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INTERACTIONS BETWEEN NEUROBLASTOMA AND THE IMMUNE SYSTEM –

CELLULAR PATHWAYS AND MEDIATORS

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Front cover: T-cell infiltration in a human neuroblastoma tumor. Photo taken by Dr Philos Baldur Sveinbjörnsson.

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”Den vägen som jag åker på, den är krokig och dan, det händer att jag stannar till ibland, men jag tar mig ändå fram ”

(Jumper, 1996)

ABSTRACT

Neuroblastoma (NB) is an embryonal tumor of early childhood arising in tissues of the sympathetic nervous system, such as the adrenal gland and paraspinal ganglia. It is the most common extra-cranial solid tumor of childhood, and 10-20 children are diagnosed with NB each year in Sweden. The overall survival rate is about 70%, but 50% of the children in the high-risk group succumb in spite of intense multimodal therapy. This warrants the search for alternative treatment strategies. One upcoming treatment option is immunotherapy, which represents a specific treatment modality with the possibility of minimizing long-term side effects in survivors.

Cellular therapies for NB have previously been discouraged due to the notion that NB is a tumor of low immunogenicity. This thesis demonstrates that differentiating agents alter the immune phenotype of primary NB tumors and cell lines such as to enhance the expression of classical HLA molecules and the adhesion molecule ICAM-1. This was paralleled by an increased ability of differentiated NB cells to bind granzyme B at the cell surface and translated into enhanced killing by natural killer (NK) cells and T-cells. These results argue in favor of differentiation and cellular immunotherapy as a combined auxiliary approach for NB patients (paper I). Furthermore, the work presented in this thesis demonstrates that tumor-non-specific activated cytotoxic T lymphocytes (CTLs) release effector molecules which facilitate immune-mediated recognition of NB. Effector molecules from CTLs upregulated HLA class I, ICAM-1 and Fas at the cell surface and restored the expression and activity of caspase-8 in primary NB tumors and cell lines. This rendered NB cells more susceptible to death receptor-mediated killing (paper II).

This thesis also demonstrates that primary human NB samples, representing all genetical subtypes, harbor tumor-infiltrating T-cells which proliferate *in situ*. Tumor-infiltrating lymphocytes were preferentially $CD8^+$, expressed high levels of the activation marker CD25 and exhibited a phenotype of memory cells. Autologous peripheral blood lymphocytes were exposed to tumor cells *in vitro* and their production of IFN-γ and TNF-α was increased, while an activated phenotype was obtained. This indicates that human NB cells do not prevent the generation of active T-cell responses (paper III). In the transgenic TH-MYCN mouse model of NB, tumor-associated inflammation was investigated and NB tumor progression was shown to be paralleled by a gradual suppression of intratumoral T-cell responses in favor of immature cells of the innate immune system. Anti-inflammatory treatment with low-dose aspirin displayed a promising efficacy in delaying tumor outgrowth with a concomitant abrogation of an inflammatory switch (paper IV).

Taken together, the work presented in this thesis demonstrates that NB can serve as a proper target for cellular immunotherapy. It argues for an early implementation of immunotherapy in clinical protocols, where differentiating agents and/or the attraction of activated CTLs to the NB microenvironment could enhance immune-mediated tumor recognition.

LIST OF PUBLICATIONS

I. **Lena-Maria Carlson**, Sven Påhlman, Anna De Geer, Per Kogner and Jelena Levitskaya. Differentiation induced by physiological and pharmacological stimuli leads to increased antigenicity of human neuroblastoma cells. *Cell Research*, 2008, 18:398-411

II. 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. *Cancer Immunology, Immunotherapy,* 2008, 57:731-743

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Jehad Charo, Jan Alvar Lindencrona, **Lena-Maria Carlson**, Jorma Hinkula and Rolf Kiessling. Protective efficacy of a DNA influenza virus vaccine is markedly increased by the coadministration of a Schiff base-forming drug. *Journal of Virology*, 2004, 78(20):11321-6.

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LIST OF ABBREVIATIONS

ACT	Adoptive cell transfer
ADCC	Antibody-dependent cellular cytotoxicity
AICD	Activation-induced cell death
ALK	Anaplastic lymphoma kinase
APC	Antigen presenting cell
APM	Antigen processing machinery
Arg-1	Arginase-1
AS	Activated supernatant
β2m	β2-microglobulin
BDNF	Brain-derived neurotrophic factor
CAD	Caspase-activated DNAse
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CD40L	CD40 ligand
CDC	Complement dependent cytotoxicity
CM	Central memory
COX	Cyclooxygenase
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DC	Dendritic cell
DD	Death domain
DISC	Death-inducing signaling complex
DNAM-1	DNAX accessory molecule-1
DR	Death receptor
EBV	Epstein-Barr virus
EFS	Event-free survival
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EM	Effector memory
ER	Endoplasmic reticulum
FADD	Fas-associated death domain
FasL	Fas ligand
FcR	Fc-receptor
FDA	Food and Drug administration
FGF	Fibroblast growth factor
Foxp3	Forkhead box P3
GD2	Disialoganglioside 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GrB	Granzyme B
HLA	Human leukocyte antigen
Hsp	Heat shock protein
ICAD	Inhibitor of caspase-activated DNAse
ICAM-1	Intercellular adhesion molecule-1
iDC	Immature dendritic cell

