activating gene
RAGE	Receptor for advanced glycation end products
RAR	Retinoic acid receptor
RIP	Receptor interacting protein
ROS	Reactive oxygen species
RXR	Retinoic X receptor
tBid	Truncated Bid
STAT	Signal transducer and activator of transcription
TAA	Tumor associated antigen
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGFβ	Transforming growth factor β
Th	T helper
TH	Tyrosine hydroxylase
TIL	Tumor infiltrating lymphocyte
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TNF-R	TNFα-receptor
TRAIL (-R)	TNF-related apoptosis-inducing ligand (-receptor)
Treg	Regulatory T cell
ULPB	UL-16 binding protein
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor

1. NEUROBLASTOMA

1.1 BIOLOGY OF NEUROBLASTOMA

Neuroblastoma (NB) is the most common extracranial solid tumor in childhood and the most frequent pediatric cancer after leukemia and brain tumors. It accounts for 8-10% of all childhood cancers and is responsible for as much as 15% of all childhood cancer deaths (1,2). This represents about 20 new NB cases per year in Sweden. The median age at diagnosis is 18 months, and moreover, NB is the most common cancer diagnosed during infancy (3). At the time of diagnosis, 70% of the children have metastases, and the most common sites of metastases are lymph nodes, bone marrow, bone, liver and skin (Figure 1) (2,4,5).

Neuroblastoma is a neuroectodermal tumor originating from neural crest cells and can arise anywhere along the sympathetic nervous system. More than 60% of the primary tumors originate from the tissues of the adrenal gland medulla or paraspinal ganglia in the abdominal cavity (5). NB usually occurs sporadically, yet in 1-2% of the cases there is a family history (6).

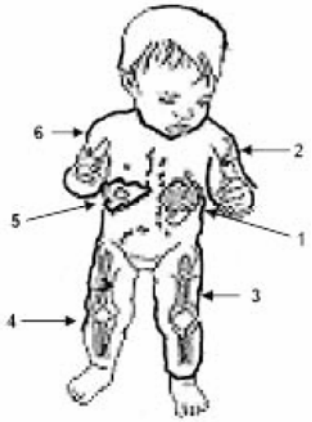

Figure 1. Neuroblastoma primary location and metastatic sites. Most primary NB tumors occur in the adrenal gland (1). Metastases spread via the lymph to distant lymph nodes (2), or via the blood; most frequently to the bone marrow (3), bone (4), liver (5) and skin (6).

NB is known for its broad spectrum of clinical behavior; some tumors undergo spontaneous regression or differentiate into a benign ganglioneuroma, while other tumors progress into an aggressive malignant form. This heterogenic nature is reflected by various genetic abnormalities. Different somatic changes such as gain of alleles, amplification of genetic material, activation of oncogenes, loss of alleles and changes in tumor cell ploidy have been reported to be implicated in the development of sporadic neuroblastoma, but so far no single genetic aberration has been found to be present in all cases and none has been identified as a causative factor in disease initiation (reviewed in (7)). One common genetic aberration in NB is the amplification of the gene *N-Myc* (8), that encodes an oncoprotein that may lead to deregulated cell proliferation when over-expressed. Amplification of *N-Myc*, seen in around 20-25% of NB patients, usually results in 50-400 gene copies per cell corresponding with high levels of protein expression (9,10). Amplification of *N-Myc* is associated with advanced

disease as well as with an unfavorable prognosis in infants with a lower stage of disease (11,12) and further correlates with resistance to certain treatment modalities (13). Therefore, *N-Myc* status is usually assessed in patients due to its strong prognostic value and is used worldwide for treatment stratification (14).

Other factors that might be responsible for regulating the malignant transformation of NB cells are the neurotrophin receptors. Neurotrophin signalling plays a central role in normal neuronal development and is mediated by the Trk family of tyrosine kinases (15). Expression patterns of the neurotrophin receptors (TrkA, TrkB and TrkC) correlate with the biological and clinical features of NB and the degree of neuroblast differentiation (16). TrkA is the cognate receptor for the nerve growth factor (NGF), and developing neurons differentiate in response to ligand or die by apoptosis when NGF is withdrawn (characteristics of a dependence receptor). Consequently, high TrkA expression is correlated with a favorable prognosis of NB (17,18). In contrary, TrkB, the receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin-4, is expressed mainly in *N-Myc*-amplified advanced-stage tumors and is thought to mediate autocrine survival in tumors which simultaneously express BDNF (19).

1.2 SPONTANEOUS REGRESSION OF NEUROBLASTOMA

Spontaneous regression of cancer is defined as the complete or partial disappearance of malignant tumor in the absence of a therapy capable of inducing anti-neoplastic effects (20). Five to ten percent of NBs that are detected regress without treatment, which is the highest frequency observed in any tumor type. Spontaneously regressing tumors are most often seen in stage 4S patients (where "S" stands for "special"). These patients are children below 1 year of age with a small primary tumor and a special pattern of disseminated disease with involvement of the skin, liver, and bone marrow (21,22). Clinically, regression of NB can be manifested as a complete disappearance of the tumor or differentiation into a benign ganglioneuroma. Relapse may develop following spontaneous regression, but is considered a rare event (23). A consistent pattern of biological factors that describes these patients has not been established, although low *N-Myc* levels and high TrkA expression are considered favorable. The three current hypotheses on the mechanism of regression is that it is due to an immunological attack on the tumor, to spontaneous differentiation/maturation, or that there is a delay in the developmental time-switch for apoptosis (20,24-26). This intriguing clinical behavior of NB has stimulated research on differentiation- and apoptosis-inducing agents for the treatment of the disease.

1.3 STAGES AND TREATMENT STRATIFICATION

The heterogeneous clinical behavior of NB prompted the development of a risk group stratification system where NB patients are assigned into low-, intermediate-, and high-risk categories based on different prognostic factors (27). The most important prognostic factors are the clinical stage, the age at diagnosis and the histological characteristics of the primary

tumor. At the time of diagnosis, tumors are staged 1-4 according to the International Neuroblastoma Staging System (INSS), governed by either a histopathologic evaluation of the tumor tissue or by the presence of tumor cells in bone marrow and the levels of urinary catecholamines (28).

In treating the low-risk patients, surgery is used as the primary approach in order to minimize the risk of chemotherapy-related morbidity (29). The survival rate for these patients is 95% (30,31). Since stage 4S patients constitute a special group of patients who often show spontaneous regression of their tumors and since they have a low risk of progression to advanced-stage disease, these patients are observed rather than operated in currently ongoing clinical trials (32). Intermediate-risk patients usually undergo surgery followed by combinational chemotherapy. It was shown that children lacking *N-Myc* amplification had a 93% 3-year event-free survival (EFS) rate while children with *N-Myc* amplification only had a 10% 3-year EFS rate, again pointing at the clinical significance of this marker (33). The majority of NB patients belong to the high-risk-group. Treatment for high-risk patients can be divided into three phases: 1) induction therapy (chemotherapy); 2) consolidation therapy (myeloablative chemotherapy and stem cell rescue (e.g. autologous bone marrow transplantation (ABMT)) and; 3) minimal residual disease therapy (e.g. administration of retinoids or targeted molecules directed against GD2 on NB cells (see below)). Up to date, the standard treatment for children with high-risk NB is intensive myeloablative therapy supported by autologous hematopoietic stem-cell transplantation and the administration of retinoids. The overall survival has improved reasonably for high-risk NB patients the last decades but cure rates remain low (10-20% long-term survival) despite intensive multimodal therapy (2). Current approaches to improve long-term survival aim at increasing dose intensity of the most active chemotherapeutic agents, the use of very high-dose myeloablative chemoradiotherapy followed by ABMT or the use of biologic therapy. Therefore, in the search for effective biological therapies for patients with high-risk NB, the development of different immunotherapeutic approaches has gained much interest.

1.4 TREATMENT OF MINIMAL RESIDUAL DISEASE

Although the outcome for high-risk patients has improved, more than 50% of the children will relapse due to drug-resistant residual disease. Recurrent tumors may arise from chemotherapy-resistant tumor cells or from tumor-cell contaminated stem cell infusions. In treating minimal residual disease, tolerable non-myelotoxic therapies must be applied. Apart from being non-myelotoxic, this therapy cannot be cross-resistant with the chemotherapy used. There are at present two approaches available in clinical trials for treatment of minimal residual disease; the use of retinoids and anti-GD2 antibodies administered with or without cytokines.

1.4.1 Retinoid therapy

The clinical observation that neuroblastomas often show regression by differentiation *in vivo* inspired researchers to study the effects of various differentiation agents on NB *in vitro* (34-

36). Retinoic acid (RA) was shown to induce growth arrest, neurite outgrowth and apoptosis in human NB cells as well as a reduction of *N-Myc* expression *in vitro* (37,38), which prompted the clinical application of RA derivatives (retinoids). Multiple studies have shown that retinoids can antagonize radiation and various cytotoxic agents (39-41). Therefore, retinoids are only applied to high-risk NB patients, targeting minimal residual disease, after completion of radiation and chemotherapy.

RA is a derivate of Vitamin A which enters the nucleus where it exerts its biological function through receptors belonging to the ligand-activated steroid/thyroid hormone superfamily of transcription factors, eventually leading to a transcriptional regulation of different target genes. The two natural isomers of RA, all-*trans*-RA (ATRA) and 9-*cis*-RA, activate different receptors. There are two families of RA receptors, namely RAR (α,β,γ) and RXR (α,β,γ).

Encouraged by the promising *in vitro* studies on ATRA-induced differentiation of NB cells, retinoid treatment was transferred into the clinic. Importantly, although the first trials did not show the excpected clinical beneficial effect it was concluded that RA did not cause myelosuppression, suggesting that RA could be used with benefit as a maintenance therapy after intensive chemotherapy and bone marrow transplantation without compromising hematopoiesis. Moreover, it was decided that RA therapy should be used to treat patients with minimal residual disease rather than patients with progressing or recurrent tumors (42,43). The seminal phase III trial that demonstrated a clinical effect of RA included high-risk patients that were randomized firstly to either myeloablative therapy (combination chemotherapy plus total body irradiation) supported by purged ABMT or to intensive non-myeloablative chemotherapy, and secondly to either no further therapy or to receive 13-*cis*-RA (44). Results from this study showed that treatment with 13-*cis*-RA showed significant benefit compared to no further treatment (EFS of 46% and 29% respectively).

Resistance to retinoids is seen in about 50% of children through an as of yet unknown mechanism (45). This resistance cannot be attributed to differences in the expression of RA receptors since the RAR and RXR receptors are expressed in most NB cell lines and primary tumors (46,47). However, the relative contribution of each receptor subtype to the effects of RA is unclear (41). In order to overcome resistance and reduce toxicity, a number of new synthetic retinoids are developed and tested for their efficacy on NB (45,48). RO 13-6307 and Fenretinide (4-HPR) represent two such synthetic retinoids (49-51).

1.4.2 Anti-GD2 therapy

Besides retinoid therapy, antibodies directed against the glycolipid GD2, expressed on the surface of NB cells, are currently in use for treatment of minimal residual disease. This form of therapy will be discussed below in the section dealing with NB as an immunotherapeutic target.

2. THE IMMUNE SYSTEM

The human immune system comprises a highly intricate network of different components. In the following section some of the components of importance for the work described in this thesis will be described.

2.1 THE MHC

2.1.1 MHC class I molecules and peptide transport

Major histocompatibility complex (MHC) class I molecules present peptides derived from intracellular proteins, including viral and tumor antigens, to $CD8^+$ T cells. MHC class I molecules consist of a trans-membrane α-chain and the non-covalently attached non-polymorphic β2-microglobulin (β2m) chain which is encoded outside the MHC locus (52). The α-chain of the MHC class I molecule is folded into three domains, α1, α2 and α3, of which the α1 and α2 domains form the peptide binding cleft. Peptides bind to MHC class I non-covalently via hydrogen bonds and ionic interactions, and are usually between 8-10 amino acids long (53,54). Most MHC class I binding peptides are generated by the degradation of newly synthesized misfolded proteins by the 26S proteasome, the major proteolytic machinery in the cytoplasm (see below) (55,56). Up to 80% of the MHC class I epitopes are believed to derive from so-called DRiPs (defective ribosomal products) that constitute defective proteins which are rapidly degraded following synthesis (57,58). While the majority of peptides generated by the proteasome are subsequently degraded into amino acids, some of these peptides will be translocated into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) complex (heterodimers of TAP1 and TAP2), encoded within the MHC locus and inducible by interferon-γ (IFNγ) (59). Each TAP monomer contains a hydrophobic domain and an ATP-binding domain. When peptides bind to TAP, a conformational change leads to ATP hydrolysis and pore opening, and peptides can be translocated into the ER lumen (60). The TAP complex optimally transports peptides of 9-12 amino acids in length with preferably hydrophobic and basic C-termini, suggesting that further N-terminal trimming of the peptides occurs in the ER (61). Indeed, it was demonstrated that aminopeptidases in the ER such as ERAP1 (ER aminopeptidase-1) and L-RAP (leukocyte-derived arginine aminopeptidase) are able to trim the N-terminal of peptides delivered by TAP (62-64).

2.1.2 MHC class I complex assembly

Newly synthesized MHC class I α-chains bind to the ER chaperone calnexin, although it has been suggested that another chaperone, the immunoglobulin binding protein (BiP), can functionally replace calnexin (65-67). The partially folded MHC molecules will upon binding to β2m dissociate from calnexin and instead bind to the "MHC class I peptide-loading complex", which includes the chaperone calreticulin, ERp57, the two subunits of TAP and tapasin (TAP associated glycoprotein) (68-70). The function of this complex is to retain MHC class I molecules within the ER until they are loaded with high-affinity peptides. Tapasin is a

trans-membrane ER-resident protein that associates TAP with the MHC class I molecule until a "proper" peptide will bind (71,72). Tapasin also recruits the oxidoreductase ERp57, which is believed to be crucial for oxidation of the class I heavy chain, and necessary for loading of class I molecules with high affinity peptides (73). ERp57 further supports formation of the intra-chain disulfide bonds within the class I molecule that are essential for its proper assembly (74). It is believed that MHC class I molecules first bind "suboptimal" peptides that are subsequently exchanged for peptides of higher affinity, mainly via the action of tapasin (75-77). Upon binding of a peptide with an optimal length and anchor residues, the MHC class I:peptide complex will dissociate from the loading complex and persist in the ER until transported via the Golgi to the cell surface through potential interactions with specific transport receptors at ER exit sites (78,79). Class I molecules that do not bind peptides will be transported back into the cytosol where they will be degraded by the proteasome (ER-associated degradation, ERAD) (80,81). Altogether, all these ER proteins serve to make sure that only properly assembled MHC class I molecules that bind peptides stably will leave the ER and be exposed on the cell surface for recognition by $CD8^+$ T cells (Figure 2) (82).

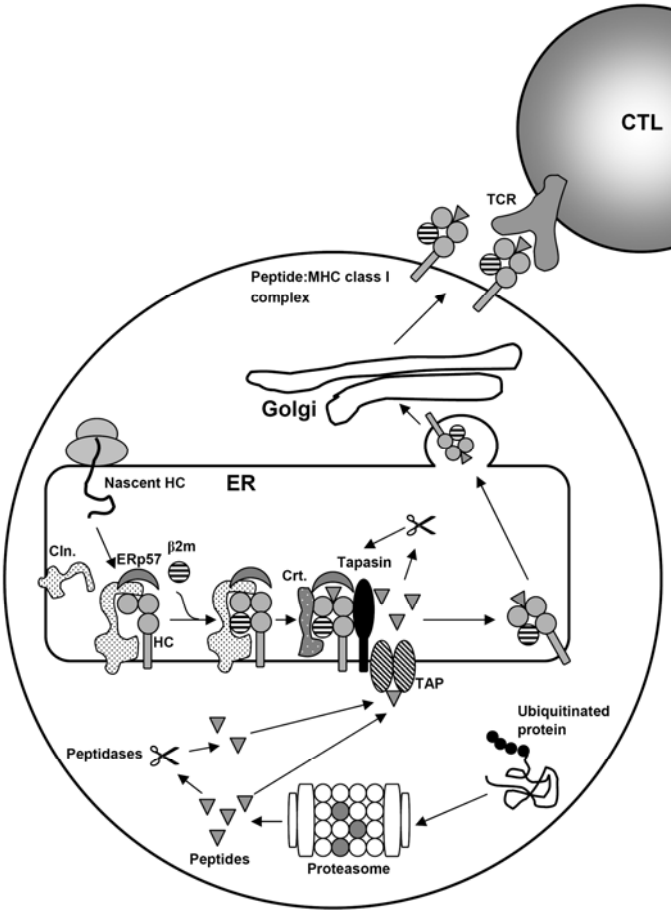

Figure 2. The MHC class I antigen processing and presentation pathway. Proteins conjugated with multiubiquitin chains are targeted to the 26S proteasome for degradation. The resulting peptides may be further trimmed by cytotoslic peptidases and then transported into the ER by the TAP complex. Within the ER, peptides may be trimmed to appropriate sizes by ER-resident aminopeptidases and bind and stabilize the MHC class I heterodimers. The assembly of MHC molecules is coordinated by molecular chaperones. The nascent MHC class I heavy chain (HC) is translocated into the ER where it interacts with calnexin (Cln.) and the thiol oxidoreductaes ERp57. Upon assembly with β2m, calnexin is replaced by calreticulin (Crt.). The TAP complex and its associated tapasin are recruited to form the peptide loading complex. Upon peptide binding, the loading complex disassembles and the peptide:MHC complex will be clustered at ER export sites. Finally, the complex is transported via Golgi to the cell surface where it can be recognised by CTLs harbouring the specific T cell receptor (TCR).

2.2 THE UBIQUITIN-PROTEASOME SYSTEM

The proteasome, a large multi-catalytic protease complex, is responsible for most of the proteolytic activity in the cytosol and it is generally believed that processing of most MHC

class I antigens is mediated by this pathway (83). The process of protein degradation is tightly regulated in order to prevent non-specific destruction of essential proteins and is kept at steady state levels. Proteins are targeted for proteasomal degradation by the conjugation of a poly-ubiquitin chain to lysine residues (reviewed in (84)).

The 26S proteasome normally consists of the barrel-shaped 20S core harboring the catalytic activity, and two 19S regulator complexes. The 19S regulators recognize poly-ubiquitin chains on proteins that are tagged for degradation and are involved in unfolding and channeling of the proteins into the 20S core (85,86). The 20S core consists of two rings composed of seven α-subunits (α1-α7) that line two rings of seven β-subunits (β1-β7). The three β-subunits β1, β2 and β5 hold the catalytically active sites on their inner surface with three different cleavage preferences; acidic-, basic- or hydrophobic- activity, respectively. In mammalian cells, a combination of 20S poteasomes either non-capped by 19S, capped by one 19S or by two 19S can be found (84,87).

2.2.1 The immunoproteasome

The major role of the protease system in the cell is to eliminate regulatory, nonfunctional, excess and potentially toxic proteins in order to maintain the cell's protein homeostasis. Therefore, most proteins are believed to be degraded into single amino acids and only as little as 1%, or less, of the peptide pool generated will be presented to the immune system (58,88). For efficient MHC class I antigen presentation, the pool of available peptides needs to be increased. One way of achieving this is by inducing the antigen-processing efficiency of the proteasome, where the best known enhancer of antigen presentation is IFNγ. Two catalytic subunits of the proteasome, LMP2 (β1i) and LMP7 (β5i), encoded within the MHC, and a third, not encoded in the MHC region, MECL-1 (β2i), were shown to be inducible by IFNγ (89). Upon IFNγ induction, these subunits replace the three catalytically active β-subunits of the constitutive proteasome to generate the immunoproteasome, which results in an altered enzymatic specificity of the complex, enhancing the cleavage after hydrophobic and basic residues and decreasing the cleavage after acidic residues (Figure 3). Since MHC class I molecules preferentially bind peptides with hydrophobic C-termini, the immunoproteasome is believed to improve MHC class I antigen presentation. It is assumed that all three IFNγ-inducible subunits have to be incorporated into the immunoproteasome for its full function (90). It was shown that cancer cells often display a nonfunctional variant of LMP7 in response to IFNγ which will result in an immunoproteasomal deficiency (91).

IFNγ also induces non-proteolytic proteasomal components such as the proteasomal activator PA28, shown to improve antigen presentation as well as the recently described proteasome maturation protein (POMP) that recruits LMP7 to the complex. (92,93). The PA28 activator is a six- or seven-membered ring composed of PA28α and PA28β subunits and attaches as a cap to both ends of the 20S proteasome. Binding of the PA28 activator to the proteasome increases the spectrum of peptides generated, possibly by inducing double peptide cleavage.

Another reason could be the increased efflux of peptides from the PA28-bound proteasome, which allows potentially antigenic peptides to escape additional processing by the proteasome (94-97).

IFNγ is a first line cytokine to be released from activated cells of the immune system, including activated T- and NK cells, and is often referred to as the "golden enhancer" of antigen processing and presentation. By upregulating several proteins involved in the MHC class I processing and presentation pathway (i.e. the MHC molecule (both heavy chain (HC) and β2m), TAP1, TAP2, tapasin, LMP2, LMP7, MECL-1, POMP, PA28 and aminopeptidases), it is ensured that peptides are presented more efficiently when needed (90). It should be noted that apart from IFNγ, other cytokines and substances have the ability to modulate the MHC class I antigen presentation machinery. While tumor necrosis factor-α (TNFα) and retinoids have a positive effect on the machinery (98,99), transforming growth factor-β (TGFβ) and IL-10 are known to inhibit antigen presentation (100,101).

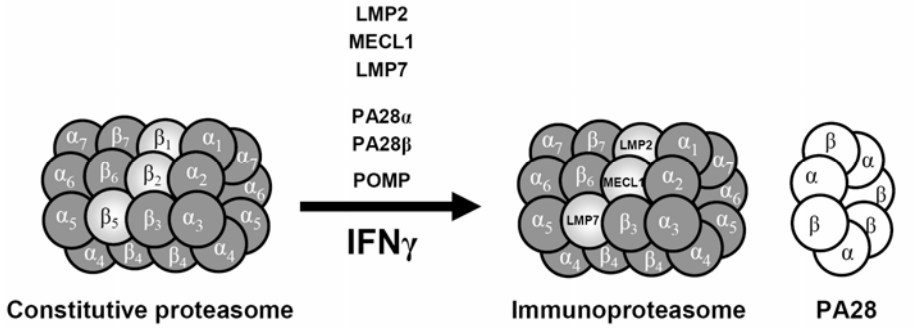

Figure 3. Formation of the immunoproteasome. IFNγ induces the synthesis of the three immunoproteasomal subunits LMP2 (β1i), MECL1 (β2i) and LMP7 (β5i), the proteasome maturation protein (POMP), and the proteasome activator PA28 subunits PA28α and PA28β. PA28 binds to the outer rings of the 20S proteasome, thereby opening the central gate and facilitating protein entry. For simplicity, the regulator complexes attached to the constitutive proteasome are omitted from the figure (Adapted from Strehl, Imunological Reviews 2005, 207: 19–30).

2.3 STABILITY OF PEPTIDE:MHC COMPLEXES AT THE CELL SURFACE

Once on the surface, the stability of the peptide:MHC class I complex partly determines the immunogenicity of the cell presenting the antigen. It is now accepted that the dissociation rate of a particular peptide, rather than a peptide's binding affinity is the major factor that determines the stability of the peptide:MHC complex. Thus a peptide with a low dissociation rate will, once bound, form a stable peptide:MHC class I complex in the ER, be transported to the cell surface, and persist there for a time sufficient to allow T cell recognition (102). Both IFNγ and TNFα induce an increased stability of peptide:MHC class I complexes on the cell surface. This is probably due to the upregulation of proteins necessary for efficient antigen processing by the proteasome, peptide transport and also some ER-resident proteins involved in quality control of MHC class I peptide loading (tapasin), thus resulting in the generation of complexes containing high-affinity peptides (98,103).

2.4 NON-CLASSICAL HLA MOLECULES

Human leukocyte antigen (HLA) molecules encoded by the A-, B-, and C-loci are usually referred to as classical HLA class I molecules whereas HLA-E and HLA-G, though mapped within the human MHC locus, are defined as non-classical HLA class I molecules. The non-classical HLA molecules present a restricted repertoire of peptides, demonstrate a low degree of polymorphism and show a more restricted tissue distribution as compared to classical HLA class I molecules (104,105). The highly polymorphic MHC class I chain related (MIC) molecules are often linked to the non-classical HLA molecules, however they do not combine with β2m and do not bind peptides (106-108).

2.4.1 HLA-G

HLA-G was initially found on placental trophoblasts, where it is involved in conferring immune privilege by suppressing immune responses against the fetus (109). However, HLA-G has now been found on other normal tissues as well as on malignant cells (110,111). HLA-G transcripts resulting from alternative splicing encode membrane-bound and soluble isoforms. Both forms of HLA-G bind the same limited set of peptides derived from intracellular proteins (112,113). HLA-G induces inhibition of effector cells, including NK cells and T cells, by binding to the receptors ILT-2, ILT-4 or KIR2DL4 present on these cells (114-116). Furthermore, HLA-G might exert its immunosuppressive effect indirectly through binding of its leader sequence to stabilise HLA-E, thus inhibiting CD94/NKG2A-expressing natural killer (NK) cells or antigen-specific cytotoxic T lymphocytes (CTLs) (117-119).

2.4.2 HLA-E

HLA-E is ubiquitously transcribed in the majority of human tissues, however, cell surface expression of HLA-E is rarely observed. The reasons for low HLA-E surface expression was suggested to be caused by an inefficient peptide loading in the ER or by a low affinity interaction between the HLA-E HC and β2m which may prevent its assembly and stability at the cell surface (113,120,121). In contrast to the classical HLA molecules, HLA-E has a very specific peptide-binding groove, preferentially binding HLA class I leader peptides (122). HLA-E serves as a ligand for the CD94/NKG2A, -B, and -C receptors, where NKG2A and -B are inhibitory and NKG2C activating (123). Although HLA-E can present peptides to HLA-E-restricted T cells and induce regulatory T cell populations (124-126), the best described function of HLA-E is its inhibitory activity on CD94/NKG2A-expressing cells which constitute most NK cells, γδ T cells and a subset of αβ $CD8^+$ T cells (117).

2.4.3 MIC

MIC mRNA expression is found in many cell types, but the expression of the MIC protein is only well documented on the cell surface of gastric epithelial cells, endothelial cells and fibroblasts. However, in response to stress stimuli such as cellular transformation, heat-shock, viral infection, or genotoxic stress, MIC proteins are induced, especially on cells of epithelial origin (108,127). It has recently been demonstrated that genotoxic stress that induces the DNA damage pathway will lead to an upregulation of MIC molecules, directly linking the

tumorigenesis process to enhanced immune recognition (128,129). The MIC proteins act as ligands for the activating NKG2D receptor expressed by NK cells, γδ T cells and αβ $CD8^+$ CTLs. In T cells, NKG2D act as a costimulatory receptor in a manner similar to CD28 (130). Consequently, expression of NKG2D ligands renders target cells sensitive to NK lysis and lead to costimulation of antigen-specific T cells. Other ligands binding to NKG2D are the family of UL-16 binding proteins ULBP1, ULBP2 and ULBP3 (131).

Whether the signal delivered by MIC is sufficient to cause activation of NK cells *in vivo* probably depends on the relative contribution of NK cell activating and inhibitory receptors expressed by effector cells as well as on the strength of MIC binding (131,132). It has been proposed that NKG2D-mediated signalling may override the inhibition mediated by MHC class I-specific NK inhibitory receptors (133). NKG2D may provide a first line surveillance against stressed or abnormal cells, which is indicated in experiments showing that certain NKG2D haplotypes are associated with a decreased risk of developing cancer (134), and was suggested as a good prognostic factor in colon cancer (135). A higher frequency of loss of membrane-bound MIC was seen in metastatic melanoma lesions as compared to primary lesions, suggesting that in its membrane-bound form, MIC can be a target for immune selection in the course of malignant disease (136). The induction of functional NKG2D ligand expression could potentially be exploited for immunotherapeutic purposes. In a study on hepatocellular carcinoma it was shown that MIC molecules were upregulated by retinoic acid, further activating NK cells, suggesting that RA may be useful for increasing innate immunity against cancer (137).

2.5 CYTOTOXIC T LYMPHOCYTES

The immune system interacts with tumors via multiple cellular subsets and effector molecules. Below, I will focus on the role of one particular component of anti-tumor immunity, namely CTL-mediated immune responses.

Given that most tumors may express MHC class I molecules, which serve as the restriction element for $CD8^+$ T cell recognition, but do not usually express MHC class II molecules which are required for $CD4^+$ T cell recognition, the predominant anti-tumor effector cells capable of direct tumor recognition are the $CD8^+$ CTLs. In addition to the expression of specific MHC:peptide complexes, the expression of adhesion molecules such as intercellular adhesion molecules (ICAMs) that bind to the integrin LFA-1 (lymphocyte function-associated antigen-1) expressed on T cells is also crucial for tumor recognition by CTLs. Upon recognition of a specific MHC complex, a conformational change in LFA-1 will increase the adhesion between the tumor cell and the CTL (138).

2.5.1 Effector functions of CTLs

Cytotoxic T cells kill their targets via two major pathways, namely the granule-mediated and the death receptor-mediated (reviewed in (139)). In addition, CTLs secrete lymphokines such as IFNγ and TNFα, which either directly or indirectly cause tumor cell death. Upon activation,

$CD8^+$ T cells upregulate their cytotoxic machinery, including the expression of granule components and the increased expression of surface death ligands (139-142). A schematic view of the effector functions of CTLs is depicted in figure 4.

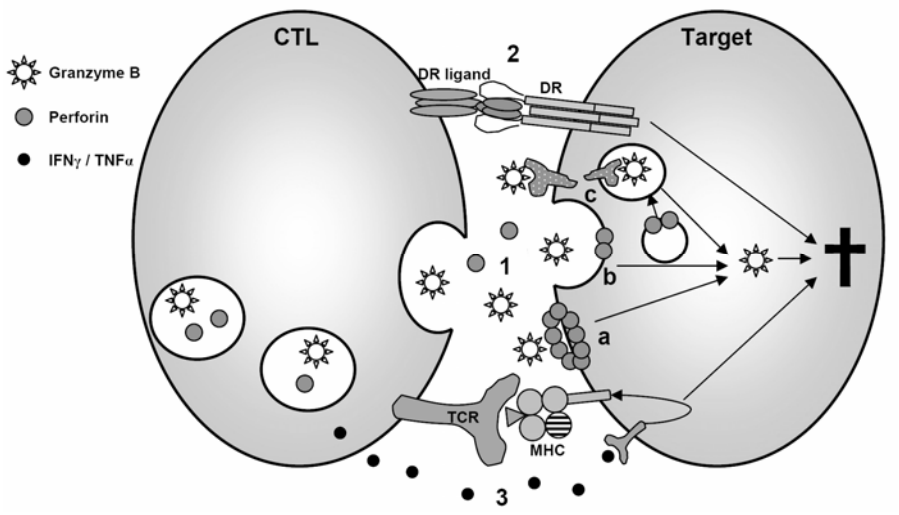

Figure 4. Effector functions of CTLs. A CTL is activated upon recognition of its target cell via specific TCR-MHC:peptide interactions and can induce target cell death via three major pathways. **(1)** The granule-mediated pathway. Modes of uptake of perforin and granzyme B in target cells; (a) perforin induces membrane damage through pore formation (b) granzyme B enters target cell via reparative endocytosis, or (c) by receptor-mediated uptake. **(2)** The death receptor (DR)-mediated pathway. **(3)** The production and secretion of pro-inflammatory cytokines such as IFNγ and TNFα may induce direct target killing and induce the levels of MHC class I on the surface of cells, resulting in better recognition by CTLs.

2.5.1.1 The granule-mediated pathway

Perforin/granzyme-mediated killing is rapid and ensures high specificity; only the cell on which the peptide:MHC complex is recognized will be eliminated as the microtubule-organizing center of the T cell is reorganized upon target recognition and granules are directed towards the immunological synapse. The cytolytic granules contain multiple proteins of which perforin and granzyme B are the most prevalent. Other components of the lytic granules include the granzymes -A, -C, -H, -K, -M, serglycin, calreticulin, granulysin, cathepsin B, cathepsin C and FasL (139,143).

2.5.1.1.1 Perforin

Once released in close proximity to the target cell membrane and in the presence of Ca^{2+}, perforin molecules polymerize in the target cell membrane. The immunological synapse creates a tight seal that prevents granule contents to leak out and damage neighbouring cells. The perforin released into the extracellular space may probably react nonspecifically with lipids in the presence of Ca^{2+}, leading to inactivation of the protein (144). This serves as an important mechanism for rescuing neighboring cells not harboring the specific peptide:MHC complex. It is believed that at least 3-4 monomers of perforin are required for pore formation. Perforin is synthesized as a 70 kD pro-form that requires proteolytic processing within the granules to generate the active 60 kD form, however, the processing enzyme has not yet been identified (145). Whether perforin binds directly to the cell membrane or to a specific receptor

in the target cell membrane is unclear. It was shown that NK cells release the platelet-activating factor (PAF) together with perforin. Perforin-sensitive cell lines expressed the corresponding PAF receptors whereas perforin-resistant cell lines did not, suggesting that PAF expression by the target cell is necessary for the lytic capacity of perforin (146).

It was previously believed that perforin is the major cytotoxic molecule, killing cells by creating channels in the target cell membrane, allowing water and salts to pass rapidly into the cell. Later, granzyme B was identified as a molecule indispensable for cytolytic capacity and perforin channels were merely looked upon as entry points for granzyme B into the target cell. However, it has now been demonstrated that the perforin channels are too narrow for granzyme B to pass through (147).

2.5.1.1.2 Granzymes

The granules also contain a group of serine proteases called granzymes, where granzyme B is the most predominant. Upon release, granzyme B is secreted complexed with the negatively charged proteoglycan serglycin (148). Serglycin is thought to act as a chaperone, protecting the positively charged granzyme and, possibly, to mediate binding of cytolytic granule components to the target cell membrane. Since it was shown that granzyme B, and especially when complexed to serglycin, is too big for entering through the perforin pores, this hypothesis of granzyme B entry is not considered anymore and others were suggested (149). One additional hypothesis is offered by the reparative endocytosis theory, which states that upon perforin binding the cell repairs itself by endocytosing the perforin-containing membrane parts, and concomitantly endocytose granzymes. Another hypothesis is that granzyme B may enter the cell through receptor-mediated endocytosis. In this model perforin is thought to mediate the exit of granzyme B from the endosomes into the cytoplasm. Although many putative granzyme B receptors have been proposed such as the cation-independent mannose-6 phosphate receptor (CI-MPR) (150), CD44 (151), heat shock protein (HSP) 70 (152), and heparan sulfate glycosaminoglycans (HS-GAGs) (153), there is no final agreement on which one plays the major role. Alternatively, as stated above, granzyme B may enter the cell with the help of serglycin binding to receptors on the target cell surface (151).

The granzymes are proteases that do not trigger apoptosis in the target cell by directly fragmenting the DNA, one of the endpoints of apoptosis. Instead granzyme B can cleave pro-caspase-3 and other pro-caspases into their active forms. Active caspase-3 subsequently activates the nuclease CAD (caspase-activated deoxyribonuclease) by cleaving an inhibitory protein (ICAD) that binds to and inactivates CAD. This enzyme is believed to be the final effector of apoptotic DNA degradation. However, it has been shown that granzyme B can induce apoptosis in the absence of functional caspases by directly cleaving ICAD to activate CAD (154). Granzyme B may also cause mitochondrial depolarization by inducing Bid cleavage, a pathway thought to merely augment the death signal. In addition, granzyme B can directly cleave other caspase substrates such as poly (ADP-Ribose) polymerase (PARP),

DNA-dependent protein kinase (DNA-PK) (both functioning in DNA repair) and lamin B (one of the main structural components in the nuclear envelope) (143).

2.5.1.2 The death receptor-mediated pathway

In contrast to the perforin/granzyme-mediated killing, death receptor-mediated killing displays slower kinetics. Once on the cell surface, the death ligands have a long half-life and may also result in bystander killing of proximate target cells that do not express the specific antigen initially recognized by the CTL. Activated cytotoxic lymphocytes upregulate a number of ligands of the TNF superfamily of receptors on their surface. The best characterized are TNFα, Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL), all of which can be produced in both membrane-bound and soluble forms. The molecular mechanism of signalling through these receptors will be discussed in section 5.1.1.

2.5.1.2.1 FasL

FasL expression is increased upon activation of CTLs, NK cells and T helper 1 (Th1) cells, and it plays an important role in both immune homeostasis and T cell- and NK cell-mediated toxicity (155-157). The role of Fas:FasL interactions in immune homeostasis was observed when mice deficient in either Fas (*lpr*) or FasL (*gld*) developed massive lymphoid hyperplasia. Fas-mediated apoptosis, mainly induced by $CD4^+$ T cells, plays a major role in regulating peripheral immune responses via activation-induced cell death (158). Moreover, experimental evidence supports a role for Fas/FasL in tumor surveillance. *Gld* and *lpr*-mice are prone to develop malignant lymphomas, especially when these mice are back-crossed to tumor-predisposed mice (159,160). Deficient Fas/FasL expression has also been correlated with a more aggressive tumor phenotype, contributing to metastatic progression in a mouse melanoma model (161).

2.5.1.2.2 TRAIL

TRAIL was also indicated as an important player in immune surveillance against tumor cells (162-164), especially in situations when tumor cells show resistance to FasL-mediated killing (165). Furthermore, TRAIL has been shown to selectively induce apoptosis in a variety of tumor cells but not in normal cells, and has therefore gained interest as a promising agent for cancer therapy (166).

2.5.1.3 Pro-inflammatory lymphokines

Upon activation, CTLs secrete a number of cytokines including IFNγ and TNFα. Besides being able to induce direct killing of tumor cells, both IFNγ and TNFα mediate upregulation of MHC class I and other proteins involved in the antigen processing and presentation machinery, leading to a more efficient recognition of the target cell (described above). Graubert et al. showed that T cell-mediated cytotoxicity could not be completely attributable to perforin/granzyme and FasL, suggesting that secretion of TNFα and IFNγ may be important (167). It should be noted that TNFα may also have a tumor-promoting role (168,169).

Taken together, it is now established that the perforin/granzyme- and Fas/FasL-mediated killing pathways are the main effector mechanisms used by activated CTLs in eliminating target cells (141,170,171). When these cytolytic functions are not involved, other killing mechanisms, such as TRAIL or the pro-inflammatory cytokines IFNγ and TNFα, may have important roles in rejection of tumors and metastases (163,172-175).

2.6 NK CELLS

NK cells constitute an essential component of innate immunity against tumors and virally infected cells (176). NK cells can induce target cell death through a directed release of cytotoxic granules containing perforin and granzymes and/or ligands engaging death receptors on the target cell such as FasL or TRAIL. Furthermore, upon stimulation NK cells can secrete various cytokines and chemokines which can have direct cytotoxic effects, activate other cells or induce cell differentiation (177). The function of NK cells is controlled by activating and inhibitory receptors as described above. The inhibitory receptors include the HLA class I-specific killer immunoglobulin-like receptors (KIRs) and the HLA-E receptor CD94/NKG2A. The interaction of HLA molecules expressed on normal cells with HLA class I-specific NK receptors results in protection of these cells from NK-mediated lysis. However, in the absence of this inhibitory interaction as in the case of HLA-defective targets such as tumor cells or virally infected cells, target cell killing depends on the binding of ligands expressed by target cells to activating receptors expressed at the NK cell surface. Important activating receptors are the natural cytotoxicity receptors (NCRs) NKp46, NKp30 and NKp44 as well as NKG2D and DNAX accessory molecule-1 (DNAM-1). Most of the ligands are so far unidentified with the exception of e.g. the MIC molecules which bind NKG2D, and the poliovirus receptor (PVR) and Nectin-2 that bind DNAM-1 (reviewed in (178)).

3. IMMUNE CONTROL OF CANCER; NEUROBLASTOMA IN FOCUS

In 2000, Hanahan and Weinberg proposed six hallmarks of cancer that need to be fulfilled in order for a tumor to grow and survive: 1) self-sufficiency in growth signals, 2) insensitivity to growth-inhibitory signals, 3) evasion of apoptosis, 4) unlimited replicative potential, 5) sustained angiogenesis, and 6) tissue invasion and metastasis (179). Whether and how cancer cells can be recognized by the immune system was for long a matter of debate. As cancer cells are inherently genetically instable (180), both genetic and epigenetic changes may potentially give rise to neoantigens which could serve as targets for effector cells of the immune system. Throughout this section some text will appear in gray-shaded areas in which I have summarised what is known about NB relevant to the respective paragraphs.