IDO	Indoleamine 2,3-dioxygenase
IDRF	Image defined risk factor
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
INPC	International neuroblastoma pathology classification
INRG	International neuroblastoma risk group
INRGSS	International neuroblastoma risk group staging system
INSS	International neuroblastoma staging system
KIR	Killer-cell immunoglobulin-like receptor
LFA-1	Lymphocyte function-associated antigen-1
Lin	Lineage
LMP	Low molecular weight protein
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
M-CSF	Macrophage colony-stimulating factor
MDSC	Myeloid derived suppressor cell
MHC	Major histocompatibility complex
MICA /B	MHC class I related chain A/B
MIF	Macrophage migration inhibitory factor
MMP	Matrix metalloproteinase
MMR	Macrophage mannose receptor
NB	Neuroblastoma
NCR	Natural cytotoxicity receptor
NGF	Nerve growth factor
NK	Natural killer
NKT	Natural Killer T
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
NT-3	Neurotrophin-3
PBL	Peripheral blood lymphocyte
PD-1	Programmed death-1
PD-1L	Programmed death-1 ligand
PG	Prostaglandin
PI-9	Proteinase inhibitor-9
PRR	Pattern-recognition receptor
PVR	Poliovirus receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
RAG	Recombination activating gene
sMICA	Soluble MICA
STAT	Signal transducer and activator of transcription
TAA	Tumor-associated antigen
TAL	Tumor-associated lymphocyte
TAM	Tumor-associated macrophage
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF-β	Transforming growth factor beta

TH	Tyrosine hydroxylase
TIL	Tumor-infiltrating lymphocyte
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
TNF-R	TNF-α receptor
TPA	12-O-tetradecanoylphorbol-13-acetate
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TRAIL-R	TRAIL-receptor
Treg	Regulatory T-cell
ULBP	UL-16 binding protein
VEGF	Vascular endothelial growth factor

1 NEUROBLASTOMA

In 1864, the German pathologist Rudolf Virchow described nodular structures arising in the adrenal medulla that contained cells of the sympathetic nervous system [1]. This is believed to be the first description of neuroblastoma (NB), but the disease was first designated by its name in 1910 by Wright [2]. Ever since, NB has fascinated researchers around the world and still today remains a biological mystery in several aspects.

Fifty percent of NBs arise in the adrenal medulla, and the remaining half originates in abdominal and/or thoracic paraspinal sympathetic ganglia [3] (Figure 1). In Sweden, 10-20 children are diagnosed with NB every year [4-5]. The median age at diagnosis is 18 months [4], and NB accounts for 6% of all childhood cancers but 9% of all pediatric cancer deaths in Sweden [5]. The 5-year overall survival rate for all children diagnosed with NB in Sweden during 2000-2008 was 74.1%, but as low as 54.5% for high-risk patients (staging of patients further discussed in section 1.3) [4].

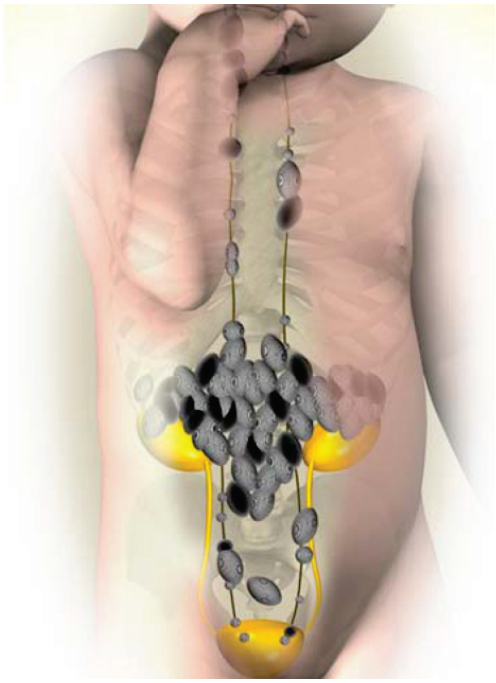

Figure 1. Localisation of neuroblastoma. Neuroblastoma primary tumors derived from the neural crest arise in the sympathetic nervous system including the adrenal medulla, sympathetic ganglia and paraganglia. Neuroblastomas mainly metastasize to lymph nodes, bone and bone marrow, and in infants also spread to liver and subcutaneous tissue.

Reprinted with kind permission from Springer Science and Business Media. Johnsen *et al.,* Embryonal neural tumours and cell death. Apoptosis, 14:424-438, 2009.