3.1 TUMOR ASSOCIATED ANTIGENS

In the 1960's it was shown that mice could be immunized against syngeneic transplantable tumors and rejected a secondary challenge with the same tumor cells. Thereby, it was concluded that tumor-specific antigens existed and that tumor-specific immune responses could occur (181,182). Following this initial finding many studies demonstrated that target structures on tumor cells were recognised by cells of the immune system. After the discovery of the role of HLA class I molecules in antigen presentation, many tumor antigen recognised by T lymphocytes have been described (183-186). Tumor associated antigens (TAAs) can be subdivided into several different groups; (i) antigens that are also expressed during some stages of differentiation in normal cells from the same lineage, e.g. MART-1 (differentiation antigens), (ii) antigens that arise as a result of mutations or rearrangements, e.g. mutated p53 (mutated antigens), (iii) antigens that are over-expressed or amplified in tumors, e.g. Her-2/neu (over-expressed/amplified antigens), (iv) antigens encoded by cellular genes, normally only expressed in male germ cells in the testis, e.g. NY-ESO-1 (cancer-testis antigens), and (v) antigens encoded by viral oncogenes, e.g. human papillomavirus (HPV) proteins E6 and E7 (viral antigens).

There are a few NB-associated tumor antigens described in the literature up to date that could potentially be used as targets for immunotherapy. In fact, NB was among the first human tumors against which a cell-mediated immunity to TAAs was detected (187). Most therapeutic approaches documented so far have been directed towards the glycolipid GD2, over-expressed in all tumors of neuroectodermal origin including NB (188). It has also been demonstrated that NB cells express antigens that may be recognised by CTLs. Among those is the proto-oncogene N-Myc that is highly amplified in advanced-stage NB tumors (8). Several studies have also demonstrated the expression of germline antigens such as the MAGE family of proteins and NY-ESO-1 in NB (189-193). Other NB-associated antigens that could be potential targets for immunotherapy include PRAME (preferentially expressed antigen of melanoma) (194), survivin (195), GD2 synthase (196), and telomerase (197). DNA-based vaccination strategies have also been directed against tyrosine hydroxylase, an enzyme which is involved in the catecholamine biosynthesis (198). Potentially TrkB could also serve as a

target for immune responses, since its expression is a marker of advanced-stage NB and it is correlated with the progression of the disease (199). In section 4, I will return to immunotherapeutic strategies targeting some of these antigens expressed by NB.

3.2 CANCER IMMUNE SURVEILLANCE

When Hanahan and Weinberg proposed the six hallmarks of cancer mentioned above, they did not stress a role for the immune system in the control of tumors. However, over the years it has now been proven that the immune system can both recognize cancer cells, and actually reject tumors. Therefore, it has been proposed that the seventh hallmark of cancer should be "the capacity of a malignant cell to evade the extrinsic tumor suppressor functions of the immune system" (179,200,201). Nevertheless, the concept of cancer immune surveillance is still a subject of debate.

3.2.1 The concept of tumor immune surveillance

The initial concept of tumor immune surveillance was first introduced as the so called "Immune surveillance hypothesis" by Thomas in 1959 (202) and by Burnet in 1970 (203), stating that tumors arise repeatedly similar to pathogen infections, and are constantly recognized and eliminated by the immune system based on the recognition of TAAs. The seminal experiments that provided evidence for the hypothesis demonstrated that a higher incidence of spontaneous tumors and chemically induced tumors was observed in IFNγR1-/- and IFNγ-/- (173,204-206), perforin-/- (171,207,208), recombination-activating gene (RAG)-/- (209,210) mice than in control mice. It was also shown that tumors developing in RAG-deficient animals were rejected in immune competent mice, strengthening the immune surveillance hypothesis (201,209). More recently, the role of the activating receptor NKG2D in immune surveillance of MIC-expressing tumors was also demonstrated (211). Thus, both innate and adaptive parts of the immune system have been shown to play a role in cancer immune surveillance.

There are obvious problems in studying the immune surveillance of non-viral tumors arising in immune compromised patients as (i) their high susceptibility to pathogens and (ii) that spontaneous tumors develop more slowly and might never be found during the relatively shorter lifetime of these patients. However, there have been some epidemiological studies reporting a higher probability to develop different tumors of non-viral etiology in immunodeficient humans (212,213). What also supports the existence of cancer immune surveillance in humans is the positive correlation observed between tumor infiltrating lymphocytes (TILs) and an increased patient survival (214-216).

Immune surveillance and cell-based immunotherapy of NB has not been studied in depth over the years. This could possibly be due to researchers being discouraged by the fact that NB cells often are devoid of MHC antigens (193,217). Up to date, there are only a few studies on NB where a correlation between the presence of lymphocyte infiltrates and patient survival was investigated. Interestingly, a positive correlation was found, suggesting that immune

surveillance of NB may take place (218-220). Furthermore, early studies suggested that one of the mechanisms behind spontaneous regression of NB could be an immune-mediated clearance of the tumor cells (24).

3.3 IMMUNOEDITING AND TUMOR ESCAPE

Even though cancer immune surveillance exists, cancer still occurs in immunocompetent individuals. As stated above, tumors formed in the absence of an intact immune system are more immunogenic than tumors that arise in immunocompetent hosts. Tumors may thus be sculpted by the immunological environment in which they develop, and they are rendered less immunogenic and more resistant to immune attack. Sculpting by the immune system is thought to be facilitated by the fact that tumors are inherently genetically instable. Following this, Dunn, Old and Schreiber proposed the usage of a broader and modernized term for cancer immune surveillance, which by itself only describes the host-protecting effects of the immune system (201). The new term "cancer immunoediting" also encompasses the findings that the immune system may facilitate tumor progression by sculpting the immunogenic phenotype of tumors as they develop. The process of cancer immunoediting includes three phases; elimination, equilibrium and escape. During the elimination phase, if successful, the tumor is eradicated. If tumor cells survive the elimination phase the equilibrium phase is initiated in which a Darwinian selection process is taking place where lymphocytes exert selection pressure on the tumor cells. Tumor cells that in some way gain a growth advantage due to this selection pressure may then enter the escape phase and become clinically detectable. Cancer cells may avoid immune surveillance through two major mechanisms; immunoselection, which is the outgrowth of poorly immunogenic tumor cell variants, and immunosubversion, an active suppression of the immune response. Below I will summarise what is known about some of these different mechanisms used by tumor cells. The interplay between tumor progression and the immune system is schematically illustrated in figure 5.

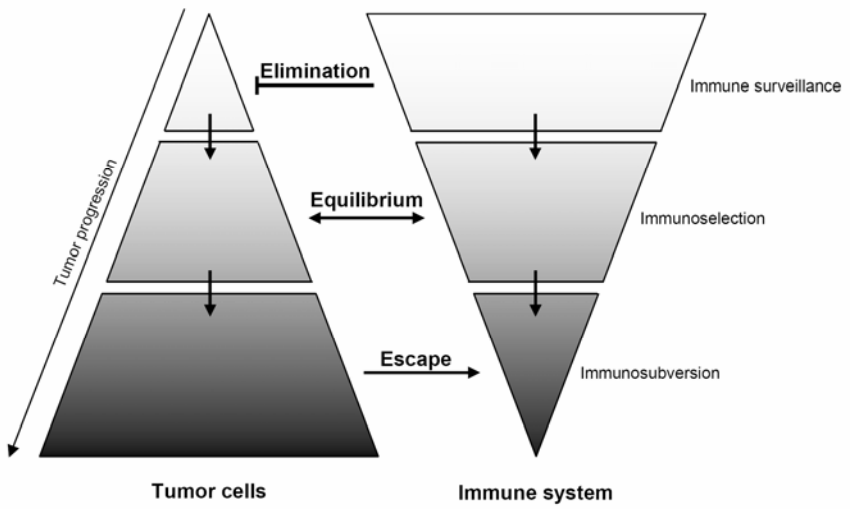

Figure 5. The interplay between tumor progression and the immune system. The figure illustrates that tumor progression results from a cross-talk between tumor cell-intrinsic and immune-mediated mechanisms. (Adapted from Zitvogel, Nature Reviews Immunology 2006, 6:715-727).

3.3.1 Immunoselection of tumor cells

3.3.1.1 Loss of antigen processing and presentation

Since HLA class I antigens have a fundamental role in presenting tumor antigens to CTLs, HLA class I antigen defects in tumor cells provide a mechanism for tumor growth in an immunocompetent host. Immunohistochemical stainings of surgically removed malignant lesions indicate that the surface expression of these molecules is frequently defective on malignant cells (221,222). Several lines of evidence indicate that defects in the HLA class I may play a role in the clinical course of the disease. A defective HLA class I expression is more common in metastases than in the primary tumor, suggesting that an immune selective pressure is actually taking place *in vivo* (223-226). Furthermore, an association between a defective HLA class I expression in the tumor and a poor prognosis of the patients can often be demonstrated (223,226-228). It was shown that either upon immunisation with melanoma-specific peptides or upon T cell based immunotherapy, a total HLA class I loss or selective haplotype loss was observed in recurrent metastases of melanoma (225,229,230). There are three levels of HLA class I defects that have been observed in human tumors of various origin, which include total HLA class I loss, total HLA class I downregulation, and selective loss or downregulation of HLA class I alleles.

While a complete loss of HLA-class I, often caused by structural defects and/or loss of heterozygocity (LOH) of the two β2m alleles, is less commonly observed in tumor cells (221,231-242), HLA class I downregulation is more frequent. A total HLA class I downregulation may be caused by several mechanisms such as a defective HC gene transcription due to either altered binding of regulatory factors or by epigenetic changes resulting in an altered chromatin structure of the HC gene promoters (243), or by a defective peptide loading of HLA class I due to different deficiencies in the antigen processing machinery (APM) components. Of the latter, the most described alteration is TAP1 downregulation, likely due to a defective regulatory mechanism or a structural defect (244-247). A total HLA class I downregulation can often be corrected by cytokines such as IFNγ and TNFα, which indicates that HLA class I downregulation is usually a result of regulatory and not structural abnormalities. Other defects in APM components such as LMP subunits and tapasin, have also been described to correlate with downregulation of HLA on tumor cells (245,248-253). Selective losses of HLA allo-specificities or haplotypes are also observed in human tumors and is usually a result of LOH or mutations inhibiting the transcription or translation of the HC genes (254-257). The reason for the LOH has been correlated to the expression profiles of various oncogenes, such as c-myc (258). Alternatively, it is merely a reflection of the naturally high recombination frequency of this genetic region.

Some tumor cells naturally express no or low levels of MHC class I, which is a reflection of the surface phenotype found in their normal counterparts. Therefore, it is not surprising that NB, which originates from HLA class I-low neuronal cells, also displays this phenotype (259,260). It has been suggested that the naturally low expression of MHC class I in neurons

and thus in most NB cell lines is due to the absence of positive regulatory transcription factors, which in turn protect neurons from T cell-mediated immune surveillance. However, this phenotype can be corrected by e.g. cytokines, inducing the appropriate transcription factors (261,262). Additionally, multiple levels of defects in the APM have been demonstrated in NB, which could account for the instability of HLA class I and its deficient expression. Notably, the majority of studies on HLA class I expression has been carried out on NB cell lines which may express different levels of HLA class I as compared to primary tumor samples. Results from studies on cell lines show defects in expression of β2m, TAP1 and TAP2 (217,262-264). The defects were suggested to be of a more regulatory nature rather than caused by mutations since IFNγ treatment induced the mRNA expression of all analysed molecules. Up-to-date there are only three published studies where primary tumor samples were analysed, and in all these reports almost no HLA expression at all was detected in the NB tissues (193,265,266). Moreover, these primary NB tumors showed defects in the expression of TAP1, TAP2, HLA class I heavy chain, β2m, LMP2 or LMP7 (265,266). Marozzi et al. found yet another type of MHC class I defect in NB cells, namely the presence of β2m-free, presumably peptide-non-bearing HLA class I heavy chains on their surface, but the functional relevance of this phenomenon is unknown (264). Lastly, alterations in MHC class I expression through immune selection following either NK cell- or T cell therapy were shown in a model of murine NB. Upon NK cell-mediated therapy, recurring metastases showed an enhanced MHC class I expression, while a decreased MHC class I expression was observed upon T cell-mediated therapy (267). This suggests that immune sculpting of NB may take place *in vivo*.

3.3.1.2 Antigenic loss variants

Besides HLA class I loss or downregulation, an altered expression of tumor antigens may also occur in tumors. The tumor antigens may be lost in the course of the disease. This phenomenon has mainly been observed in metastatic lesions in malignant melanoma (225,229,268-270). In addition, any defect in the APM might result in poor presentation of certain epitopes even when HLA class I levels are not affected (245). For instance, tumor cells with a defective tapasin expression displayed an altered repertoire of peptides presented on HLA class I (77,271). Alterations in the expression of proteasome subunits may interfere with the epitope presentation by HLA class I molecules, for example, induction of the immunoproteasomal subunits lead to a decreased presentation of a MART1-derived peptide (272), while presentation of TRP-2-derived peptides was decreased in melanoma cells that had lost the capacity to upregulate the proteasome activator PA28 (273).

In NB, high N-Myc levels correlate with low HLA class I levels, suggesting an involvement of N-Myc regulation in HLA expression (274,275). However, it is tempting to speculate that low N-Myc levels in HLA class I high cells may constitute an immune escape mechanism The expression of MAGE antigens which are considered as potential targets for CTLs was correlated with the absence of metastases and a more favourable clinical outcome in patients with NB, indirectly suggesting that tumors which lost their MAGE expression may have

escaped an anti-tumor immune response (190). Although GD2 can be effectively targeted by different therapy modalities, its loss following antibody treatment was seldom found (276).

3.3.1.3 The expression of non-classical HLA molecules

Although tumor cells might express functional HLA class I:peptide complexes, they may still evade CTL lysis due to the expression of inhibitory HLA molecules such as HLA-G and HLA-E.

3.3.1.3.1 HLA-G

HLA-G expression has been described on malignant cells, and the expression inhibited the activity of CTLs against several tumor types (277-280). HLA-G can be upregulated by cytokines present in the microenvironment such as interferons and IL-10 (281-283). Other immunomodulatory effects of HLA-G has also been described such as induction of apoptosis in cytotoxic lymphocytes via binding to CD8 and triggering of the Fas/FasL pathway (284), inhibition of $CD4^+$ T cell proliferation (285), and induction of a shift towards a Th2 cytokine profile (111). HLA-G may be shed by tumor cells, and its soluble form may induce apoptosis of CTLs and inactivate NK cells (284,286). Although these findings remain to be investigated *in vivo*, a correlation has been observed between elevated levels of soluble HLA-G in the serum or ascites of cancer patients and a poor prognosis (287-289).

3.3.1.3.2 HLA-E

HLA-E is expressed in tumors of various origins (290,291). Because of its capacity to bind the inhibitory receptor CD94/NKG2A, expressed by NK cells and a subset of CTLs, (123,292,293), HLA-E expression may contribute to tumor escape from CTL and NK cell immune surveillance *in vivo*. This has been supported by *in vitro* experiments showing that both NK cell- and T-cell mediated lysis of tumor cells was increased or restored by blocking the CD94/NKG2A receptor (117,278,294-296).

Interestingly, although the ligands for HLA-E, such as peptides derived from signal sequences of HLA class I-A, -B, -C and -G molecules, are usually available, protein expression of HLA-E is seldom seen. This may potentially be explained by the relatively weak capacity of HLA-E to bind β2m which may be competed out by other HLA molecules with higher affinity for the HLA class I light chain (120). In addition, it can not be excluded that other so far unknown tumor cell-derived peptides might stabilise HLA-E and rescue them from NK cell-mediated lysis. Similar to virus-infected cells, tumor cells may perhaps express peptides with homology to HLA class I leader peptides, thus escaping NK cell-mediated lysis by stabilising HLA-E (297). However, it should be noted that the functional outcome of HLA-E expression on cytotoxic lymphocyte activation may depend on the nature of the bound peptide ligand and necessarily does not need to lead to inhibition of the immune response, and has to be investigated in each particular case (298).

Interestingly, HLA-E can be induced by IFNγ and was shown to protect targets from NK cells (291,294). In a study performed in our lab it was also demonstrated that upregulation of HLA-E by IFNγ protected short-term ovarian carcinoma cell lines from lysis by peptide- and allospecific $CD8^+$ T cells (278). IFNγ might induce HLA-E membrane expression either by upregulating HLA-E transcription (299), or via the supply of peptides from newly produced classical HLA class I and HLA-G molecules. HLA-E may be shed by tumor cells, particularly upon IFNγ treatment. However, the functional impact of this phenomenon is unknown and requires further studies (294).

3.3.1.3.3 MIC-friend or foe?

The stress-induced MIC and ULBP ligands are found on transformed cells of various types, particularly in those of epithelial origin, such as tumors of the breast, lung, ovary, prostate, colon and kidney (108,136,137,300-302). This observation is intriguing since the expression of ligands for NK cell activating receptors may increase tumor susceptibility to immune surveillance. However, malignant cells develop means to control the expression of ligands and the respective activating and inhibitory receptors on immune cells in order to avoid immune surveillance. It was elegantly demonstrated that tumor cells may release a soluble form of MIC into the circulation which may impair tumor cytolysis through the induction of endocytosis and degradation of NKG2D on both NK cells, TILs and PBLs in cancer patients (303-305). In agreement with this, elevated MIC levels in serum were associated with a poor prognosis of patients with prostate cancer (306). It was proposed that the production of soluble MIC molecules, or alternatively, a prolonged exposure to membrane-bound ligands, may desensitize NKG2D on lymphocytes and thus constitute a tumor-evasion strategy (307,308). Moreover, MIC-expressing tumor cells were shown to induce a unique suppressive population of $NKG2D^+CD4^+$ T cells that were dependent on NKG2D ligands and FasL for their suppressive activity (309). Some viruses produce certain proteins, specifically retaining MIC and ULBP molecules within the cell, preventing them to reach the cell surface and thus for the cell to be recognised and killed by NKG2D-expressing cytotoxic lymphocytes (310). Whether tumor cells also exploit this elegant "mechanism of disguise" remains to be unraveled. In conclusion, although an initial expression of MIC by emerging tumors might to some extent be effective in mobilising responses from effector T cells and NK cells and result in removal of tumor cells, its subsequent proteolytic shedding at progressive stages of tumor growth will rather promote immune evasion.

Although there are no descriptions so far in the literature of surface HLA-G and HLA-E expression by NB tumor cells, it was shown that NB cells may downmodulate their expression of NKG2D ligands, such as MIC and ULBPs, providing them with a potential to escape death mediated by NKG2D-expressing effector cells. However, it needs to be established whether these low levels are tissue- or tumor specific. Furthermore, it was shown that sera from NB patients, as compared to healthy controls, contained soluble MICA which could impair NKG2D expression on cytotoxic cells and consequently their cytotoxic potential (311).

3.3.1.4 Tumor escape from death receptor-mediated signalling

Increasing evidence is provided for a role of death receptor-mediated killing in immune surveillance of tumor cells (159,312). Multiple studies have shown that tumors may acquire resistance to death receptor-mediated signalling. The resistance mechanisms may inflict at different levels in the death-promoting pathway. Tumor cells may loose their surface expression of either Fas or TRAIL-receptors. This may result from either mutations or deletions of the genes or from a defective transport of the receptor to the cell surface, and has been described both for Fas (313,314), and for TRAIL-receptors (315-321). Interestingly, the genes for TRAIL-R1 and -R2 are located on a chromosomal segment which is one of the most common sites for LOH in several types of cancer (322). Tumors may also escape death receptor-mediated apoptosis by expressing decoy receptors, competitively binding FasL or TRAIL (323-325). It was long thought that the balance between surface death-mediating and decoy receptors for TRAIL could determine the sensitivity of tumor cells to TRAIL, however, no convincing correlation has been found so far (326). Another mechanism of escaping Fas-mediated killing was discovered in our lab when we showed that the autocrine secretion of FasL from uveal melanoma cells shields tumor cells from Fas-mediated killing by cytotoxic lymphocytes (327). Tumor resistance to the death receptor-mediated pathway may also be due to a functional impairment of Fas-associated death domain (FADD) by inactivating mutations (328,329), over-expression of inhibitors of apoptosis such as cFLIP (cellular FLICE inhibitory protein), an inhibitor of caspase-8 (330-333), IAPs (inhibitor of apoptosis proteins) (334), and also the Bcl-2 family of proteins acting at the level of mitochondria (see below) (335,336). Furthermore, loss of caspase-8 expression, a key molecule in TRAIL-, FasL- and TNFα-induced apoptosis, has been shown to correlate with TRAIL resistance in many tumor types, due to gene deletion or hypermethylation of the promoter (337-339). Finally, it has been postulated that the tumor microenvironment can influence the sensitivity of tumor cells to TRAIL (340). Both the hypoxic milieu and IL-8 secreted into the microenvironment may lead to an increased tumor cell resistance to TRAIL-mediated apoptosis (341,342).

The deficiency of caspase-8 expression in NB cells confers resistance to apoptosis (338,343). This defect, which usually results in resistance to TRAIL, is in the majority of cases due to hypermethylation of the promoter region of the caspase-8 gene, and can be restored by demethylation agents or IFNγ (337,344-348). Furthermore, the levels of expression of the death receptors TRAIL-R1, Fas and TNF-R1 have been observed to be higher in tumors with favourable prognosis, suggesting that the downregulation of these receptors by progressing tumors may give an advantage of tumor immune escape (349).