1.1 THE BIOLOGY OF NEUROBLASTOMA

NB is an embryonal tumor derived from immature precursor cells present in the neural crest. This is a transient structure present during development which harbors multipotent progenitor cells that will give rise to diverse mature cell populations such as melanocytes and cells of the peripheral nervous system. During the development of the nervous system, the balance between apoptosis and proliferation is tightly regulated, and a deregulation in this process could promote transformation towards the development of NB [6]. Yet no single causative agent has so far been identified for NB. It was recently demonstrated that familial NBs (1-2% of all cases) with autosomal dominant inheritance were dependent on activating mutations in the anaplastic

lymphoma kinase (*ALK*) oncogene [7]. Similarly, somatic mutations leading to *ALK* activation were detected in 5-15% of sporadic NBs [7-8]. Germline mutations in *PHOX2B* also predispose to NB, and may present in combination with central hypoventilation and Hirschsprung´s disease [9]. Loss of the tumor suppressor *NF1* occurs in the genetic syndrome neurofibromatosis, and this condition is also associated with an increased risk of developing NB [10-11]. Intriguingly, children with Down´s syndrome do not develop NB [11].

Divergent biological features of NB underlie the heterogeneous clinical course seen in NB patients. Symptoms are largely variable and depend on the location and the pattern of metastatic spread of the tumor [12], and approximately 40% of NB patients display metastatic disease at diagnosis [4]. The outcome of the disease covers all ranges, from spontaneous regression of tumors with certain favorable features, to treatment-resistant phenotypes with fatal outcome in spite of intense multimodal therapy. Approximately 5-10% of detected NBs undergo regression completely without therapeutic intervention, which is the highest rate observed in all human cancers. The regression may lead to complete disappearance of disease or maturation of the tumor into a ganglioneuroma [3, 13-14]. Furthermore, NB *in situ* is a subclinical tumor-like condition in the adrenal medulla with unproven relation to clinical NB and detectable in 1/250 newborns dying from non-neoplastic causes [15]. Screening for NB, by biochemical detection of catecholamine metabolites in the urine of healthy infants, was shown to increase the detection rate of early NB without decreasing the number of late aggressive tumors or the total number of deaths from NB [16-17].

Underlying mechanisms for the spontaneous regression of NB are still not completely understood, but differentiation via nerve growth factor (NGF) /trkA signaling or immunological mechanisms have been proposed [18-19].

1.2 PROGNOSTIC FACTORS

The age of the patient and the clinical stage of the disease have since long been known to correlate well to the outcome in NB patients [20]. In a consensus report by the International Neuroblastoma Risk Group (INRG) in 2009, age above 18 months was proposed as a cut-off for classifying certain patients into higher risk groups [21]. Apart from that, basic research has contributed to disclosing molecular, genetical and histological patterns that all aid in predicting the behavior of the tumor and the individual need for treatment in NB patients [3, 21].

Amplification of the *MYCN* oncogene remains one of the most important prognostic factors in NB. Overexpression of *MYCN* leads to deregulated growth and proliferation following transcription of target genes [3]. Amplification of *MYCN* in NB was first described by Schwab *et al.* in 1983 [22] and subsequent studies by Brodeur *et al.* and Seeger *et al*. revealed its correlation to advanced stage of disease as well as rapid disease progression [23-24]. In the Swedish cohort, 27% of investigated NB tumors were shown to be *MYCN* amplified [4].

Other genetical aberrations with particular interest locate to chromosome 1, 11 and 17. Deletion of the short arm of chromosome 1 was early discovered to be associated with *MYCN* amplification and later on with worse overall disease outcome [25-26]. On the contrary, deletion of 11q was proven to be inversely correlated to *MYCN* amplification, but still linked to decreased event-free survival (EFS) [26-27]. Gain of genetic material on chromosome 17q is detected in 40-50% of NB tumors, and is associated with an aggressive phenotype of the tumor [3, 28]. The overall DNA content of the tumor is also prognostic in the case of infants with NB, with triploid tumors being linked to a less advanced stage of the disease [29]. Hence, genetic subsets of significance for clinical prognosis include *i)* tumors with numerical gains and losses but no structural aberrations as the most favorable subset; *ii) MYCN*-amplified and 11q-deleted tumors as two different high-risk subsets; and *iii)* remaining tumors with other segmental aberrations including 17q-gain as an intermediate prognostic NB subset [27].

Another factor of prognostic value is the histological phenotype of the tumor. NB is generally described to consist of small, round cells and sometimes displays partial traits of differentiation. A system for detailed analyses of histological parameters of importance to the outcome of NB was suggested by Shimada *et al*. in 1984 [30] and in 1999, it served as the basis for the International Neuroblastoma Pathology Classification (INPC) [31] which is now used as the standard histological classification of NB tumors.