NB cells may over-express molecules with anti-apoptotic activity. Increased levels of survivin, a member of the IAP family, have been associated with increased resistance to TRAIL-induced apoptosis in NB as well as with a high-risk prognosis and reduced survival (195,349-352). Increased levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL have been observed (353,354) and their over-expression correlated with factors of poor prognosis like high *N-Myc* and unfavorable histology (355). A possible microenvironmental factor that could

induce both survivin and Bcl-2 expression in NB was suggested to be the vascular endothelial growth factor (VEGF) (356,357).

It is known that the deregulated N-Myc can promote both cell proliferation and p53-dependent apoptosis (358). The latter can be viewed as a safeguard mechanism to prevent oncogene-driven tumor formation. Enigmatically, p53 mutations are uncommon in NBs, strongly suggesting that an alternative mechanism prevents the oncogene-driven induction of apoptosis (359). It was demonstrated that a transcription factor called Twist, is constantly over-expressed in N-Myc-amplified neuroblastomas and is responsible for the inhibition of the ARF/p53 pathway involved in the N-Myc-dependent apoptotic response (360). Alternatively, it was suggested that N-Myc induces apoptosis through activating caspases or by upregulating TRAIL-R2, which can be counteracted by the frequent defects in caspase-8 expression observed in NB (361,362).

3.3.1.5 Resistance to perforin and granzyme-mediated killing

Tumor cells may resist killing by cytotoxic lymphocytes by interfering with the perforin/granzyme pathway. It has been demonstrated that tumor cells may express certain inhibitors of perforin and granzyme B, originally described to protect the cytotoxic lymphocytes from suicide killing. The first inhibitor to be described was cathepsin B, that is upregulated on the surface of CTLs after T cell receptor (TCR) stimulation and can then cleave and inactivate perforin (363). Although an increased expression and/or activity of cathepsin B has been observed in tumors of different origin, its role in protecting against perforin-mediated death has not been elucidated (364). Another inhibitor that is expressed in cytotoxic lymphocytes to protect them from the granule exocytosis-mediated death is the serine protease inhibitor PI-9/SPI-6, which inhibits granzyme B (365). Expression of this inhibitor in tumor cells resulted in resistance to lysis by cytotoxic lymphocytes, leading to immune escape (366). Recently, a novel inhibitor of granzyme B, serpina3n, was found to protect testis Sertoli cells from killing by granzyme B. However, it remains to be determined whether this inhibitor could also be induced by tumor cells as an escape mechanism (367).

Tumor cells may also escape death by cytotoxic effector cells by preventing perforin binding to the surface (368). This finding is consistent with results from our laboratory, where we showed that IFNγ-treated uveal melanoma cells were resistant to CTL-lysis due to impaired perforin action (Hallermalm et al., submitted). It is possible that factors released in the tumor microenvironment could influence the membrane composition of tumor cells, rendering the tumor cells unreceptive to perforin and granzyme. In fact, IFNγ was shown to affect both the density and fluidity of the plasma cell membrane in cells, which could potentially affect perforin polymerisation (369-371). This notion is supported by studies demonstrating that it is the membrane composition of CTLs that mediates the resistance to perforin, a characteristic that could possibly also be adapted by tumor cells (369,372,373). One molecule that could mediate this effect is calreticulin which is also stored in granules of CTLs and was proposed to diminish the effects of perforin by stabilizing the membrane (374). Recently, Kroemer and

co-workers demonstrated that calreticulin exposure on tumor cells treated with certain cytotoxic drugs improves the immunogenicity and thereby the immune response towards the tumor (375). In this particular study the protective role of calreticulin was overrriden by its immune stimulatory capacity. However, it can not formally be excluded that in other tumor models the induction of calreticulin by chemotherapeutic agents could confer tumor resistance to granule mediated cytotoxicity, thus providing tumor cells with an escape mechanism.

3.3.1.6 Loss of responses to IFNγ and its negative effects on immune recognition

According to the current dogma, the presence of IFNγ in the tumor microenvironment is expected to facilitate tumor elimination via MHC class I restricted T cell responses. Some tumors, however, loose their capacity to upregulate APM components in response to IFNγ due to downregulation of the transcription factor binding to the interferon response sequence element (IRSE) or to an abnormal expression of IFNγ-signalling components such as IFNγ-R1, Jak1 or Jak2 (376-379), In addition, as mentioned earlier, the immunoproteasome assembly upon IFNγ treatment may result in the destruction, rather than the generation of at least some MHC class I-restricted CTL epitopes (272). Furthermore, IFNγ may have a negative impact on tumor cell recognition by cytotoxic lymphocytes. IFNγ-mediated upregulation of HLA-E leads to an inhibition of CTL-mediated killing (278), and might also provide an inhibitory signal for NK cells. Another inhibitory molecule, HLA-G, can be upregulated by IFNγ (110,282). Zhang et al. showed that IFNγ can have a negative impact on NK cell cytotoxicity by downmodulating the activating NKG2D receptor, while upregulating the inhibitory NKG2A receptor (380). IFNγ can also promote tumor escape by downregulating the expression of endogenous tumor antigens. This phenomenon was demonstrated in colon carcinoma and led to a reduced lysis by tumor specific CTLs (381). Finally, exposure of tumor cells to IFNγ may, through a yet unknown mechanism, lead to a resistance to perforin-mediated lysis (Hallermalm et al., submitted). Collectively, all negative effects of IFNγ described above might explain why application of IFNγ has not been successful in clinical trials of cancer therapy (382,383).

3.3.2 Subversion of the host immune system

Tumor escape might not only depend on changes which concern tumors as targets, reducing their recognition by the immune system. Clinically advanced tumors have likely developed active mechanisms to shift the balance of immunity from surveillance to tolerance, allowing the tumors to expand. Different tumor-derived factors may be involved in this process of immunosubversion, and I will discuss some of the proposed mechanisms below. In figure 6, a number of the escape mechanisms that are discussed are depicted.

3.3.2.1 Suppression of immune responses by tumor-derived factors

Tumor cells or tumor-associated cells, such as stromal and vasculature cells, may secrete factors that have a negative impact on the immune response. TGFβ may inhibit immune responses in many different ways and can target almost every effector cell of the immune system. It suppresses T cells through inhibition of T cell activation, effector function,

proliferation and differentiation (384-386) and can drive the expansion of regulatory T cells *in vivo* (387). TGFβ may down-modulate the expression of activating NKG2D on both NK- and T cells (388) and induce inhibitory CD94/NKG2A receptors (389). The impact of this cytokine on immunosuppression is highlighted in studies showing a negative correlation between high serum levels of TGFβ and prognosis of patients with prostate, bladder and gastric carcinoma (385,390) and in experiments where blockade of TGFβ can restore tumor-specific CTL-responses (391). IL-10 released by tumor cells may also exert multiple negative effects on the immune system. IL-10 inhibits production of Th1 type cytokines including IFNγ and IL-2, impairs dendritic cell (DC) function, and reduces antigen presentation in tumor cells (392-394). High serum levels of IL-10 have been correlated to poor prognosis in a number of malignancies (395,396). It was recently discovered that the expression of the constitutively active transcription factor STAT3 (signal transducer and activator of transcription 3) by both tumors and immune cells in the tumor microenvironment inhibits the inflammatory responses necessary for immune activation against tumor cells. IL-10 signalling is one of the mechanism constitutively activating STAT3 in the tumor milleu (397,398). VEGF is another immunosuppressive factor released from tumor cells, that, in addition to its pro-angiogenic effects, has been shown to inhibit the functional maturation of DCs (399). Another strategy used by tumors to evade an immune response is the secretion of metabolic factors that limit T cell function. The best described factor so far is IFNγ-inducible indoleamine-2, 3-dioxygenase (IDO) that mainly suppresses T cell function through tryptophan depletion. Inhibition of IDO improves T cell-mediated tumor control *in vivo* (400).

NB cells may secrete both TGFβ and IL-10 which can prevent the activation and expansion of tumor-infiltrating lymphocyets (401-403). Another immunosuppressive molecule expressed by NB, B7-H3, confers protection of tumor cells from NK cell-mediated lysis by interacting with a yet uncharacterised NK inhibitory receptor (404). Furthermore, NB cells may produce high levels of macrophage migration inhibitory factor (MIF) which may cause activation induced T cell death as demonstrated in a murine model of NB (405,406). Moreover, MIF was shown to contribute to NB tumor progression and to induce VEGF production by NB cells; hence, neutralising MIF in the NB microenvironment would have positive effects on both eliminating tumor cells and restoring cellular immunity (407,408). Another factor secreted by NB cells is the pro-angiogenic chemokine IL-8 which has the potential to inhibit TRAIL-mediated apoptosis (341,402,408,409). Interestingly, norepinephrine produced by NB cells is a potent inhibitor of NK cell cytotoxicity (410). The same neurotransmitter was also shown to inhibit anti-tumor CTLs (411).

3.3.2.3 Recruitment and activation of suppressive cell populations

Apart from secreting immunosuppressive factors themselves, tumor cells may cause immunosubversion by either promoting the expansion of or attracting immunosuppressive cell populations such as regulatory T cells (Tregs) or myeloid suppressor cells (MSCs). Tregs suppress immune responses against tumors and depletion of Tregs improves T cell-based tumor clearance (412,413). The recruitment of Tregs into tumor lesions has been

demonstrated for many types of tumors and is considered as a negative prognostic factor (414-417). It is not entirely clear how tumor cells recruit naturally occurring Tregs into the tumor milleu; the secretion of the chemokine CCL22 has been implicated as one potential mechanism (414,415). Other mechanism of Treg induction are mediated by TGFβ and IL-10 produced by tumor cells directly (418,419) or indirectly through IL-10-induced immature DCs (420-423). The suppressive effects of Tregs can be exerted via cell-to-cell contact and via soluble factors, affecting both effector lymphocytes and antigen presenting cells (APCs) (for review see (417)).

MSCs found in tumor infiltrates are potent suppressors of both $CD4^+$ and $CD8^+$ T cells in mice (424). MSCs represent a heterogeneous population of myeloid cells that include immature macrophages, granulocytes, DCs and other myeloid cells at earlier stages of differentiation. In humans they still represent a quite elusive cell population which has not been characterised in detailed yet. Numerous studies indicate that tumor-derived factors promote both MSC recruitment and their maturation towards an immunosuppressive phenotype. Tumor-derived factors that have been implicated in MSC recruitment include macrophage colony stimulating factor 1 (M-CSF), IL-6, IL-10, VEGF, and granulocyte-macrophage CSF (GM-CSF) (424-427). The mechanisms by which MSCs suppress immune responses include production of the metabolic enzymes IDO and arginase, and inducible nitric oxide synthase (iNOS). MSCs can also promote tumor growth by inducing angiogenesis in response to matrix metalloproteinase-9 (MMP9)-dependent release of VEGF from the extracellular matrix (428). Importantly, there are indications that MSCs may also indirectly cause immunosuppression by inducing Tregs (429).

It was reported that NB cells secrete IL-6 which could have an effect on both MSC recruitment and prevent DC maturation as discussed below (405,430). However, so far no studies have investigated the role of MSCs in neuroblastoma; so, the impact of IL-6 secretion by NB tumor cells remains to be elucidated in further studies.

3.3.2.4 Tumor counter-attack

It has been postulated that expression of death receptor ligands such as FasL or TRAIL by the tumor cells may induce death of death receptor-bearing tumor-specific T cells (431-434). This finding is supported by the negative correlation seen between FasL expression by the tumor and the prognosis of patients with e.g. carcinomas of the cervix, breast and colon (435-437). However, in other reports, the effect of FasL was rather pro-inflammatory and anti-tumoral (438,439). Furthermore, there is a general belief in the field that in the early studies demonstrating the expression of FasL by tumor cells, reagents with non-sufficient specificity were used. When proper antibodies were applied to monitor FasL expression by tumor cells, a relatively poor expression of FasL by tumor cells was documented (440,441). Still, provided that some tumor cells might express FasL, there are recent studies claiming that the type and level of expression of FasL determines whether FasL mediates immune privilege or inflammation (438,442,443).

The expression of FasL has been demonstrated in NB tumors (444-447). Moreover, at least in one study a negative correlation between the amount of FasL-expressing tumor cells and the numbers of $CD3^+$ lymphocytes was found in tissue sections from NB patients. In *in vitro* assays, FasL-expressing NB cells could kill Jurkat T cells in a partly FasL-dependent manner (446,447). Although this phenomenon was only described in one study, it indicates that the expression of FasL by NB cells could constitute a mechanism of immune evasion. In contrast, in line with the pro-inflammatory role of FasL, FasL-expressing NB cells were shown to mediate neutrophil infiltration and subsequent $CD8^+$ T cell-mediated tumor rejection in a murine model (448).

3.3.2.5 Recruitment and induction of disabled antigen presenting cells

Whether or not presentation by DCs will lead to T cell priming or tolerance induction is largely decided by the microenvironment in which the DCs will encounter the antigens. If antigen capture happens in a non-inflammatory environment characterised by the lack of a costimulatory capacity by the DCs, the end result will rather be induction of tolerance in effector cells (445). In addition, when a tumor progresses, secretion of immunosuppressive cytokines will have a further negative impact on the DC function. It has been demonstrated that DCs found within the tumor microenvironment have an immature phenotype (449). The immature DC populations may, in turn, induce Tregs (399,450) or T cell unresponsiveness (420,422). In addition, DC subsets with regulatory immunosuppressive properties might be induced (451,452). These regulatory DCs might tune T cell responses via e.g. IDO- or IL-10 production. There are indications that tumor cells themselves can promote a faulty differentiation of myeloid and lymphoid precursor cells into different immature and regulatory DC populations. Tumor-derived factors such as IL-10, TGFβ, VEGF, IL-6 and M-CSF have been implicated in preventing DC maturation or causing a switch towards a more immature DC phenotype (453,454). The importance of STAT3 signalling in suppressing immune responses is highlighted here as well, as tumor-derived factors such as IL-10 and VEGF may activate STAT3 signalling in DCs and thereby inhibit their activation (455).

It was shown by Valentino et al. that high circulating levels of the ganglioside GD2 was associated with a rapid disease progression and lower survival rate in patients with NB (456). The authors speculated that shedding of gangliosides by NB tumor may play a role in accelerating tumor progression. Other reports have shown that NB tumor gangliosides had a potent immunoregulatory activity and could inhibit cellular immune responses (457). A mechanism behind the immune suppressive activity of gangliosides was demonstrated in reports that described the ability of NB cells to impair DC function (458,459). *In vitro* studies have further shown that ganglisosides, expressed on the surface of NB cells, have the ability to suppress the development and function of monocyte-derived DCs (460-462). Putting these observations together, it is likely that shedding of gangliosides by NB cells may subvert the immune system by preventing DC maturation, and may therefore constitute a mechanism of NB immune escape.

The inflammation-induced enzyme cyclooxygenase-2 (COX-2) is over-expressed in many cancers and has been implicated in resistance to apoptosis as well as in induction of metastases and angiogenesis. Furthermore, COX-2 promotes prostaglandin E2 (PGE2) production in the tumor microenvironment (463). PGE2, synthesized by COX-2-over-expressing tumor cells, contributes to cellular immune suppression in cancer patients, probably, by directly inhibiting T cells and through the induction of inhibitory DC populations secreting high levels of IL-10, and the concomitant induction of Tregs (464-466). Since NB cells were shown to express COX-2, it can contribute to immunosuppresion (467).

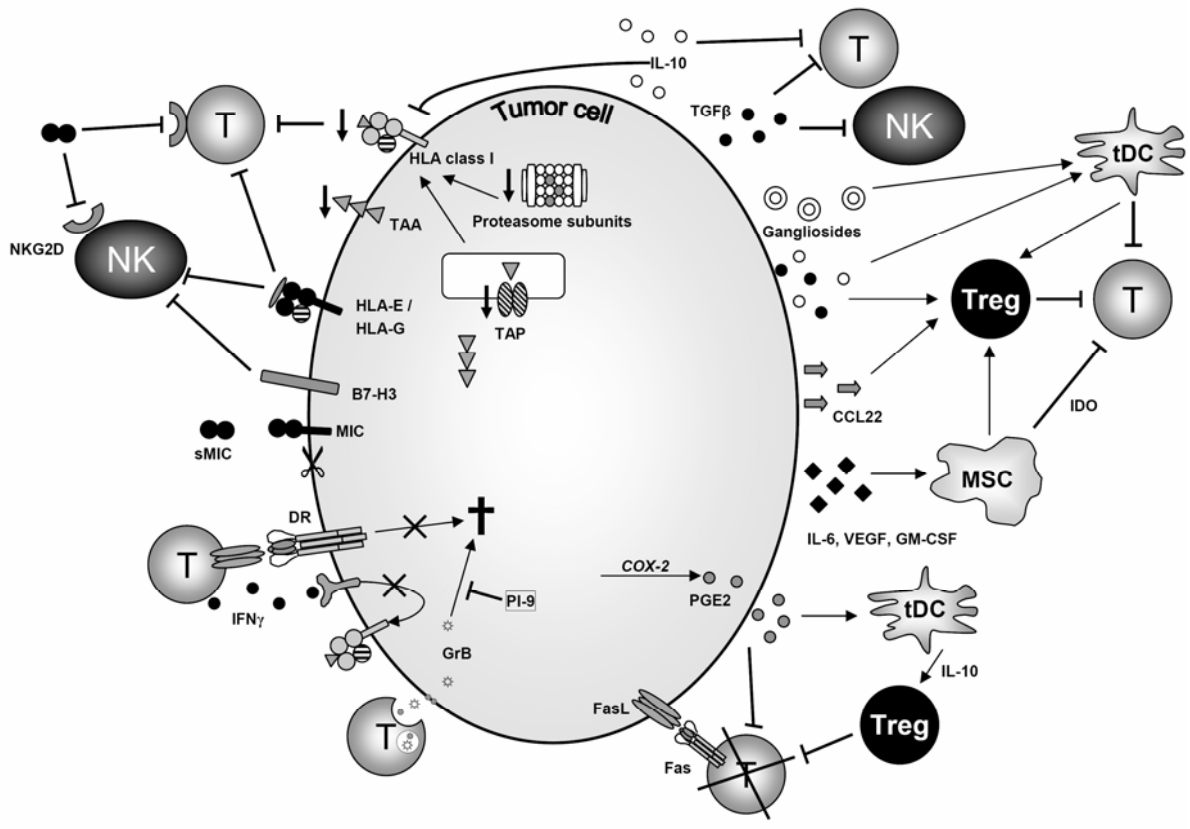

Figure 6. Tumor immune escape mechanisms. In this figure I have summarised some of the mechanisms that may lead to tumor immune escape and that are discussed in the text. On the left hand side I have depicted (from the top to the bottom); HLA class I downregulation (which could result from deficiencies in various APM components), tumor antigen loss, expression of inhibitory HLA-G and HLA-E, expression of the NK inhibitory ligand B7-H3, shedding of soluble MIC molecules, tumor cell resistance to death receptor-mediated killing, tumor cell loss of responses to IFNγ and resistance to granzyme B-mediated death via the expression of the serine protease inhibitor PI-9. On the right hand side various tumor-derived factors that are involved in the process of immunosubversion are depicted (from the top to the bottom); IL-10 may reduce HLA class I antigen presentation, inhibit T cells and induce tolerogenic DCs (tDC) and Tregs, TGFβ may inhibit activation of both NK- and T cells, induce tolerogenic DCs and Tregs, gangliosides may inhibit the maturation of DCs, tumor-derived CCL22 may recruit Tregs, various tumor-derived factors such as IL-6 and VEGF have been implicated in MSC recruitment, COX-2 over-expressed in tumor cells produce PGE2 that can both inhibit T cells and induce Tregs via the induction of tolerogenic DCs producing IL-10, and lastly expression of FasL by the tumor cells may induce apoptosis of T cells expressing the Fas receptor.

3.4 ”THE STORY OF A SUCCESSFUL IMMUNE RESPONSE”

To give you a picture of how the components of the immune system could collaborate together to eliminate a rising tumor when everything is working properly, I will illustrate a scenario of how a successful immune response could look like (Figure 7). Let’s say that we are dealing with a melanoma in the skin. If the tumor expresses a neoantigen that will be presented to the immune system in the context of the pro-inflammatory environment, according to the “danger model” of immune activation, it will be seen as foreign, inducing a strong T cell response that together with innate immune responses could eliminate the tumor (468).