The trk family of neurotrophin receptors is important for proper development of the central and peripheral nervous system. Of special importance for the development of sympathetic neurons is the signaling by NGF through trkA [32]. The expression of trkA in NB is correlated to favorable outcome and a lack of *MYCN*-amplification, and trkA signaling is suggested to enable differentiation as well as programmed cell death in the absence of NGF [33-34]. TrkB, on the contrary, is expressed in aggressive NBs and coincides with *MYCN*-amplification. The ligand for trkB, brain-derived neurotrophic factor (BDNF) can also be expressed by NB cells, providing an autocrine survival loop [35]. Similarly to trkA, trkC is also expressed in low-stage NBs without *MYCN* amplification and is the receptor for neurotrophin-3 (NT-3) [36].

1.3 STAGING AND TREATMENT STRATIFICATION

Considering all prognostic variables that should be taken into account when deciding on the treatment for NB patients, several staging systems have been suggested through the years. In 1971, Evans *et al*. proposed the first staging system [37]. This system is solely based on the local and/or metastatic extent of the disease, and applies the roman numerals I-IV, where infants with metastases confined to the liver, skin or bone marrow are designated as stage IV-S. In 1988, the International Neuroblastoma Staging System (INSS) evolved as a consensus approach to stratify NB patients into risk groups [38]. This system classified patients into stages 1-4, with the 4s category still remaining for infants with favorable pattern of metastatic spread. The system was revised in 1993 with more biological parameters taken into account [39]. However, the INSS relied upon post-surgical evaluation of the remaining tumor, and was hence dependent on the skills of the surgeon. In 2009, so called image defined risk factors (IDRFs), based on

the growth pattern of the tumor and of relevance for surgical intervention were described [40]. Together with other prognostic factors, including age, histology, *MYCN* status, 11q status and DNA ploidy, the IDRFs provide the basis of the INRG staging system (INRGSS) which evolved in 2009, and that allows for pre-surgical risk-classification of all NB patients [21].

1.4 TREATMENT

Prior to any treatment decision, NB patients are stratified into risk groups according to INRGSS. This will avoid over-treating patients with favorable prognosis and aid in identifying patients that need intense multimodal therapy. In general, low-risk patients are treated with surgery alone, whereas patients at intermediate risk receive chemotherapy prior to therapy if IDRFs exist [12, 41].

Children stratified into the high-risk group are treated intensively. Initial chemotherapy is administered as an induction phase [42-43], followed by harvesting of peripheral blood stem cells and surgery. Post surgery, the patients receive high-dose myeloablative chemotherapy and a subsequent reinfusion of peripheral blood stem cells, and radiotherapy is applied to the area of the primary tumor [44]. However, patients may still suffer from residual disease in the bone marrow (minimal residual disease). In the 1980´s, it was shown that retinoid compounds could induce differentiation of NB cells with concomitant reduction of growth and downregulation of *MYCN* [45-46]. Matthay *et al*. demonstrated that the addition of isotretinoin (13-*cis*-retinoic acid) improved the outcome for high-risk patients, and isotretinoin is now incorporated as a maintenance therapy for all high-risk patients following radiotherapy [44].

Considering the fact that 45-60% of all high-risk patients still succumb due to disease progression [4, 12], the search for alternative regimens is warranted. One such upcoming approach is immunotherapy based on monoclonal antibodies (mAbs) targeting disialoganglioside 2 (GD2). This will be separately discussed in section 6.4.2.

2 THE IMMUNE SYSTEM – AN OVERVIEW

The work presented in this thesis touches upon some, but far from all, of the mechanisms, cells and mediators that constitute the immune system. The following section is meant to give a brief introduction to the immune system, in order to facilitate further reading.

2.1 INNATE VERSUS ADAPTIVE IMMUNITY

In vertebrates, innate and adaptive immunity co-operate to protect their host from what is recognized as foreign. The innate immune system entails dendritic cells (DCs), monocytes/macrophages, mast cells, granulocytes and natural killer (NK) cells. Of these, neutrophil granulocytes and macrophages provide the first line of defense upon bacterial infections. In contrast to the adaptive immune system, the innate immune system relies upon germline encoded pattern-recognition receptors (PRRs) that do not rearrange upon stimulation [47]. As a consequence, the response mounted is immediate, but less specific. The actual structures recognized by the innate immune system are molecular motifs that have been conserved through the evolution, such as lipopolysaccharide (LPS) from gram-negative bacteria through toll-like receptor (TLR) 4 [48], or unmethylated CpG dinucleotides present in bacterial DNA through TLR 9 [49]. Upon stimuli, macrophages can engulf invading bacteria and/or secrete biologically active cytokines or chemokines, which are proteins with an intrinsic ability to activate and attract other cells of the immune system. Likewise, these initial mechanisms, together with histamine release from mast cells, underlie the cardinal features of inflammation; *calor, dolor, rubor* and *tumor* (heat, pain, redness and swelling) [50].

However, not all bacteria carry structures enabling this pathogen-associated molecular recognition, and some may mask such motifs behind capsule-like structures. In addition, viruses are rarely eradicated exclusively by innate immunity [51]. Therefore, the sole presence of the innate immune system is not enough to eliminate all foreign threats, and its shortcomings appear.

The major players in the adaptive immune system are the T- and B-lymphocytes, with abilities to recognize details of a molecular structure, such as peptides or proteins. These cells carry receptors which are encoded within somatic gene segments, and that undergo random rearrangements to produce a broad repertoire of divergent receptors. T-cells undergo a tightly regulated selection process in the thymus, whereas B-cells mature in the bone marrow, and each lymphocyte carries receptors of a single specificity. Upon encounter of a “non-self” structure, DCs initiate a cross-talk between the innate and the adaptive immunity, by engulfing the foreign structure and migrating to secondary lymphoid organs where an adaptive immune response is mounted. Thereupon, a clonal expansion of T- and B-cells occurs, leading to the establishment of specific immunological memory, which is solely attributed to the adaptive immune system. Hence, adaptive immunity initially generates a delayed response, but upon re-encounter with the antigen, the response is rapid and effective [52-53].

2.2 THE MAJOR HISTOCOMPATIBILITY COMPLEX

The major histocompatibility complex (MHC) is a genomic region encoding proteins of relevance for the adaptive immune system, such as the MHC class I and II genes. In humans, the MHC genes are termed Human Leukocyte Antigens (HLA), yet the term MHC is often used instead of HLA. The MHC molecules provide an interface for interactions between innate and adaptive immunity, and constitute the basis for antigen-restricted T-cell recognition of target cells as well as T-cell mediated initiation of B-cell responses.

Certain features are shared between MHC class I and MHC class II, but major differences exist. MHC class I is present on the cell surface of all nucleated cells, and preferentially binds peptides of 8-10 amino acids [54-55]. MHC class II, on the other hand, is expressed on professional antigen presenting cells (APCs) such as B-cells, DCs and macrophages, and bind longer peptides of variable length, usually of 13-17 amino acids [56-57]. The peptides presented in the context of MHC class I are derived from intracellular proteins present in the cytosol, and functional MHC class I molecules assemble with their corresponding peptides and the β2-microbglobulin (β2m) chain in the endoplasmic reticulum (ER). The peptide-MHC class I complex will be recognized by $CD8^+$ T-cells, thus enabling a survey of the interior of the cell from the outside. In contrast, MHC class II molecules are transported out of ER towards endosomes where they assemble with peptides derived from proteins that have been endocytosed from the extracellular environment. Peptides present in the pocket of MHC class II molecules will be recognized by $CD4^+$ T-helper cells, which are needed for the generation of proper B-cell and $CD8^+$ T-cell responses [55, 57].

Of particular relevance for the generation of an anti-tumor immune response, proteins engulfed from the extracellular environment may also be presented in the context of MHC class I, through a process named cross-presentation. Hereby, proteins acquired from the tissue, for example from apoptotic tumor cells, are endocytosed by APCs and degraded into peptides that assemble with MHC class I molecules. This enables proteins derived from tumor cells to evoke $CD8^+$ T-cell responses, which will be further discussed in section 4.4.1 [58].

In addition to the so-called classical MHC molecules mentioned above, non-classical MHC molecules exist which have important immunoregulatory functions. The non-classical MHC molecules HLA-E and HLA-G present a limited repertoire of peptides, and display a restricted pattern of tissue distribution [59-60]. HLA-E and HLA-G, and their respective roles in tumor-induced immune dysfunction, will be separately discussed in section 5.2.3.

3 CANCER-RELATED INFLAMMATION

3.1 THE CONCEPT OF CANCER-RELATED INFLAMMATION

The linkage between inflammation and carcinogenesis was postulated by Rudolf Virchow as early as 1863, when the appearance of leukocytes in tumor samples made him hypothesize that chronic inflammation predisposes a tissue to neoplastic transformation [61]. More than a century later, in 1986, Harold Dvorak suggested that tumor development should be viewed upon as a wound that does not heal, yet constantly commences the healing process, including the deposition of extracellular matrix and the ensuing influx of inflammatory cells [62]. Although the concept of cancer-related inflammation dates long back, the underlying molecular events and interactions that connect inflammatory changes with tumor progression are only recently beginning to be unraveled. The cardinal features of cancer-related inflammation include the presence of inflammatory mediators, an infiltrating hematopoietic component, tissue remodeling, angiogenesis and tissue repair [63]. In fact, cancer-related inflammation, including these features, was recently suggested to be the seventh hallmark of cancer [64], in addition to the previous six hallmarks postulated by Hanahan and Weinberg in 2002 [65].

Conceptual proof for the role of inflammation in tumor development is found in a number of studies disclosing a reduced incidence of several cancer types, including colorectal, breast, prostate and lung cancer in populations reporting frequent use of non-steroidal anti-inflammatory drugs (NSAIDs) [66-71]. Furthermore, chronic inflammatory conditions, such as inflammatory bowel disease, hepatitis and Helicobacter pylori infection predispose the host to suffer from tumors originating in these inflamed tissues [63, 72-74].