The tumor itself represents a tissue with disturbed architecture that will give rise to pro-inflammatory signals in the form of cytokines and chemokines. The physical damage to tissues can be due to either angiogenesis or tissue-invasive growth of the tumor. Moreover, heat shock proteins expressed by cells undergoing damage or necrotic death may serve as such a signal. In turn, the pro-inflammatory signals act as activating signals for the innate immunity. The first effector cells to be activated are the tissue resident macrophages which will augment the pro-inflammatory signals. TNFα released from macrophages can induce adhesion molecules on endothelial cells to recruit more cells. Due to the inflammation, vessels are also dilated and blood flow is slowed down, which helps leukocytes to extravasate into the tissue. Furthermore, chemokines function as chemoattractants, recruiting leukocytes from the blood to the site of the tumor. Neutrophils are the first cells to arrive. Neutrophils may be activated to release their lysosomal contents by the pro-inflammatory cytokines present in the tumor milieu, and thus further contribute to the tissue destruction (469). Recruited NK cells are activated in response to macrophage-derived cytokines, especially IL-12. The transformation process will likely also result in the expression of stress-induced ligands on tumor cells which can be recognised by NK cells that may even eliminate the tumor, provided an optimal balance of inhibitory and activating receptors. NK cells will further produce high levels of IFNγ which may have multiple effects such as enhancing the antigen-presenting properties of tumor cells, being anti-proliferative, pro-apoptotic, and inhibiting angiogenesis. The induced innate immune responses can either succeed in clearing the tumor or contain it while an adaptive immune response develops.

Next, the inflammatory response leads to the recruitment of DCs. Immature DCs recruited to the site of the tumor will take up antigens and be activated by cytokines produced during the inflammatory response or by interacting with NK cells, and respond by migrating to the lymph nodes and inducing the expression of MHC-, adhesion-, and costimulatory molecules. In addition, the inflammation as such leads to an increased entry of plasma and a consequent drainage of these tissue fluids into the lymph which will speed up the transport of antigens to the local lymphoid tissue. In the lymph node the DCs will present antigen to both $CD4^+$ and $CD8^+$ T cells via cross-priming. Naïve $CD8^+$ T cells will become effector CTLs and naïve $CD4^+$ T cells will preferentially be induced to differentiate into Th1 cells in order to promote cell-mediated immunity. The armed effector T cells are then ready to home to the tumor site

via adhesion molecule interactions and can be activated upon encounter with the tumor antigen without the need for costimulation. $CD8^+$ CTLs will be maintained by cytokines produced by Th1 cells and will kill tumor cells via both direct and indirect mechanisms. In a perfect scenario the tumor will then be eliminated through the concerted action of the components of the innate and adaptive immune systems (470-472).

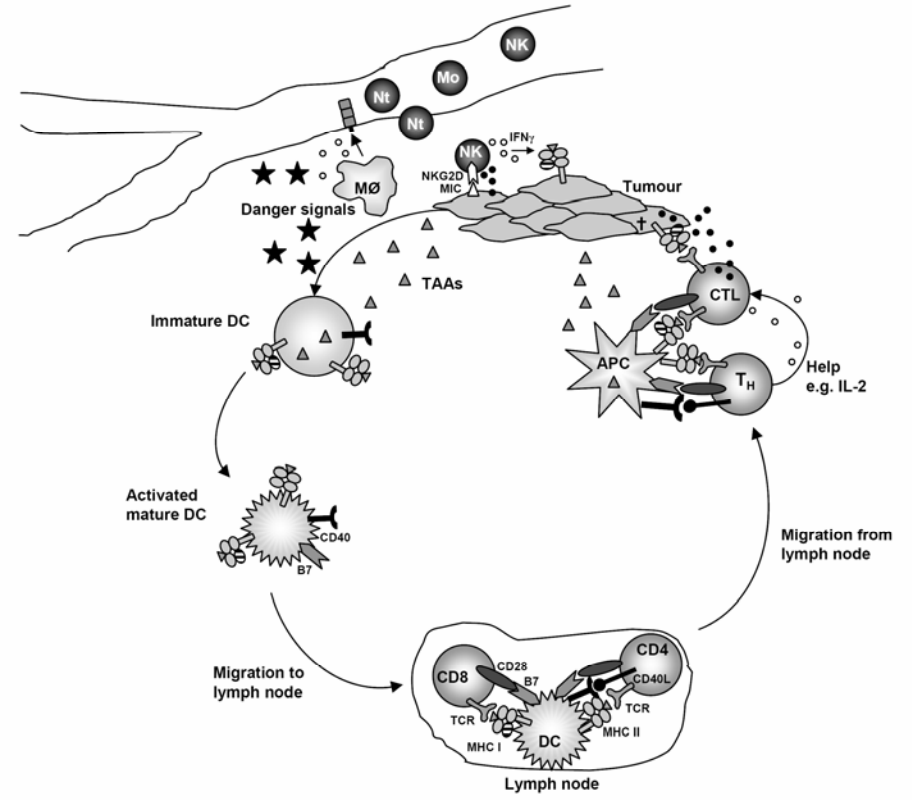

Figure 7. A successful anti-tumour immune response. Cytokines released from tissue-resident macrophages (MØ) induce the expression of adhesion molecules on the endothelium. The first cell types to be recruited to the tumor site are neutrophils (Nt), monocytes (Mo) and NK cells (NK). NK cells are activated through NKG2D and kill some tumor cells, and produce IFNγ that induces MHC expression on tumor cells. DCs capture and present TAAs at the tumor site. Upon activation by danger signals secreted into the tumor microenvironment, immature DCs mature into activated DCs that express costimulatory ligands and migrate to the lymph nodes. In the lymph nodes, naïve T cells are activated into effector cells which have the ability to migrate and home to the tumor site and eventually kill the tumor cells (Adapted from Smyth, Nature Immunology 2001, 2:293-299).

3.5 SOME NOTES ON IMMUNOTHERAPY OF CANCER

Regardless of whether or not tumor immune surveillance is occurring *in vivo*, the immune system may be exploited for the purpose of therapeutical intervention against cancer. So far, immunotherapeutic approaches have not gained the expected clinical success, most likely due to the mechanisms of tumor immune escape and immune suppression, which must be taken into account when designing immune therapeutic strategies.

4. IMMUNOTHERAPY OF NEUROBLASTOMA

NB tumors and cell lines are characterized by their low or absent expression of MHC class I (193,217) which questions the ability of this tumor to serve as a sufficient target for MHC class I-restricted CTL-mediated responses (473,474). Despite this, early studies demonstrated that NB cells can elicit both humoral and cellular immunity, suggesting that tumor-specific antigens and relevant HLA elements are available (187). It was also demonstrated that the presence of TILs correlates with a better prognosis (218,475). In addition, the relatively frequent spontaneous regressions observed for NB suggested a role of the immune system in the control of this tumor. This was further supported by Squire et al. who found that stage 4S tumors had a higher MHC class I expression, supporting the hypothesis that spontaneous regression of 4S NB might be immunologically mediated (476). In agreement, a recent study showed that stage 4S patients with tumors undergoing spontaneous regression displayed increased levels of serum granulysin, indicating that an immune attack had taken place (477). Due to its usually low toxicity, immunotherapy may become a promising approach for the treatment of NB, especially in cases of chemotherapy resistant disease. The low immunogenicity of NB should be considered when designing immunotherapeutic modalities; thus, NK cell-mediated therapies could be exploited for targeting of MHC class I-low tumors; or alternatively, strategies to increase the immunogenicity of NB cells must be applied aiming at rendering NB cells sensitive to CTL-mediated immunotherapy. Below, I will summarise the immunotherapeutical strategies that has been exploited for NB up to date.

4.1 IL-2-BASED IMMUNOTHERAPY

Pre-treatment of NK cells and PBMCs with IL-2 lead to an increased recognition of NB cell lines *in vitro* (478-480). These experiments, as well as promising results from trials on IL-2-treated melanoma patients, encouraged trials involving systemic IL-2 treatment of NB patients. A general conclusion from these studies was that IL-2 treatment was well tolerated by the NB patients and induced both the proliferation of circulating NK- and T cells as well as the activity of NK cells and/or LAK (lymphokine activated killer) cells. However, these clinical trials were performed on relatively few patients, and no major clinical responses were demonstrated (481-488). IL-2 has also been given to NB patients in combination with infusions of autologous LAK cells, however, quite severe toxicity and no major response rates were observed (489,490).

Systemic administration of IL-2 alone at clinically effective doses may, in some patients, be associated with serious side effects that limit its use in cancer immunotherapy (491). Moreover, it may not be sufficient enough to induce anti-NB immunity, and therefore the cytokine should rather be used in combination with other immune therapeutic approaches. Instead, administrating IL-2 as an adjuvant to anti-GD2 therapy or direct delivery of IL-2 to the tumor microenvironment by fusing it with anti-GD2 antibodies could be more effective. Several studies have shown the potency of IL-2 to activate NK cells resulting in increased antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by anti-GD2 antibodies

(492-497), however this treatment modality achieved limited clinical potential (498,499). Immunization of NB patients with IL-2-transduced autologous tumor cells achieved some clinical responses which correlated with a detectable anti-tumor CTL activity (500). In another trial, allogeneic NB cells were transduced to secrete both the chemokine lymphotactin and IL-2. Out of six patients, two patients demonstrated complete responses and one patient a partial response (501). A similar strategy was demonstrated in a murine model of NB using fractalkine (CX3CL1)-secreting tumor cells combined with targeted IL-2 treatment where a reduction in primary tumor growth and a complete eradication of experimental liver metastases mediated by T- and NK cells were achieved (502).

4.2 DENDRITIC CELLS IN NEUROBLASTOMA THERAPY

The potent antigen-presenting capacity of DCs for therapy of NB has been investigated in several studies where the DCs were pulsed with apoptotic or necrotic tumor cells, tumor lysates, or transduced with tumor mRNA. An efficient induction of CTLs could be demonstrated (503,504). Collectively, *in vitro* and *in vivo* studies have shown that it is feasible to isolate DCs from NB patients and safe to administer the DC-based vaccine (503-507). In a phase I trial, DCs pulsed with tumor lysate were administered with no toxicity and induced specific T cell responses (506). Although studies on DC-based therapy of NB have shown some promising results, it has to be taken into account that DC function may be impaired, possibly due to shedding of gangliosides from NB cells that contribute to NB immune escape (as discussed above).

4.3 CTL-MEDIATED THERAPY

Early and somewhat primitive studies showed that NB cell lines remained resistant to allogeneic CTL-mediated lysis even after IFNγ-mediated upregulation of MHC class I, suggesting that other levels of resistance were present as well (473). However, in these studies allogeneic CTLs specific for only one HLA class I determinant on the NB cell line surface were used. Later more refined studies on the same NB cell lines, but using more appropriately primed CTLs, suggested that they were indeed susceptible to CTL-mediated killing (508). Moreover, xenogeneic NB cells formed tumors in T cell-deficient, but not in T cell-competent mice, suggesting that T cells may play a role in the control of the NB tumor growth (509).

A few attempts have been made to generate NB-specific CTLs. Sarkar et al. generated CTL lines from two NB patients that specifically killed autologous tumor cells *in vitro* (510), demonstrating that NB cells are susceptible to CTL-mediated killing and implying that these tumor cells can present endogenous antigens to T cells. The same group generated CTLs specific to N-Myc (511). Although these CTLs efficiently killed *N-Myc*-amplified NB cells *in vitro*, the *in vivo* application of such CTLs might be hampered due to low MHC class I levels in N-Myc over-expressing NB cells, as over-expression of N-Myc leads to decreased levels of the p50 subunit of NF-kB, a transcription factor binding to the HLA class I gene promoter (274,275). Furthermore, the N-Myc-derived peptide identified in this study is restricted to the

not so common HLA-A1 allele which limits its clinical application. Subsets of NB cells express the germline antigens MAGE-1, MAGE-3 and NY-ESO-1 (192,193). NY-ESO-1-specific $CD4^+$- and $CD8^+$ T cell immune responses were observed in PBLs from 50% of the patients after *in vitro* stimulation with either the NY-ESO-1 recombinant protein or with an HLA-A2-restricted peptide. Moreover, NY-ESO-1-specific T cells were able to recognize NY-ESO-1 expressing NBs (191). Morandi et al. used another strategy to induce NB-specific responses. DCs were generated from patients, transfected with mRNA from pooled NB cell lines and used to stimulate autologous $CD8^+$ lymphocytes. The generated CTLs specifically recognized HLA-matched NB cell lines as measured by IFNγ release and HLA-restricted cytotoxicity (503). Other reports demonstrated that NB patient-derived CTLs recognize peptides from Alk, PRAME, survivin, telomerase and tyrosine hydroxylase (TH) (503). Similarly, NB-specific CTLs recognizing survivin could be generated by stimulating PBMCs from NB patients with autologous RNA-transfecetd CD40-activated B cells (512). In a recent follow-up study it was shown that NB patients harbor functional circulating survivin-specific T cells (513).

Attempts to induce T cell-mediated immunity to NB were also made using a mouse model. In a poorly immunogenic murine model of NB, mice that received a TH-based DNA vaccine were protected against a subsequent tumor challenge and displayed a partial reduction in subcutaneous tumor growth. $CD8^+$ cells were involved in the immune response (514). Combining the vaccine with tumor-targeted IL-2 treatment resulted in even better response rates (515). Another study involving protective immunity against murine NB was based on tumor cells transfected with the costimulatory molecules CD80 and CD86 (516).

In conclusion, the generation of NB-specifc CTLs from patients is feasible, at least *in vitro*, and these CTLs could potentially be used in an adoptive therapeutic setting. Even though NB cells express low amounts of HLA class I on their surface, it does not prevent from recognition by CTLs. Indeed, in the studies by Morandi et al. it was demonstrated that even NB cells expressing low amounts of HLA class I were lysed by CTLs (503). On the other hand there are agents capable of enhancing HLA class I expression by NB cells, e.g. IFNγ and retinoids, which have been part of the focus of my studies.

4.3.1 Manipulated CTLs as targeted therapy

The ability to facilitate the delivery of adoptively transferred NB-specific CTLs to the tumor milieu will not only minimize possible toxicity but may also be more efficient in eliminating tumor cells. For this purpose, T cells harboring chimeric receptors, comprised of an extracellular single-chain antibody specific for NB-associated antigens fused to the intracellular signalling domain of the TCR complex ζ-chain, have been generated (517,518). These chimeric receptors expressed in T cells have the ability to redirect antigen recognition based on the specificity of the antibody. In addition, these T cells retain their ability to lyse target cells and to secrete cytokines (519). Importantly they will bind antigen in an HLA-independent manner, making it possible to target even MHC-low immune escape variants. It

was shown in a recent phase I trial that autologous CTLs expressing an antibody directed towards the NB-expressed antigen L1-CAM, could be generated and safely administered to NB patients (520,521). Of special interest for the studies included in the thesis is the report where Epstein-Barr visus (EBV)-specifc CTLs, transduced with the anti-GD2/TCR-ζ-chimeric receptor, were shown to lyse tumor cells (518). The EBV-specific CTLs, which can be easily produced from NB patients, constitutes a population of pre-activated CTLs that can persist in the circulation for years, provided that the patient is seropositive for EBV (518,522).

Alternatively, pre-existing T cells can be targeted to the NB sites using bi-specific antibodies directed towards NB-expressed antigens and CD3 expressed by T cells. Although so far only *in vitro* and a few *in vivo* studies in mice have been conducted, bi-specifc antibodies may hold promise for future therapeutic use (523,524).

4.4 NEUROBLASTOMA CELLS AS NK CELL TARGETS

The low HLA class I expression of NB cells suggests that they could potentially serve as targets for NK cell-mediated immunotherapy. Although the NK cells derived from NB patients appeared to be functional and mediated tumor regression in different therapeutical settings, NB cells are heterogeneous in their susceptibility to NK-mediated killing (525-528), suggesting that the lack of HLA class I is not the only determinant defining NB sensitivity to lysis by NK cells.

A few attempts were made to understand the molecular interactions involved in NK cell-mediated killing of NB cells. It was demonstrated that the cell adhesion molecules LFA-3 and ICAM-1 were involved (526), while another report questioned the importance of these molecules for NK cell killing of NB (525). While NKG2D does not seem to be involved in NK cell killing of NB cells in some of the experimental settings (301), other studies have shown that the activating natural cytotoxicity receptors NKp46, NKp44 and NKp30 were involved, however, the ligands expressed by NB cells are unknown (529). In a follow-up study by the same authors it was demonstrated that the susceptibility of freshly isolated NB cells to lysis by NK cells was heterogeneous and correlated with the surface expression of PVR that acts as a ligand for the DNAM-1 activating receptor expressed on NK cells (525). Since the PVR expression was lost in later stages of the disease it was suggested that its downregulation could constitute an escape mechanism from NK cell-mediated surveillance of NB. Moreover, it was shown that stage 4 NB cells express the B7-H3 molecule, which confers resistance to NK cell-mediated lysis by interacting with a still unidentified receptor (404). In addition, it was shown that norepinephrine produced by NB cells is a potent inhibitor of NK cell cytotoxicity (410). The same neurotransmitter also inhibited anti-tumor CTLs (411).

No NK cell-based immunotherapy has been applied to NB patients so far, apart from the indirect induction of NK cells via IL-2-mediated therapy. Nevertheless, recent data from a

study in mice demonstrated that NK cell infusions increased the survival time of NB tumor-bearing mice, a strategy which may hold promise for future clinical applications in humans (530).

4.5 TARGETING OF GD2

The disialoganglioside GD2 is highly expressed by virtually all NB cells and, to a lesser extent, by normal cells (188,531,532). Loss of GD2 in response to therapy is a rare phenomenon, further strengthening its potential as a target for immunotherapy of NB (276). Immunotherapeutic targeting of GD2 aims at directing the hosts' Fc-receptor-positive NK cells, granulocytes and macrophages, against the tumor cells. It has been shown that anti-GD2 antibodies induce killing of NB cells by both ADCC and complement dependent cellular cytotoxicity (CDC) (533). There are currently three variants of monoclonal antibodies developed for clinical use; the murine IgG3 mAb 3F8, the murine IgG2a mAb 14G.2a and the human-murine chimeric mAb ch14.18. Since no hematopoietic toxicity can be demonstrated, anti-GD2 therapy is used after myeloabative therapy to treat minimal residual disease. Passive immunotherapy using either of these three antibodies has demonstrated complete remissions and prolonged EFS in some patients in phase I/II trials (534-540). To further stimulate the immune system, combinations of anti-GD2 therapy with cytokine administration (IL-2 and GM-CSF) have been employed with variable outcome (498,541,542). GD2 has also been exploited to target other cytotoxic molecules into the tumor microenvironment (543-546).

Although it was possible to elicit CTLs that were specific for the glycolipid GD2 *in vitro*, there are no published examples of GD2-specific CTLs recognizing tumor in NB patients (547). A novel strategy for GD2 targeting by T cells, where the nature of the antigen was changed from glycolipid to a peptide, was recently developed in a mouse model of NB. It was based on the immunization with DNA encoding a GD2-mimicking peptide, which induced both a humoral and a NK-mediated immune response upon a lethal challenge with NB tumor cells (548,549).

5. THE PATHWAYS TO DEATH

During decades, while describing cell death, researchers used to discriminate only between apoptosis and necrosis. While apoptosis is characterized by changes in cellular morphology, such as early chromatin and cytoplasmic condensation, resulting in cell shrinkage, DNA fragmentation, phosphatidylserine externalization and formation of apoptotic bodies, necrosis is described by swelling of organelles and cytoplasm, clumping of chromatin, breakdown of plasma membrane and, finally, total cell disintegration. In contrast to necrosis, cells undergoing apoptosis induce negligible inflammation in the surrounding tissue (reviewed in (550)).

The terms programmed cell death and apoptosis are often used interchangeably. "Programmed cell death" describes an active process that is dependent on signalling events within a cell, while the true meaning of "apoptosis" refers to a particular cell morphology that is a result of caspase activation. Apoptosis plays a central role in the regulation of tissue homeostasis, and an imbalance between cell death and proliferation may result in tumor formation. Tumor cells attacked by T cells mostly die by apoptosis; hence this type of programmed cell death plays a fundamental role in tumor immunology. Likewise, γ-irradiation and the majority of chemotherapeutic agents induce apoptosis in tumor cells. However, as the molecular understanding of apoptosis increases, evidence is emerging that tumor cells treated by chemotherapy, irradiation or immunotherapy do not always die by apoptosis. Apart from classical apoptosis, other types of programmed cell death may exhibit only some features of apoptotic or even necrotic morphology (reviewed in (551,552)).