As will be further discussed, cancer-related inflammation is governed by the presence of inflammatory molecules and inflammatory cells of the immune system.

3.2 MEDIATORS IN CANCER-RELATED INFLAMMATION

The search for molecules contributing to shaping an inflammatory, tumor-promoting microenvironment has intensified in parallel to the upcoming evidence for the role of inflammation in malignant transition. A number of subgroups of mediators are now recognized as messengers in this interplay; some of the most important reviewed below.

3.2.1 Cytokines

Pro-inflammatory cytokines are major players in tumor development and cancer-related inflammation; the most recognized being interleukin (IL) -1β, IL-6 and TNF-α.

3.2.1.1 IL-1β

The involvement of IL-1β in cancer-related inflammation stems from its ability to activate host alarm signals, resulting in the induction of pro-inflammatory enzymes and other mediators such as cyclooxygenase (COX) -2, inducible nitric oxide synthase (iNOS) and IL-6 [75]. Recently, IL-1 β has also been demonstrated to facilitate the accumulation of inflammatory myeloid derived suppressor cells (MDSCs) in the tumor-bearing host [76]. Evidence for the role of IL-1β in tumor progression is further strengthened by reports demonstrating a reduced number of metastases in IL-1β knockout mice [77] and an augmented metastatic spread upon intravenous IL-1β injection [78]. However, IL-1β seems to have a dual role in shaping the inflammatory microenvironment; low levels can indeed contribute to the acquisition of a localized, favorable inflammatory setting with the initiation of a specific anti-tumor response [75].

3.2.1.2 IL-6

IL-6 not only holds the properties of a growth-signal blocking apoptosis, but also possesses pro-inflammatory abilities and is capable of promoting tissue damage and enhanced cell proliferation [79-81]. The implication of IL-6 in tumor growth has been demonstrated in several cancer types, including multiple myeloma [82], colorectal cancer [83], Kaposis sarcoma [84], hepatocellular carcinoma [79], breast cancer [85] and recently, NB [86-87].

3.2.1.3 TNF-α

Tumor necrosis factor α (TNF-α) was given its name due to the finding that it could induce necrosis of transplantable sarcomas in the 1970´s [88]. However, subsequent studies revealed that TNF-α was a prominent inducer of shock-related symptoms and possessed pro-inflammatory abilities. The effects exerted by TNF-α are conveyed via two different receptors, TNF-R1 and TNF-R2, of which TNF-R1 is ubiquitously expressed and mediates most pro-tumorigenic properties of TNF-α. The biological activity of TNF-R2, on the other hand, is not completely understood, but TNF-R2 is not associated with intracellular death domains (DDs) [89]. The pro-inflammatory properties of TNF-α are vast and, in the context of cancer, entail induction of angiogenesis [90], subversion of infiltrating macrophages to a tumor-promoting phenotype [91], induction of suppressive regulatory T-cells (Tregs) [92], contribution to remodeling of extracellular matrix and promotion of expression of other inflammatory mediators [89]. Furthermore, direct effects of TNF-α are also exerted on malignant cells, such as induction of further DNA damage [93], promotion of tumor growth [94] and induction of immune evasion and resistance to chemotherapy [89]. However, as discussed in section 4.4.1.4, TNF-α also possesses pro-apoptotic properties.

3.2.2 Chemokines

The name chemokine derives from the combination of “chemotactic cytokine” into chemokine, and these secreted, cytokine-like molecules are key mediators in the migratory patterns of leukocytes but also of tumor cells [95-96]. As ensues, chemokines regulate cancer-related inflammation, invasion and metastasis and the cellular source

may be tumor cells as well as stromal components. The "CC" and "CXC" chemokines constitute the majority of chemokines, and are so termed due to the adjacent or separated positioning of their first two cysteine residues, respectively, and the receptors are equally termed CCRx/CXCRx. In terms of cancer-related inflammation, the most well defined chemokines and their respective inflammatory outcomes are CCL2/5; infiltration of tumor-associated macrophages (TAMs) [97], CCL22; infiltration of Tregs [98] and indirect stimulation of angiogenesis [99], CXCL9/10; infiltration of lymphocytes [100] and CXCL8; infiltration of neutrophils [101]. In the case of NB, tumor cells have been suggested to utilize the CCL12/CXCR4 pathway in their invasive behavior and dissemination to the bone marrow [102-103].

3.2.3 Prostaglandin E_2 in cancer-related inflammation

Prostaglandin (PG) E_2, a lipid mediator derived from arachidonic acid, is a member of the eicosanoid supergroup which is in turn further classically divided into leukotrienes and prostanoids, the latter including prostaglandins [104]. The synthesis of PGE_2 requires the presence of either COX-1 or COX-2 to direct the conversion of arachidonic acid into prostaglandins. The importance of these pathways in tumor progression has been verified in several studies unveiling high expression of both COX-1 [105-106] and COX-2 [107-113] in a number of cancer types, including NB, mesothelioma, colorectal, breast, ovarian, and bladder cancer.