5.1 CASPASE-MEDIATED “CLASSICAL” APOPTOSIS

Most of the morphological changes characteristic for apoptosis are mediated by caspases, a family of **c**ysteine **as**partate-specific **p**rote**ases**. Substrates for caspases that directly mediate the morphological changes compatible with apoptosis include; ICAD (inhibitor of caspase-activated DNase), an inhibitor of the nuclease CAD which cuts genomic DNA between nucleosomes generating DNA fragmentation; nuclear lamins, which, when cleaved, contribute to nuclear budding; cytoskeletal proteins, the cleavage of which lead to loss of overall cell shape. The DNA repair enzyme PARP is another substrate that is inactivated by cleavage (551). Caspases are synthesized as inactive zymogens and can be divided into two groups, the initiator caspases (e.g. caspase-2, -8, -9, -10) and the execution caspases (e.g. caspase-3, -6, -7). The initiator caspases are usually activated through regulated protein-protein interactions. In contrast, execution caspases are typically activated proteolytically, either by other caspases or auto-catalytically. The most prevalent caspase in the cell is caspase-3 which is ultimately responsible for most apoptotic effects (551,553). There are two main pathways by which apoptosis can be initiated in the cell; the extrinsic death receptor pathway and the intrinsic mitochondrial pathway (depicted in Figure 8).

5.1.1 The extrinsic death receptor-mediated pathway

The best known ligands of the death receptor family are FasL/CD95L, TNFα and TRAIL/Apo-2L. The ligands act as homotrimers and, upon binding, cause trimerization and aggregation of their respective death receptors Fas/CD95, TNF-R1 and TRAIL-R1/DR4, TRAIL-R2/DR5. The death receptors contain an intracellular death domain (DD) that upon ligand binding recruits procaspase-8 via adapter proteins. The recruited adapter proteins may differ between the three ligands, which may also result in different outcomes. Below, I will discuss the prototype signalling events occurring upon FasL binding to its receptor.

Following association of FasL with Fas, the DD-containing adaptor protein FADD is recruited to the intracellular death domain of Fas. The FADD adaptor also contains a so-called death effector domain (DED), which will recruit the DED-containing procaspase-8. All these molecules together constitute the so-called death-inducing signalling complex (DISC). When two or more procaspase-8 molecules are present, they become activated to cleave other proteins, including procaspase-3. Fas-mediated apoptosis may be blocked by c-FLIP, which is a molecule that resembles procaspase-8 but lacks the protease active site. Hence, if c-FLIP is recruited to FADD, the death signal cannot be conveyed (554,555).

The TRAIL pathway is more complex at the receptor level. So far, five receptors for TRAIL have been identified, including TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (decoy receptor-1), TRAIL-R4 (decoy receptor-2) and osteoprotegrin (OPG). Both TRAIL-R1 and TRAIL-R2 contain a DD motif and hence provide apoptosis signalling through binding of FADD and recruitment of procaspase-8. The other three receptors, TRAIL-R3, TRAIL-R4 and OPG, act as decoy receptors and inhibit TRAIL-induced apoptosis when over-expressed. TRAIL-R3 and OPG completely lack the cytoplasmic region and TRAIL-R4 has a truncated non-functional DD (556).

5.1.2 Susceptibility of neuroblastoma to death receptor ligands

5.1.2.1 Susceptibility of neuroblastoma to FasL

Fas is constitutively expressed by most NB tumors and NB cell lines (557-559). However, some studies have shown no or weak Fas expression by NB cells, where this low expression of Fas was correlated with a poor prognosis of NB (446,560). Despite the presence of Fas, NB cells often resist Fas-mediated apoptosis. Several studies have reported that chemotherapy-induced apoptosis of NB cells is mediated via the Fas/FasL-pathway and that resistance to Fas confers cross-resistance to chemotherapy, suggesting an important role for Fas-mediated signalling in NB eradication (561,562). In some NB cell lines that were resistant to apoptosis mediated by anti-Fas antibodies or soluble FasL, apoptosis could be induced by the addition of mediators acting downstream of the Fas-receptor, suggesting that this resistance may operate at the receptor level (563). So far, Fas-resistance of NB cells has mainly been attributed to silencing of the gene for caspase-8 and not to the expression of Fas (343,564). In addition, resistance may be due to the over-expression of c-FLIP and Bcl-2 which block both

the initial steps of caspase-8 activation and the subsequent steps of mitochondrial activation, respectively (565). However, Fas expression on NB cells may be upregulated by IFNγ and TNFα (558,566-568). Bernassola et al. showed that apoptosis induced by IFNγ correlated with an upregulation of both Fas and FasL, and it was suggested that death of NB cells occured via triggering of a suicide apoptotic mechanism (567).

5.1.2.2 Susceptibility of neuroblastoma to TRAIL

Most NB tumors and NB cell lines express TRAIL-R2 and few express TRAIL-R1 (569-571). In fact, the effect of TRAIL on NB cells was shown to be primarily mediated via TRAIL-R2 (572). In contrast to other cancer cell lines, most NB cell lines are resistant to TRAIL-induced apoptosis (573,574). The TRAIL decoy receptors are expressed quite widely in both TRAIL-sensitive and resistant NB cells and, hence, do not probably confer resistance to TRAIL (344). The resistance rather correlates with caspase-8 deficiency, which is mainly attributed to methylation of the gene (338,345,347), and sensitivity to TRAIL can be completely re-established after ectopic restoration of caspase-8 expression or by the use of demethylating agents or IFNγ (337,345,346,569,575-578). However, in cell lines that were not sensitized to TRAIL by IFNγ, a deficiency in TRAIL-receptor expression may be involved (569). Instead, it was suggested that TRAIL-receptor expression could be upregulated and TRAIL-sensitivity restored by combining IFNγ and chemotherapy (569,574). It was also reported that TRAIL could induce killing of NB cells deficient for caspase-8 by activating the caspase 9/caspase-7 pathway (571).

In addition, over-expression of anti-apoptotic molecules such as IAPs and Bcl-2 (see below) has been reported to confer resistance to TRAIL-mediated killing (351,579,580). One hundred percent of recurrent NBs showed an expression of the IAP protein survivin, in contrast to "cured" NBs which did not express survivin at all (349). Upon down-modulation of survivin expression, TRAIL-induced apoptosis was enhanced (580). An interesting approach to circumvent anti-apoptotic mechanisms employed by NBs is the use of IAP-inhibitory Smac peptides. The addition of Smac peptides enabled bypassing of the Bcl-2 block and sensitized NB cells to TRAIL (581). Histone deacetylase (HDAC) inhibitors were shown to sensitise NB cells to TRAIL-mediated apoptosis through an upregulation of pro-apoptotic proteins (582). Interestingly, an anti-diabetic drug (Troglitazone) could sensitise NB cells to TRAIL by simultaneous action at different levels; by downregulating cFLIP and survivin while upregulating TRAIL-R2, and caspase-8 (583).

5.1.2.3 Susceptibility of neuroblastoma to TNFα

TNFα exerts its effect by binding to two cell surface receptors; namely the 55 kDa TNF-R1 and the 75 kDa TNF-R2 (584). TNF-R1 is the main mediator of TNFα-induced cytotoxicity and is usually involved in most of other TNFα-mediated effects in NB cells and other cell types (reviewed in (585)). No extensive analysis of TNF-receptor expression on NB has been performed. It has been reported that cell lines usually express TNF-R1 and, to a lesser extent,

TNF-R2 (586). A higher expression of TNF-R1 (100%) was observed in the cured tumors as compared to the tumors that recurred (57%) (349). Besides inducing growth arrest and differentiation, TNFα may induce apoptosis and necrosis in NB cells (587,588). IFNγ has a synergistic effect, possibly by inducing the upregulation of TNF-receptors (589). Also retinoids may upregulate the expression of TNF-Rs (586). Of note, TNFα can stimulate the proliferation of some NB cells ((590,591), Levitskaya J., De Geer A., unpublished observation). The proliferative effect of TNFα was mediated by TNF-R2 on NB cells (591).

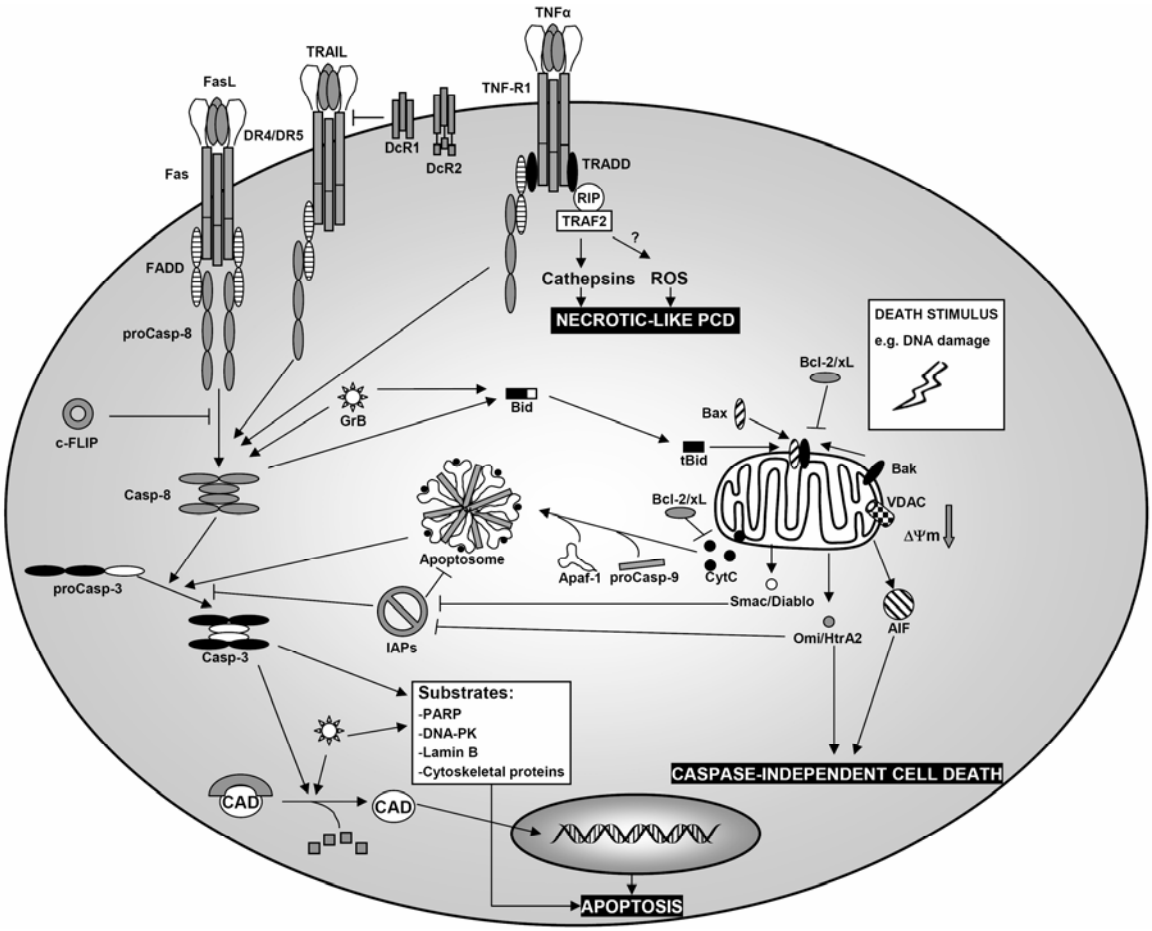

Figure 8. Extrinsic and intrinsic pathways leading to cell death. The figure illustrates the different pathways of cell death described in the text. While the Fas- and TRAIL-R-signalling complexes include the death domain (DD)-containing adaptor molecule FADD, the TNF-R1 signalling complex also includes the adaptor molecule TRADD (TNF-R-associated DD). Moreover, TNF-R1 signalling can result in binding of the adaptor proteins RIP (contains a DD) and TRAF2 (TNF-R-associated factor 2) that may, through the release of ROS or activation of lysosomal cathepsins, lead to necrotic-like PCD rather than classical apoptosis. (GrB; Granzyme B).

5.1.3 The intrinsic mitochondria-mediated pathway.

The intrinsic pathway is usually activated in response to cellular stress stimuli such as DNA damage, oxidative stress, heat shock, growth factor withdrawal and hypoxia. These diverse stimuli induce signalling cascades which converge on the mitochondria and involve the activation of the pro-apoptotic members of the Bcl-2 family of proteins. The mitochondria contain many pro-apoptotic factors including cytochrome c, which normally functions as an

electron carrier in the electron transport chain, and plays a primary role for intrinsic apoptosis. The release of cytochrome c is controlled by the pro-apoptotic Bcl-2 family members Bak and Bax by not yet completely understood mechanisms. It may either occur via pore formation by merely inserting in the outer mitochondrial membrane, or via an interaction with the voltage-dependent anion channel (VDAC) inducing a conformational change leading to channel opening (592,593). If the balance between pro- and anti-apoptotic members of the Bcl-2 family is in favor of apoptosis, cytochrome c is released. Once in the cytosol, cytochrome c associates with the adaptor Apaf-1 (apoptosis protease-activator factor 1), dATP and subsequently procaspase-9 to form the apoptosome. Procaspase-9 is activated and, in turn, activates the execution caspase-3. Caspase-3 can then activate other procaspases, such as -8 and -10, resulting in an amplification of the signal (594).

Cytochrome c is not the only protein released from the mitochondria during apoptosis. Other mitochondrial proteins that are or can be released from the mitochondria are Smac/Diablo, Omi/HtrA2, and apoptosis inducing factor (AIF). The apoptogenic factor Smac/Diablo is released concomitantly with cytochrome c and binds to inhibitors of apoptosis (IAPs) to prevent their anti-apoptotic activity. Smac/Diablo primarily inhibits XIAP (X-linked IAP), which is bound to caspase-9 and caspase-3. This serves as a safe-guard mechanism; the IAPs have to be removed before caspases can be fully active. Omi/HtrA2 exerts a similar function by inhibiting IAPs, but may also induce cell death independent of caspase activation or interaction with IAPs by a yet unknown mechanism. AIF also translocates to the nucleus where it causes large-scale DNA fragmentation but no oligonucleosomal DNA laddering. Over-expression of AIF may cause a drop in the mitochondrial potential and exposure of phosphatidylserine. The effects of AIF are caspase-independent but it is currently unclear how it exerts its functions (595).

5.1.4 Regulators of mitochondria-mediated apoptosis

The Bcl-2 family of proteins constitutes an important group of apoptotic regulators. All family members possess at least one of four conserved BH domains (Bcl-2 homology domains; BH1-BH4) and are divided into three groups: 1) anti-apoptotic members (e.g. Bcl-2, Bcl-xL); 2) pro-apoptotic members (e.g. Bax, Bak); and 3) the "BH3-only" pro-apoptotic members (e.g. Bid, Bad, Bim). The members of the Bcl-2 family regulate apoptosis at the level of the mitochondria. The pro-apoptotic members may either be present in the cytosol or reside on the mitochondrial membrane, while the anti-apoptotic members are usually confined to the mitochondrial membrane. They form a complex network of interactions since most of the proteins can interact with each other. Ultimately, the balance between anti- and pro-apoptotic members of the Bcl-2 family “decides” if the cell will stay alive or die (596). Another large group of apoptotic regulators is the inhibitors of apoptosis proteins (IAPs), which bind to and inhibit caspases. IAPs may also function as ubiquitin ligases, mediating degradation of the caspases they bind. Some of the members include XIAP, cIAP1, cIAP2 and survivin (597).

The intrinsic and extrinsic pathways can communicate. Caspase-8 mediates cross-talk with the mitochondrial pathway by directly cleaving Bid. Activated truncated Bid (tBid) translocates to the mitochondrial membrane where it induces cytocrome c release. The relative importance of this mitochondrial amplification loop differs between cell types and is commonly used to discriminate between type I and type II cells, the latter requiring Bid cleavage for apoptosis. The death in type I cells is independent of the mitochondria and cannot be blocked by over-expression of the anti-apoptotic member Bcl-2. In contrast, type II cells rely on the mitochondrial amplification and death can be prevented by over-expression of Bcl-2.

5.2 NON-APOPTOTIC PROGRAMMED CELL DEATH

Caspase activation was for decades regarded as a hallmark of all types of programmed cell death (PCD), not only for apoptosis. However, increasing evidence suggest that PCD can occur in the absence of caspase activation and that caspase-independent cell death is more common than previously considered. Furthermore it would be detrimental for a cell to rely only on caspases for induction of death. The types of alternative cell deaths are typically divided into apoptosis-like PCD and necrosis-like PCD. (598)

During apoptosis-like PCD the chromatin does not condense to the same extent as in classical apoptosis. Phagocytosis recognition molecules are displayed prior to lysis of the plasma membrane. To complicate the picture, any degree or combination of other apoptotic features can be present. AIF is one of the effector molecules which may mediate apoptosis-like caspase independent cell death (598,599). During necrosis-like PCD there is either no chromatin condensation or little condensation appearing as a spotted pattern. Also, in necrosis-like PCD phosphatidylserine exposure at the cell surface occurs prior to lysis and varying degrees of other apoptotic features may also take place. Autophagic degeneration also belongs to this group and is characterized by extensive cytoplasmic vacuolization and formation of autophagolysosomes (598,599)

If caspases are not mediating the apoptotic features seen in these types of deaths, other molecules must be responsible. The caspase-independent forms of death also depend on proteases and loss of mitochondrial membrane potential. Various proteases have been suggested in different caspase-independent models, and cathepsins can be mentioned as an example. Cathepsins are a group of serine proteases that reside in the endosomal-lysosomal compartment and may, upon activation, enter the cytosol and/or nucleus where they exert their function. Cathepsins may be induced by death receptors, p53 and retinoids (600), and their substrates include Bid, PARP and procaspase-6, and -7. In addition to specific proteases, other molecules may mediate caspase-independent PCD. As discussed earlier, AIF may, when released from the mitochondria, induce caspase-independent DNA fragmentation. Upon TNFα, FasL or TRAIL binding to their respective receptors, an adaptor molecule called RIP (receptor-interacting protein) may be recruited. TNFα-induced necrotic-like PCD involving

RIP was shown to be mediated by mitochondria-derived reactive oxygen species (ROS). Altogether, depending on the incoming signal and the status of the caspase machinery within a cell, death receptor-mediated signalling can mediate both classical apoptosis and necrotic-like PCD. If the caspase-mediated pathway is somehow inhibited, necrotic-like PCD may be favored (553,598,599).

In many situations, several death pathways are activated at the same time, but the ultimate mechanism mediating death is dependent on the nature of the stimulus, the relative rate of each pathway and the types of antagonists that are present within the specific cell type. According to Mathiasen and Jäättelä, "the optimal cancer treatment requires a simultaneous activation of several independent death pathways" (598). A suboptimal cancer therapy may contribute to the selection of apoptosis-resistant tumor cells. In the case of NB, which often shows defects in the caspase machinery, simultaneous attack through different death pathways would be a necessary prerequisite for efficient elimination of the tumor.

5.3 HMGB1 – LINKING CELL DEATH TO AN IMMUNE RESPONSE

Although apoptosis and necrosis were originally defined based on morphologic criteria, some soluble molecules have been identified to distinguish between these two forms of cell death. One such molecule is high mobility group B1 (HMGB1). HMGB1 is a nuclear protein present in almost all eukaryotic cells where it binds loosely to chromatin and, among other functions, acts to stabilise the nucleosomal structure and to regulate gene transcription (601). It was found that HMGB1 is released into the extracellular milieu from necrotic cells (602). A recent study suggested that prior to diffusing out from necrotic cells, HMGB1 needs to be released from the chromatin following PARP-dependent chromatin modifications (603). Importantly, this does not occur when cells die by apoptosis, during which HMGB1 remains immobilised in the nucleus by a series of yet uncharacterised changes (602). This distinction between the two modes of cell death may explain the differences in the ability of necrotic and apoptotic cells to activate an inflammatory response. Upon release, HMGB1 binds at least three receptors; the receptor for advanced glycation end products (RAGE), the toll-like receptor-2 (TLR-2) and TLR-4 (604,605). When HMGB1 binds to its receptors on target cells, it triggers an inflammatory response by initiating a cascade of events leading to the release of cytokines and even more of HMGB1.

Importantly, HMGB1 links cell injury to immunity and could, therefore, have an important implication in immunotherapy. Recent observations suggest that HMGB1 released from cells acts as an endogenous immune adjuvant through the activation of DCs, macrophages, and T cells (606). It was shown that HMGB1 can cause maturation of myeloid DCs and also promote a Th1 response by inducing IL-12 production by DCs and IL-2/IFNγ by T cells (607). Moreover, HMGB1 may promote the proliferation of activated T cells and have a positive effect on $CD4^+$ T cell survival (608). Thus, HMGB1 functions as an immune cytokine to enhance both innate and adaptive immune responses. When released, it could

break tolerance to self-antigens as was shown in a model of lymphoma, where adding HMGB1 turned a poorly immunogenic apoptotic lymphoma into an efficient vaccine (606). Although classically linked to necrosis, preliminary observations by Zeh and Lotze et al. indicated that tumor cell death induced by T- and NK cells showed classical markers of apoptosis that were often also associated with a significant HMGB1 release (609). The authors speculate that this could be due to the fact that tumor cells are inherently resistant to apoptosis and therefore, most likely, die by a non-apoptotic pathway characterised by HMGB1 release, again emphasizing the importance of not being too strict when classifying different modes of cell death.

Interestingly, Guido Kroemer et al. recently demonstrated that certain chemotherapeutic drugs, though described as inducers of apoptosis and non-immunogenic death, promoted the exposure of the ER-resident protein calreticulin on the surface of target cells, thus activating the immune response in a "bystander" way to eliminate possibly chemoresistant tumor cells (375). Unpublished data from the same group demonstrate that these chemotherapeutic drugs may also trigger apoptosis-induced release of HMGB1 that activated DCs through TLR4, further strengthening the role of HMGB1 in alerting the immune system (data communcated by Guido Kroemer to the audience during the symposium "New frontiers in cancer research and therapy", March 15-16, 2007).

Apart from on cells of the innate immune system, the HMGB1-receptor RAGE is also expressed on neuronal cells (604). It was shown that HMGB1 treatment could stimulate RAGE expression on NB cells (610). As expression of RAGE was required for T cell infiltration into the central nervous system (611), stimulating HMGB1 release from NB cells, and therefore, induction of RAGE on NB cells could possibly promote lymphocyte recruitment also into the tumor milieu. Controversially, HMGB1 has been suggested to promote tumor cell growth and metastases when co-expressed with its receptor RAGE, especially under conditions of chronic inflammation (612), therefore blocking of HMGB1 would instead be beneficial in antitumor therapeutical settings (613). The role of HMGB1 in NB biology needs to be further established.

AIMS OF THE THESIS

The overall aim of this thesis was to characterise human neuroblastoma (NB) as a target for T cell-mediated immunotherapy. More specifically, the aims of the distinct projects were to;

- investigate the effects of retinoids, currently in clinical use for treatment of high-risk NB patients, on the HLA class I antigen processing and presentation machinery in NB cells and to assess the compatibility of retinoid treatment and CTL-mediated immunotherapy of NB (Paper I).

- evaluate the effects of IFNγ and TNFα on the HLA class I processing and presentation pathway in NB cell lines and primary tumor samples, and to explore the susceptibility of cytokine-treated NB cells to lysis by HLA class I-restricted CTLs (Paper III).

- assess the potential of using CTL therapy for targeting NB in an HLA class I non-restricted manner (Paper II).

- investigate the effect of soluble factors released by activated CTLs on the levels and composition of cell surface molecules involved in HLA-restricted and HLA-independent NB cell recognition as well as on the susceptibility of NB cells to death receptor-mediated killing (Paper IV).

RESULTS AND DISCUSSION

MODULATING THE IMMUNOGENICITY OF NB CELLS (Paper I and III)

Accumulating evidence demonstrates that primary NB tumor cells are devoid of or express very low levels of HLA class I on the surface, suggesting that these tumor cells may fail to present TAAs to CTLs. Therefore, MHC class I-restricted T cell-based immunotherapy may not be successful unless the expression of HLA class I on tumor cells is induced. Classically, IFNγ has been "the inducer of choice" as it efficiently upregulates surface MHC class I complexes in different cell types, including NB tumor cells (262,263,614,615). However, results from studies on the effects of IFNγ on the recognition by CTLs have been somewhat disparate. While some *in vitro* and murine *in vivo* studies have shown that IFNγ-treated NB cells become more susceptible to CTL-mediated killing (508,511,616), other reports suggest that they do not (617).

As a single agent, IFNγ has been used in one clinical trial on NB. Although HLA class I was induced on the patients' tumor cells and no severe toxicity was observed, no clinical responses were seen (618). Besides that administration of high-dose IFNγ may be associated with serious side effects *in vivo*, tumor cells may also escape cytokine-mediated effects by developing mutations in different proteins involved in IFNγ-signalling (376,377).

Manipulating the NB immunogenicity by retinoids

In the search for biological compounds capable of increasing the immunogenicity of NB cells we focused on retinoic acid derivatives (retinoids), agents which proved to be non-toxic and that are already approved for clinical use in NB patients.

Upon *in vitro* treatment with different retinoids, we consistently observed an increased expression of surface HLA class I on five out of eleven NB cell lines tested, which we further referred to as "responding" cell lines. A cell line was considered as a responder if HLA class I expression was increased 1.5-fold above the values seen in control cells. This arbitrary threshold was chosen based on the consistency in upregulation of HLA class I. The induction of HLA class I was not specific for one particular derivate, but was seen using two natural retinoids, 9-*cis*-RA and all-trans-RA (ATRA) as well as the synthetic retinoid RO-13-6307, all known to act on different receptors.

The non-responsiveness of the remaining cell lines could not be explained by a general resistance of these cells to retinoid treatment, since morphological changes compatible with retinoid-induced cell differentiation were observed upon culturing of non-responders in the presence of different RA derivatives. It may be possible that different RA receptor subtypes, differentially distributed in the NB cell lines, mediate the differentiation- and the antigen presentation effects. However, this is an unlikely explanation since it is generally assumed that all NB cell lines express the whole panel of RA-receptors, at least upon RA treatment (46). To provide a definite answer to this question, a detailed expression analysis of the RA

receptor subtypes needs to be performed for the specific cell lines analyzed. It is also possible that the NB cell lines defined by us as "non-responding", might upregulate HLA class I upon treatment with other retinoic acid derivatives.

The increased levels of HLA class I on the surface could result from an enhanced transcription or translation of the heavy chain and β2m as well as an increased stability of surface HLA complexes. The total pool of HLA class I heavy chain was increased in "responding" NB cell lines. The stability of surface HLA class I complexes as measured by flow cytometry as well as the stability of the total pool of class I heavy chains measured in cell lysates of metabolically labeled cells, was also increased. The stability of HLA class I complexes is influenced by the amount and the repertoire of bound peptides, which in turn is influenced by the proteolytic machinery generating the HLA class I-presented peptide epitopes. Interestingly, monitoring the expression of the immunoproteosomal subunits LMP2, LMP7 and MECL-1 and the proteasomal regulator subunit PA28α revealed that they were all upregulated in retinoid-treated responding cell lines but not in non-responders.

Expression of immunoproteasomal subunits can also be upregulated by IFNγ and TNFα. To rule out that the effect of retinoid treatment was mediated via an endogenous production of IFNγ or TNFα, we monitored MHC class I expression upon treatment with retinoids in the presence of either IFNγ- or TNFα-blocking agents. Blocking either of these cytokines did not affect the increase in HLA class I expression seen upon retinoid treatment, suggesting a specific pathway mediated by the latter.

To further explore the effect of the increased surface HLA class I on the susceptibility of NB to HLA class I-restricted lysis by $CD8^+$ CTLs, we set up a model system where HLA-$A11^+$ NB cells pulsed with an EBV-derived peptide, were used as targets for peptide-specific $CD8^+$ HLA-A11 restricted CTLs. Retinoid-treated NB cells showed a higher susceptibility to lysis when pre-pulsed with a peptide at concentrations ranging from 10^{-11} to 10^{-6} M. The question arose whether retinoid-treated NB cells simply acquire a higher sensitivity to death including lysis induced by CTLs. However, several aspects argue against this; (i) since the level of lysis correlated with the peptide concentration, there is an apparent dependence on the peptide:HLA complex availability, (ii) non-pulsed retinoid-treated cells are only slightly, if any, more susceptible to effector functions of CTLs as compared to control cells, and (iii) non-responding cell lines, which undergo differentiation upon retinoid treatment without upregulating HLA class I, were not more sensitive to lysis by CTLs than their control counterparts.

Expression of ICAM-1 is mainly observed in low-stage NB tumors without *N-Myc*-amplification, suggesting that its expression may facilitate NB interactions with leukocytes and, thus, anti-tumor immune responses (619). In addition to MHC class I, ICAM-1 was upregulated upon retinoid treatment, which may also result in an increased interaction

between the NB cells and the CTLs, that is in line with previously published data (620,621). Notably, retinoid treatment of non-responding cell lines lead to an increase in surface expression of ICAM-1 (data not shown), but did not result in an increased sensitivity to CTL-mediated killing. This argues against a primary role for ICAM-1 in mediating a higher sensitivity to CTL-mediated killing, and suggests that the HLA-restriction element must play a key role.

In addition to inducing HLA-dependent death, activated CTLs may kill bystander targets in an HLA class I non-restricted manner. Therefore, we also investigated the susceptibility of control and retinoid-treated NB cells to CTL-mediated bystander killing in 16 hour cytotoxicity assays and found that retinoid-treated cells were more sensitive to bystander killing by T cells. This may result from the increased levels of ICAM-1, extending the time of interaction with the CTLs, or alternatively, could be caused by an altered expression of death receptors on retinoid-treated NB cells. The latter hypothesis is supported by studies demonstrating the ability of RA to induce both TRAIL-Rs and Fas in tumor cells with a concomitant increase in the sensitivity to apoptosis (622-624). However, it remains to be elucidated whether retinoid treatment affects the expression levels of the death receptors in NB cells, or molecules such as caspase-8 that mediates the effect of death receptor ligands. Nevertheless, the finding that RA-treated NB cells were rendered more sensitive to bystander killing provides a failsafe mechanism whereby tumor cells can be killed in the event of tumor variants escaping HLA class I-restricted killing.

Despite an upregulation of total HLA class I on the surface of retinoid-treated NB cells, NK-mediated killing was not reduced. These results correlate with previously published results on the susceptibility of IFNγ-treated NB cells to NK cells and suggest that the increased expression of HLA class I antigens on the target cells does not preclude an increased susceptibility to NK cells (625). The mechanisms for this phenomenon remains to be elucidated; but it is possible that increased levels of ICAM-1 could override the inhibitory effect of the increased MHC class I levels on NK cell activation (626). Another possibility could be that retinoid treatment may induce the expression of NKG2D ligands. Although we did not observe any increase in MICA expression by the retinoid-treated NB cells (data not shown), we cannot exclude that the induction of other NKG2D ligands could contribute to the activating signal.

To our knowledge, our data demonstrate for the first time the effect of retinoids on the $CD8^+$ CTL lysis of NB cells, suggesting that a combination of retinoids with CTL-based immunotherapy may be an effective treatment for high-risk NB patients. Besides the effect on NB immunogenicity and CTL recognition, and the well-known differentiation- and apoptosis-inducing capability, retinoid treatment might have other beneficial effects. A recent study demonstrated that *in vivo* administration of ATRA dramatically reduced the presence of myeloid suppressor cells in different murine tumor models, and in addition, MSCs were

shown to differentiate into functional APCs (627). Thus, RA treatment may promote anti-tumor responses in multiple ways.

The effects of IFNγ and TNFα on NB susceptibility to CTL-mediated killing

Though numerous studies have demonstrated the ability of IFNγ to induce components of the APM in NB, the impact on subsequent tumor recognition by CTLs has not been investigated in detail. Therefore, we aimed at investigating the IFNγ-induced effects on selected components of the APM in NB cells as well as on CTL recognition. In addition, encouraged by previous results on promoting effects of TNFα induction of APM components and CTL-mediated recognition of melanoma cells (98), the effect of TNFα on NB cells was monitored in parallel.

In general, the NB cell lines and primary tumor samples analysed, heterogeneously expressed the immunoproteasomal components LMP2, LMP7 and MECL1 at steady-state. Although little information is available about the tissue distribution of the constitutive proteasomes versus immunoproteasomes, it is generally accepted that in non-lymphoid cells the immunoproteasome is usually expressed only upon inflammatory stimuli such as IFNγ. In addition, it is known that the immunoproteasome displays a much shorter half-life than the constitutive proteasome, ensuring a dynamic and transient response that permits a rapid return to the constitutive situation once the immunoproteasome function is no longer required (628,629). Thus, the presence of immunoproteasomal subunits in NB cells that have not been treated with pro-inflammatory cytokines could illustrate a deregulated expression pattern due to tumor-derived signals. One explanation for the presence of steady-state expression of immunoproteasomal subunits in NB cells could be due to their production of TNFα (Levitskaya J., De Geer A., unpublished observation), a known inducer of the immunoproteasome (98). Interestingly, LMP2, which is the subunit that showed the highest steady-state expression levels, was shown to be involved in NFκB signalling, protecting cells from TNFα induced apoptosis (630).

In contrast to LMP2, only a few NB cell lines and primary tumor samples expressed LMP7 at steady-state. This is in line with the data demonstrating that various malignant tumor cells are often characterized by the loss or downregulation of LMP7 and instead express a non-functional variant, LMP7E1. Consequently, cells preferentially expressing LMP7E1 are devoid of a fully functional immunoproteasome, a phenotype that is not reversible by IFNγ and that may contribute to immune escape (91). However, it remains to be investigated whether those cell lines and primary NB samples that did not express LMP7, even upon IFNγ-treatment, instead express the LMP7E1 variant.

Several lines of evidence suggest that unless all immunoproteasomal subunits are present, a fully functional immunoproteasome is not generated which might negatively affect the recognition of some potentially important CTL epitopes (631-635). Although IFNγ induced the immunoproteasomal subunits, the TAP complex and HC in most cell lines, there were

some exceptions (see table 1). In contrast to IFNγ, TNFα induced APM components only in some cell lines, suggesting that this immunomodulatory cytokine is not optimal for augmenting the immunogenicity of NB cells (see table 2). In conclusion, some of the NB cell lines exhibit alterations in the expression of APM components that cannot be corrected by pro-inflammatory cytokines. Importantly, none of the cytokine-treated primary NB tumors analysed in this study was capable of assembling a functional immunoproteasome. The data on the effect of cytokine treatment and on defects in HLA class I antigen presentation observed in NB samples are summarised in tables 1 and 2.

	LMP2	LMP7	MECL1	TAP1	TAP2	HC
SK-N-BE(2)	Longer t	(+)	+	+	TL defect	+
SK-N-DZ	+	+	+	+	+	+
SK-N-FI	TL defect	TL defect	+	+	+	+
SK-N-SH	+	+	+	+	+	+
IMR-32	+	+	+	+	+	+
SH-SY5Y	+	+	+	+	+	+
SK-N-AS	+	+	+	+	+	+
LAN-1	+	+	+	+	+	+
LAN-2	TL defect	TL defect	+	+	+	+
LAN-5	+	+	+	(+)	+	+
MC-IXC	+	+	+	+	(+)	+
SHEP-1	+	TL defect	+	+	+	+
CHP-212	+	+	+	+	+	+
FL-2	+	+	+	+	+	+
NB-KI-1	Longer t	Longer t	?	ND	(+)	?
NB-KI-5	Longer t	TL defect	ND	ND	ND	+
NB-KI-6	+	TS defect	+	ND	+	+
NB-KI-8	Longer t	TS defect	Longer t	ND	(+)	?

Table 1. Effect on protein expression on APM components upon IFNγ treatment.

	LMP2	LMP7	MECL1	TAP1	TAP2	HC
SK-N-BE(2)						
SK-N-DZ				+		
SK-N-FI			(+)	(+)	+	+
SK-N-SH			+			+
IMR-32						
SH-SY5Y						
SK-N-AS			+			
LAN-1						
LAN-2					(+)	
LAN-5						
MC-IXC						+
SHEP-1						
CHP-212		+	+			+
FL-2			(+)	(+)		+
NB-KI-1				ND		
NB-KI-5			ND	ND	ND	
NB-KI-6			(+)	ND		(+)
NB-KI-8				ND	(+)	

Table 2. Effect on protein expression on APM components upon TNFα treatment.

+: Upregulated protein upon cytokine treatment

(+): Minor upregulation of protein upon cytokine treatment

Longer t: mRNA is upregulated after 24h, but not the protein, even after 48h. Longer incubation time may be needed. The protein is expressed constitutively.

TL defect: mRNA is upregulated after 24h, but not the protein, even after 48h. A translational defect is suggested. The protein is not expressed constitutively.

TS defect: Neither mRNA nor protein is upregulated, no constitutive expression of protein. A transcriptional defect is suggested.

?: No protein expression constitutively or after IFNγ treatment. Lack mRNA data

ND: Not done

In agreement with previously reported results, the levels of total surface HLA class I complexes, detected by the W6/32 antibody, and total HLA class I HCs were elevated by IFNγ and, less prominently, by TNFα in all NB cell lines except for SH-SY5Y, where TNFα had no effect, probably due to low surface expression levels of TNF receptors (Paper IV). In order to find out whether the increased surface HLA class I levels could render NB cells more sensitive to CTL-mediated killing, we took advantage of the same model system used in the previous study, where NB cells harboring the appropriate restriction elements were pulsed

with EBV-derived peptides and used as targets for peptide-specific $CD8^+$ HLA-A11- and HLA-A2-restricted CTLs. Although NB cells were rendered slightly more sensitive to CTL-mediated killing following the moderate upregulation of HLA restriction elements in response to TNFα, the considerably higher HLA levels induced by IFNγ did not translate into a corresponding increase in HLA-restricted killing.

The W6/32 antibody used to monitor the surface HLA class I levels also recognises the non-classical HLA molecules HLA-E and HLA-G. Since IFNγ may induce both HLA-G and HLA-E at the surface of cells, we hypothesised that an IFNγ-induced expression of the inhibitory HLA molecules could account for the modest CTL activation. We did not observe any steady-state expression or induction of HLA-G in NB cells prior to or after IFNγ treatment. In contrast, we observed a steady-state expression of HLA-E both in cell lines and freshly isolated tumor samples. Hence, a constitutive HLA-E expression in NB cells could constitute an immune escape mechanism. Moreover, the total pool of HLA-E was induced in all cell lines analysed (except LAN-5), and slightly in the NB-KI-6 tumor sample upon IFNγ treatment. Unfortunately, we were unable to analyse the surface expression of HLA-E due to the lack of commercially available reagents. Instead, we performed a functional analysis, where we blocked CD94 in cytotoxicity assays. We observed an increased killing of NB tumor cells by CTLs upon blocking of CD94, suggesting that NB cells resist CTL-mediated killing via the expression of inhibitory HLA-E, both constitutively, as well as to a large extent after exposure to IFNγ. Of note, it cannot be formally excluded that the effect of CD94 blocking could be due to the elimination of inhibitory interactions with other molecules distinct from HLA-E. However, so far no other ligands for inhibitory CD94-heterodimeric receptors are known. Interestingly, blocking of CD94 induced killing of non-treated NB cells where no steady-state expression of HLA-E was observed by Western blot, suggesting that apart from a possible effect on unknown inhibitory ligands, NB cells could express low surface levels of HLA-E undetectable by our methods that could mediate an inhibitory effect.

The question arises on how HLA-E is stabilised on the cell surface of IFNγ-treated NB cells. We could rule out a possible stabilisation by leader sequences from HLA-G. Classical HLA class I molecules were induced by IFNγ in NB cells as well and could, therefore, compete out HLA-E molecules through their higher affinity binding to β2m molecules. One possible explanation could be a relative increase of HLA-E as compared to that of HLA-ABC, sufficient enough to compete for β2m. Alternatively, HLA-E could be stabilised through an induction of unknown peptide ligands preferentially induced by IFNγ in NB cells.

Interestingly, blocking of CD94 did not augment the killing of IFNγ-treated MC-IXC cells, although HLA-E expression was highly induced, suggesting that another level of resistance to CTL-mediated killing was induced in this particular cell line upon IFNγ-treatment. Previous data obtained in our laboratory demonstrated that IFNγ can modulate the capability of tumor cells to bind granzyme B as well as to interfere with the sensitivity of tumor cells to perforin-mediated permeabilisation (K. Hallermalm et al., submitted). Indeed, we observed that IFNγ-

treated MC-IXC cells, although displaying increased binding of granzyme B to the cell surface, showed a reduced intracellular activity of this protease as well as a reduced permeabilisation by perforin as compared to the non-treated counterparts.

The increased surface binding of granzyme B could result from an IFNγ-induced expression of putative granzyme B receptors or, alternatively, from changes in the hydrophobicity of the plasma membrane. The current literature on granzyme B uptake by mammalian cells is controversial. Although several receptors have been implicated as important for uptake, granzyme B might as well attach to target cells by electrostatic interactions with negatively charged surface structures in the membrane, that could include phospholipid headgroups, sulfated lipids, gangliosides, or heparin sulphate proteoglycans (153,636,637). Indeed, IFNγ treatment of NB cells was shown to induce GD2 expression, which could explain the increased surface binding of granzyme B to IFNγ-treated NB cells (638). Moreover, since granzyme B is a highly hydrophobic molecule, any alterations in the lipid composition could influence its binding to target cells. It should be noted that in these experiments we used purified monomeric human granzyme B, which, and in contrast to the form that is secreted from cytotoxic lymphocytes, is not complexed with serglycin and is thus much more positively charged. Therefore, it remains to be elucidated whether the same phenomenon is observed for granzyme B released from CTLs.