Bearing the correlation between the COX/PGE_2 pathway and tumor development in mind, it is intriguing to face the pro-inflammatory properties of this molecule. PGE_2 contributes to creating an inflammatory tumor microenvironment by inducing the production of growth factors, angiogenic mediators and other pro-inflammatory factors [104]. The PGE_2- mediated recruitment of blood vessels nurturing the tumor occurs via stimulation of vascular endothelial growth factor (VEGF) production by stromal cells [114], immune cells [115] and tumor cells [116] as well as via CXCL1 production by tumor cells [117] and via enhanced motility in endothelial cells [118]. Likewise, the presence of PGE_2 leads to the extravasation of hematopoietic cells from the circulation and, as they face the pro-inflammatory milieu within the expanding tumor tissue, they acquire a dysfunctional state, further establishing a chronic inflammatory setting [104]. In fact, PGE_2 has been suggested to contribute to all of the hallmarks of cancer [119].

The specific immunosuppressive effects of PGE_2 will be further discussed in section 5.3.3.

3.3 CELLS INVOLVED IN CANCER-RELATED INFLAMMATION

The immune system possesses a dual role in cancer development, with an intrinsic ability to eradicate established tumors, yet often failing this task and instead being undermined by the tumor to promote its progression and expansion by creating a chronic inflammatory setting in which the tumor can prosper [63]. In general, anti-tumor responses by immune cells are generated in the acute inflammatory phase early on in tumor establishment, whereas the later stages of carcinogenesis are associated with subverted, tumor-promoting immune cells [120]. Within the concept of cancer-

related inflammation, the innate immune system, normally serving as a rapid, first-line barrier against foreign pathogenic agents, holds a prominent position as a promoter of tumor development. The ability of the immune system to control cancer is discussed in section 4, and the tumor-promoting capabilities of infiltrating inflammatory cells are discussed below.

3.3.1 Tumor-associated macrophages

Circulating monocytes may enter the tumor tissue by extravasating in response to, among others, tumor- and stroma-derived CCL2 [121-124], VEGF [125] and granulocyte-macrophage colony-stimulating factor (GM-CSF) [126-127]. Following infiltration into the tumor territory, monocytes are entrapped in an environment inflicting phenotypical and functional changes upon the settling cells, resulting in the generation of TAMs [124]. Accumulating evidence underscores the pivotal role of TAMs in tumor progression, and uncovers an association between the infiltration of TAMs and poor prognosis in follicular lymphoma [128], breast [129], prostate [130], and thyroid cancer [131].

Analogous to the classical T_H1/T_H2 classification of T-helper cells [132], macrophages exist as M1 or M2 subsets (Figure 2). M1 macrophages are activated by microbial alarm signals, or in response to interferon (IFN)-γ, and constitute a potent first-line defense against intracellular pathogens. These macrophages exhibit properties of potent APCs, promote T_H1 responses, generate high amounts of toxic compounds such as nitric oxide (NO) and reactive oxygen intermediates, and even possess tumoricidal capacities [133-134]. M1 macrophages are defined by their high expression of MHC class II, iNOS, TNF-α, IL-12 and IL-23, as well as a low expression of IL-10 [124, 133-136]. The presence of M1 macrophages within a tumor tissue is a rare event, virtually only to be expected at early phases of tumor development [133].