However, only the internalised pool of granzyme B determines its function as a death inducing molecule. The reduced intracellular activity of granzyme B in IFNγ-treated cells could result from both a decreased rate of granzyme B internalisation as well as from an intracellular inhibition of its protease activity. To my knowledge there are no studies demonstrating that IFNγ could directly affect the protease activity of granzyme B. Moreover, this cytokine was not present during our experimental assays involving granzyme B. IFNγ-treated MC-IXC cells also resisted perforin-mediated permeabilisation, suggesting that IFNγ might indeed perturb the membrane of NB cells, and thereby prevent the internalisation and/or insertion of granzyme B and perforin, respectively, by changing the membrane composition of the tumor cells. This hypothesis is supported by data suggesting that IFNγ affects both the composition, density and fluidity of the plasma cell membrane of cells (370,371).

Interestingly, the same effects on the susceptibility of NB cells to human purified granzyme B and perforin was seen upon incubating NB cells with supernatant from activated CTLs, containing a plethora of cytokines including IFNγ. Thus, it seems plausible that during a continuous immune response at the tumor site, tumor cells can resist granule-mediated CTL killing.

In conclusion, although IFNγ is usually referred to as a positive modifier of antigen presentation in NB cells, this study highlights the importance of investigating the functional consequence of IFNγ-modified changes. Although the effects of RA on the susceptibility of

NB cells to perforin and granzyme B remain to be elucidated, our cytotoxicity results indicate that RA is a better choice of immunomodulatory agent for the clinical use in NB patients.

BYSTANDER CTL-MEDIATED KILLING AS A NEW APPROACH FOR IMMUNOTHERAPY OF NB (Paper II and IV)

An alternative immunotherapeutical strategy based on CTL usage for low HLA class I-expressing tumors is to bypass HLA class I-restriction. Therefore, we sought to investigate the potential of using CTLs activated on third party targets in the elimination of NB cells in an HLA non-restricted bystander manner.

Induction of caspase-dependent and -independent NB cell death by CTLs

We observed that NB cells could be killed by activated virus-specific CTLs, independently of direct antigen recognition of NB cells. Tumor cells were not lysed in 4h chromium release assays, which argue against a role for granule-mediated killing, and underwent death only after a prolonged incubation time. NB cell death by CTLs did not depend on particular HLA alleles and was not clone-specific, since both HLA-matched and mismatched cell lines were defined as sensitive to a number of CTL effectors with different specificities derived from different donors. Moreover, some of the NB cell lines, initially defined as non-sensitive to bystander killing, were lysed in co-incubation experiments upon further activation of the CTLs on third-party targets expressing relevant peptide:HLA complexes, which highlights the importance of using highly activated CTLs for an efficient death induction. Still, some non-sensitive cell lines remained resistant to killing and the mechanism for this non-responsiveness remains to be elucidated.

Both membrane-bound and/or soluble factors could potentially mediate death triggered by CTLs. To investigate the role of soluble factors we used supernatant from activated CTLs and found that those NB cell lines which were sensitive to CTLs, were also sensitive to “activated supernatant” (AS), indicating that soluble effector molecules can cause death independently of direct CTL-tumor contact. “Control supernatant” (CS) from resting CTLs did not induce death, which indicates the necessity of pre-activating the CTLs for efficient killing of NBs. Notably; the supernatant constituted only 10% of the incubation medium, ruling out that the NB cell viability was affected merely by differences in the pH or the nutritional status of the medium.

Activated CTLs express and release a plethora of different effector molecules. Besides the contents of cytotoxic granules, the most prominent death inducers are the death receptor ligands FasL, TRAIL and TNFα, all of which can be produced both in membrane-bound forms and released as soluble molecules. Another cytokine that is usually released upon CTL activation is IFNγ, which apart from its immunomodulatory effects, can induce apoptosis and differentiation of target cells (639). Moreover, IFNγ can augment death receptor-mediated killing by inducing caspase-8 expression and upregulate Fas receptors on target cells

(567,577). In order to identify the role of the different effector molecules expressed on, or released by, activated CTLs, we performed blocking experiments using neutralizing agents for FasL, TRAIL, TNFα and IFNγ. Four NB cell lines tested displayed various patterns of sensitivity to the blocking reagents upon co-incubation with CTLs. The degree of blocking of the different effector molecules varied between the experiments and might reflect the variable activation status of the particular CTLs used in the individual experiments. However, in all four cell lines, a reproducible reduction in lysis could be seen upon blocking of TNFα, IFNγ and/or FasL, suggesting that these effector molecules contribute to NB cell death. When experiments were performed with AS, TNFα-blocking agents significantly reduced the lysis of all four cell lines tested, which led us to conclude that TNFα is the most important mediator of NB death triggered by AS.

The chromium release assay has its obvious limitations. First, cell death cannot be monitored for longer periods of time since the spontaneous chromium release will ultimately reach high levels. Second, death monitored by chromium release does not discriminate between the molecular pathways leading to cell loss. To further monitor the effect of the AS on NB cells at later time points, we assessed cell death by trypan-blue exclusion, Annexin V/PI and Annexin V/TMRE double stainings followed by flow cytometry. Upon prolonged exposure to AS, NB cell viability was continuously reduced; cells became permeable, displayed phosphatidyl serine on the surface and lost the mitochondrial potential.

Since TNFα is capable of inducing both caspase-dependent and -independent cell death we monitored the effect of the activated supernatant in the presence of the pan-caspase inhibitor Z-VAD-fmk. Death of the NB cells induced by AS within 24 hours was significantly inhibited in the presence of Z-VAD, suggesting that this cell loss was largely caspase-dependent, at least at early time points. Inhibition of caspases in NB cells incubated with CTLs was not performed due to the apparent difficulty of distinguishing the Z-VAD-fmk effect on NB targets from its effect on CTLs. When monitoring NB death upon prolonged exposure to AS at later time points than 24 hours, Z-VAD-fmk had no effect and cell death occurred even when typical caspase-dependent apoptotic changes such as the activation of caspase-3, cleavage of the pan-caspase substrate PARP and DNA fragmentation were inhibited by Z-VAD-fmk. These results demonstrate that the “delayed” cell death monitored in the presence of Z-VAD at 48-72 hours post exposure to AS may occur in a caspase-independent fashion. Thus, those NB cells that either escape the caspase-dependent death or those in which caspases are not expressed or have been inhibited, may still be killed at later time points in a caspase-independent manner by soluble factors released by activated CTLs. This finding is of special importance for NB, a tumor that often lacks caspase-8 expression and is resistant to caspase-mediated death, suggesting that CTL-mediated therapy of NB may still represent an efficient therapeutic approach. Our results are in line with increasing evidence suggesting that the absence of caspase activity favor the induction of other types of cell death such as autophagy or necrosis-like cell death (640-642). Which death program

ensues when apoptosis is prevented may depend on the cell type and the death-inducing stimuli. In fact, it was shown that TNFα elicits a "backup" necrotic cell death in the absence of caspases (643).

In agreement with this observation, TNFα-blocking agents partially reduced the death of NB cells in the presence of caspase inhibition at later time points, suggesting that TNFα contributes to the caspase-independent death. However, when NB cells were treated with corresponding amounts of human recombinant TNFα, during the same period of time, death could not be induced, implying that other factors present in the AS are necessary for the effector function of TNFα. Alternatively, TNFα is a potentiator of another death inducing molecule present in or induced by AS. Another potential explanation could be that the form of TNFα that is released from activated CTLs differs from the recombinant TNFα used to treat NB cells, the latter being more inefficient in forming trimeric complexes. Although, it remains to be explored, it is possible that by blocking TNFα, we are rather looking at an effect of inhibiting the function of TNFα produced by the tumor cells themselves (data not shown), since it was demonstrated that TNFα produced at low amounts by tumor cells promote cancer progression rather than regression (168). Neutralization of either IFNγ, FasL or TRAIL did not rescue NB cells from the AS-induced delayed death, suggesting that cell death is promoted by another yet unidentified factor(s). One reason for the strong impact of TNFα as compared to FasL and TRAIL-mediated death could be that the released form of TNFα is much more efficient in inducing death than the other two soluble death ligands. In fact, it was demonstrated that the soluble form of FasL is 1000 times less efficient in inducing apoptosis than its membrane bound variant (644), which could also explain why we observed a greater effect of FasL blocking in cell-mediated death of NB.

We attempted to characterize the type of caspase-independent death induced by AS in NB cells. TNFα could trigger caspase-independent death in various experimental systems by inducing mediators such as cathepsin B, cathepsin D and reactive oxygen species (645). However, we failed to demonstrate any involvement of the above mentioned mediators. In addition, studies on NB have reported that spontaneously regressing tumors showed signs of caspase-independent autophagic death (646). Despite our extensive efforts, no characteristics of autophagy were observed. Another factor that may cause caspase-independent death is AIF, however, we could not demonstrate any AIF translocation to the nucleus where it performs its functions.

To further characterize the AS-induced caspase-independent death we produced NB cells transfected with the anti-apoptotic proteins Bcl-2 and Bcl-xL. Over-expression of these proteins in NB cells either inhibited or reduced death induced by AS. Although it suggests that mitochondrial depolarization is required for caspase-independent death of NB, it remains to be investigated what factor(s), released in the course of mitochondrial depolarization that is/are essential. Alternatively, the disruption of the cellular metabolism upon mitochondrial

depolarization by AS may lead to NB cell death. At any rate, NB cells over-expressing Bcl-2 were resistant to caspase-independent cell death induced by activated CTLs, suggesting that CTL therapy of NB could be potentiated by drugs inhibiting the function of Bcl-2 in NB cells (647).

The discrimination between apoptotic and necrotic types of PCD induced in NB by soluble factors released from CTLs is of special interest, since the type of cell death may determine the outcome of the immune response in the tumor milieu. We observed that HMGB1 was released upon incubation of NB cells with AS, consistent with a necrotic-like cell death. Inhibiting caspases did not prevent HMGB1 release, whereas blocking TNFα did. One possible explanation for the considerable effect of TNFα inhibition on AS-induced death could be that TNFα mediates HMGB1 release, which upon binding to RAGE receptors on NB cells promotes further TNFα and HMGB1 release, thus augmenting the signal. However, it remains to be determined if the cell lines analyzed in this study express RAGE. In addition, as discussed previously, HMGB1 may activate DCs via its interaction with TLRs and may thereby contribute to the induction of an adaptive immune response to NB tumor antigens.

In this study we took the advantage of using EBV-specific CTLs as a model for highly activated T cells. We propose that EBV-specific CTLs could serve as an optimal source of activated T cells due to several reasons: i) 95 % of the human population are seropositive for EBV and already have EBV-specific CTL precursors (this treatment modality would preferably be given to older high-risk NB patients that most probably are already seropositive for EBV), ii) autologous LCLs can be produced and used to stimulate CTL precursors *in vitro,* iii) EBV-specific CTLs generated *in vitro* were shown to persist and function for up to 6 years *in vivo* (648), iv) clinical trials have demonstrated the possibility to generate large numbers of CTLs from chemotherapy-treated patients as well as adoptive transfer of the EBV-specific CTLs without serious side effects. A phase I trial is currently recruiting NB patients who will receive EBV-specific CTLs, transduced with the GD2-specific chimeric T cell receptor. In this respect, our study contributes to the molecular understanding of the sensitivity of NB cells to the effector mechanisms of these EBV-specific CTLs.

CTLs modify the immune phenotype of NB cells

As discussed previously, the two most commonly described features of NB cells that may render them resistant to both HLA-restricted and death receptor-mediated elimination by CTLs are the low expression of HLA class I and caspase-8 deficiency in these cells. In our study we aimed at investigating; (i) the surface immune phenotype of NB cells, (ii) the NB cell sensitivity to death receptor mediated killing, and (iii) how exposure to factors released from activated CTLs influence the surface immune phenotype and sensitivity to death receptor-mediated killing of NB cells. The term “surface immune phenotype” was defined by us as an expression pattern of surface molecules determining the sensitivity of NB cells to effector mechanisms of cytotoxic lymphocytes including HLA class I, ICAM-1, the receptors

for the death ligands TNFα, FasL and TRAIL as well as the inhibitory non-classical HLA molecules HLA-G and MICA.

We observed that the molecules comprising the surface immune phenotype were heterogeneously expressed both by the long-term *in vitro* propagated cell lines and the primary tumor cells. Surprisingly, we found that the primary tumor cells analysed at the day of tumor excision expressed, though variable, levels of MHC class I, that is in contrast to previously published data on the HLA status in NB samples, suggesting that NB cells could be used as targets for HLA class I-restricted CTL responses. Importantly, similar to the NB cell lines, each of the primary NB cell samples expressed, although at variable levels, at least one of the death domain-containing surface receptors, implying that NB cells can be targeted by death receptor-mediated mechanisms.

To analyze whether soluble factors, released by CTLs upon activation, can modulate the sensitivity of the tumor cells to death receptor-mediated pathways, we first compared the surface expression of death receptors in a panel of NB cell lines and freshly isolated NB tumor samples prior to and after exposure to AS. We found that AS induced TNF-R2 and Fas, but not TNF-R1 and TRAIL-receptors both in NB cell lines and primary NB cells. The reason for the selective upregulation of TNF-R2 remains to be investigated. As discussed above, TNF-R2 can mediate the proliferation in response to TNFα; however, it was not the case for NB cells as no such indication was observed (Paper II). Several factors released from activated CTLs could potentially cause the induction of death receptor expression. For example, IFNγ has been shown to upregulate both Fas and TNF-receptors (567,589,649). Also, the death ligands may potentiate the effect of each other, as exemplified in experiments demonstrating that TNFα can sensitize cells to Fas-mediated death by facilitating FADD recruitment to the receptor complex (650). Hence, factors released from activated CTLs offer a network of interactions, potentiating the effects of each other, which makes CTLs a particularly good source of immunomodulatory- and death promoting factors.

We observed that HLA class I and ICAM-1 molecules were upregulated on NB cells by AS, indicating that the presence of CTLs in the tumor microenvironment may “improve” the quality of NB cells as targets for HLA class I-restricted CTL responses. However, according to the findings in paper III, it does not necessarily guarantee that the tumor cells will be efficiently killed. It is possible though, that the induction of MHC class I from the concerted action of several candidate cytokines in the AS may selectively upregulate classical HLA class I molecules only and not HLA-E, but this statement remains to be experimentally investigated. Nevertheless, it still holds true that some NB cells exposed to soluble factors released from activated CTLs will resist granzyme and perforin mediated death (Paper III).

Another positive outcome of the AS-treatment was the induction of TrkA on the NB cell surface. High or induced TrkA expression was correlated to a good prognosis in NB patients and has been explained as one mechanism behind spontaneous regression of NB (651). NGF,

which is the ligand for TrkA that induces differentiation, can be produced by NB cells themselves (652), or by stromal cells surrounding the tumor (653). The results presented here suggest a potential role for by-stander immune activation in the NB vicinity in facilitating the NGF-induced differentiation of NB cells. Both IFNγ and TNFα present in the AS has been demonstrated to induce differentiation of NB cells, with or without a concomitant TrkA upregulation (588,654-656). Accordingly, IFNγ and NGF work synergistically to induce NB cell differentiation (654,655), suggesting that a combination of NGF treatment/induction and CTL therapy could have a beneficial effect for NB patients.

Certain features of NB cells, like the deficiency in caspase-8 expression, might limit the possibility of using death receptor-mediated CTL immunotherapy for NB. Importantly, we showed that AS induced the expression of caspase-8 in cell lines devoid of this protein. This suggests that if CTLs are used as the source of death receptor ligands, specific demethylating agents do not need to be applied to induce caspase-8 expression in NB cells.

In order to understand whether the increased levels of surface death receptors as well as the induction of expression and activation of casapase-8 in response to AS would have functional consequences, we examined the susceptibility of NB cells to death induced via surface death receptors with and without pre-treatment with AS. We found that NB cells treated with AS were rendered more sensitive to death triggered by TRAIL and Fas-agonistic antibody (CH-11), but not by TNFα. Our data indicate that the expression and activation of caspase-8, rather than the surface levels of TRAIL receptors-1 and -2 play a role in the enhanced cytotoxic effect exerted by TRAIL on NB cells (reviewed in (657)). The increased sensitivity of NB cells to the Fas-agonistic antibody achieved by the pre-treatment with AS could potentially result from the increased surface pool of Fas molecules and the increase in caspase-8 expression by the tumor cells. Interestingly, NB cells remained resistant to TNFα-induced cell death even following AS-treatment, which correlated with the lack of an effect of AS on the surface TNF-R1 expression.

Altogether, data from our studies suggest that CTLs recruited to the tumor site could mediate NB cell death in a bystander fashion. In addition to the direct cell-mediated cytotoxic effect, soluble factors released from activated CTLs increase the levels of surface molecules and mediators central to both HLA class I-restricted killing and death receptor-mediated killing. Although NB might *per se* be MHC class I negative and lack the expression of caspase-8, recruiting activated CTLs to the tumor microenvironment might alleviate this apparent deficiency and render it a better target for CTL-mediated responses.

Systemic administration of TNFα, IFNγ or FasL may be toxic for the patient, limiting their clinical use in the form of recombinant molecules. Administration of TRAIL is relatively non-toxic compared to the other effector molecules, but is often inefficient in NB due to the caspase-8 deficiency. Instead, the adoptive transfer of autologous activated CTLs will provide

all these effector molecules that may act in concert and reinforce each other, thus taking advantage of the efficient death inducing machinery packaged into one cell type.

It is known that IFNγ acts synergistically both with RA and TNFα to induce differentiation of NB cells (589,658-660). In addition, RA was shown to upregulate TNF-Rs (586). Therefore, combining targeted CTL therapy with RA treatment and/or NGF treatment could prove even more efficient in eliminating tumors. In addition, our data indicate that the over-expression of pro-survival Bcl-2 homologues, frequently observed in NB, may prevent the caspase-independent death to occur. Therefore, providing agents aiming at reducing the Bcl-2 levels could improve this immunotherapeutic strategy even further (647).

Since advanced-stage NB patients are often immunosuppressed as a result of the chemotherapy applied, we propose the usage of adoptive T cell transfer rather than active immunotherapeutic strategies. If CTLs, non-specific for NB are targeted to the tumor by means of chimeric receptors, the granule-mediated pathway could still be induced at the tumor site. The CTLs activated via this route may cause both caspase-dependent and -independent NB cell death. Returning to the quote of Mathiasen and Jäättelä stating that “the optimal cancer treatment requires a simultaneous activation of several independent death pathways” (598), we believe that CTL-mediated immunotherapy combined with administration of differentiating agents could provide such a strategy for patients with NB.

GENERAL CONCLUSIONS

Efficient strategies aiming at targeting tumor cells of low immunogenicity by T cell-mediated immunotherapy may not only include approaches of enhancing the tumor sensitivity to T cell effector mechanisms, but may as well exploit the efficient death-inducing machinery of CTLs, bypassing the prerequisite for specific immune recognition of the tumor, i.e. bystander immune activation.

The results presented in this thesis show that NB, although previously referred to as a tumor of low immunogenicity, may represent a good target for CTL-based immunotherapy, especially if combined with retinoid treatment. The positive effect of retinoids on the HLA class I-restricted NB recognition becomes even more important in the context of the inhibitory effect of IFNγ on perforin/granzyme-mediated killing of NB cells. Further *in vitro* and *in vivo* studies will have to be carried out to explore whether the IFNγ-mediated inhibition of CTL lysis of NB cells by can be overcome by retinoid treatment.

In addition, we propose that bystander immune activation could circumvent the problems associated with HLA class I-restricted T cell-mediated recognition of NB cells, as tumor non-specific CTLs are able to induce both caspase-dependent and -independent cell death of NB cells at least *in vitro*. Furthermore, this indicates that NB tumor cells, that often acquire resistance to classical caspase-mediated apoptosis, may nevertheless be eliminated by T cells. Importantly, we demonstrate that soluble factors secreted by activated CTLs are able to induce the expression of molecules essential for HLA class I-restricted T cell-mediated death as well as molecules significant for the susceptibility of tumor cells to death receptor-mediated killing.

In conclusion, I believe that the results presented in this thesis have increased our understanding of the immunogenicity of NB and its interaction with the immune system. We demonstrated that retinoids serve as effective immunomodulatory agents that improve the recognition of NB cells by cytotoxic lymphocytes. We also propose that recruitment of activated tumor-non-specific CTLs into the vicinity of NB cells may have a therapeutic effect, through multiple activities executed in the tumor milleu by triggered T cells that include; a simultaneous activation of different death pathways and an increase of the immunogenicity of NB cells.

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