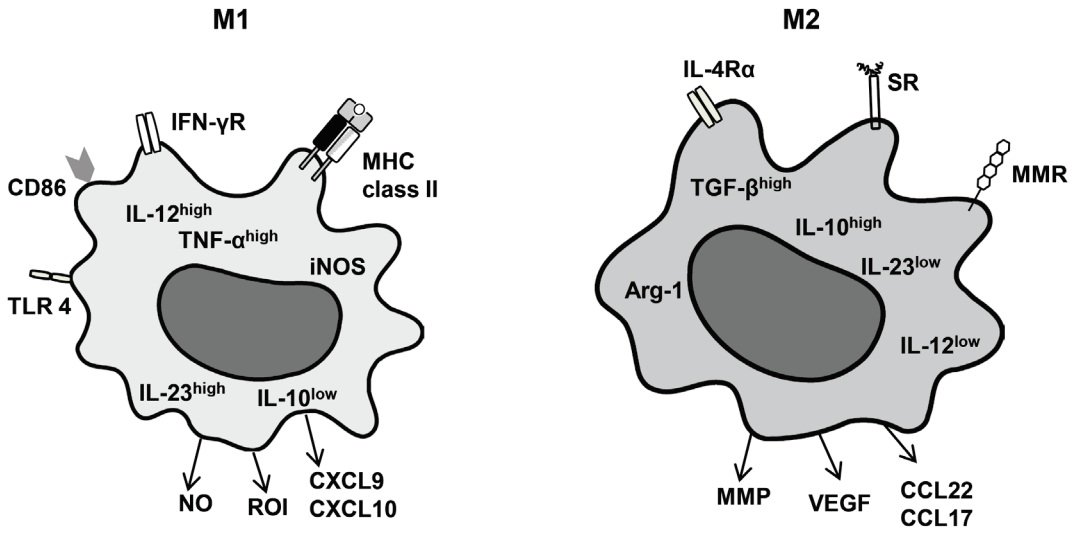

Figure 2. The M1 and M2 phenotypes of tumor-associated macrophages. M1 macrophages promote NK-cell activity via secretion of IL-12, and T_H1 responses by producing CXCL9 and/or CXCL10. M1 macrophages also express co-stimulatory molecules and MHC class II, thus functioning as APCs. They also produce toxic compounds such as NO and ROI. M2 macrophages contribute to tissue remodeling via MMP expression and to angiogenesis by secreting VEGF. T_H2 responses and Treg recruitment are promoted by M2 TAMs via secretion of CCL17 and/or CCL22. SR=scavenger receptor, ROI=reactive oxygen intermediate.

In the course of tumor growth, TAMs subvert from an M1 to an M2 phenotype, due to an inhibited NF-κB signaling [137] and external influences in the presence of IL-4 [133], IL-10 [133, 137-138], IL-13 [133], transforming growth factor β (TGF-β) [127, 137] and PGE_2 [124, 133, 137]. M2 macrophages are defined by their low expression of IL-12, IL-23 and MHC class II as well as a high expression of IL-10, TGF-β, macrophage mannose receptor (MMR), arginase-1 (Arg-1) and scavenger receptors [136, 139-140]. The pathways exploited by TAMs to promote tumor growth are several, and include *i*) tissue remodeling via secretion of matrix metalloproteinases (MMPs) and other tissue degrading enzymes [124, 133, 141]; *ii*) production of angiogenic and growth promoting substances such as VEGF, platelet-derived growth factor, epidermal growth factor (EGF) and thymidine phosphorylase [142-145]; *iii*) promotion of enhanced migration and ability to metastasize [134, 146-150]; and *iv*) inhibition of adaptive anti-tumor responses via induction [151] and recruitment [98] of Tregs, inhibition of T_H1 effector cells by the expression of indoleamine 2,3-dioxygenase (IDO), TNF-α and nitric oxide (NO) [152-153] and inhibition of DC maturation [124]. The importance of the M1 and the M2 phenotypes of macrophages in tumor development was elegantly demonstrated by a complete eradication of established tumors upon restoration of the M1 phenotype in TAMs [154].

3.3.2 Myeloid derived suppressor cells

The presence of hematopoietic cells with immunosuppressive activity as a parallel phenomenon to tumor progression has been recognized since the 1980´s [155], yet the experiments enabling to pinpoint these cells as immature myeloid cells were pursued in the 1990´s [156-157]. Today, these cells are recognized as MDSCs, a heterogeneous population of immature myeloid progenitor cells, containing precursors of DCs, macrophages and granulocytes [158]. A number of markers have been exploited to identify MDSCs and still today, different molecular characterizations indeed exist. In mice, the co-existence of Gr1 and CD11b is preferentially used to define these cells, although subsets of monocytic or granulocytic MDSCs with diverse suppressive mechanisms occur within this definition [158-160]. In humans, the definition of MDSCs has been somewhat more complicated, since there is no homologous marker to Gr1 [158, 161], and today the most common, but not exclusive, definition is based upon the expression pattern $lineage^-(Lin^-)HLA\text{-}DR^-CD33^+$ [158, 162].

In a chronic inflammatory condition, such as cancer, the differentiation of MDSCs into functional immune cells is prevented, and instead, these cells are activated in their immature state [158]. The expansion and recruitment of MDSCs into tumor tissue have been reported to occur mainly due to tumor-derived factors, such as PGE_2 [163], IL-6 [164], GM-CSF [165], VEGF [166], TLR ligands [167] and IL-1β [76]. The activity of MDSCs, on the contrary, is promoted mainly by stroma- and T-cell-derived factors, such as IL-4 [168-169], IL-13 [168-169] and IFN-γ [170].

MDSCs master a number of immunosuppressive modalities hampering adaptive and innate immune responses, including *i*) depletion of L-arginine via Arg-1 and iNOS, resulting in downregulation of the T-cell receptor (TCR) [171]; *ii*) production of reactive oxygen species [172]; *iii*) induction of Tregs [173]; *iv*) perturbation of cysteine

uptake by T-cells [174]; *v*) production of suppressive cytokines [175]; and *vi*) curtailing the expression of L-selectin on T-cells and thereby blocking their trafficking to lymph nodes and sites of inflammation [176]. Non-immunological mechanisms utilized by MDSCs favoring tumor development include the promotion of angiogenesis [177]. There are now several reports demonstrating elevated levels of circulating MDSCs in patients with various cancer diagnoses [166, 178-180], and the frequency of MDSCs has been shown to correlate to clinical tumor stage and metastatic tumor burden [162].

3.3.3 Dendritic cells

DCs normally serve as professional APCs in their mature state, regulating adaptive immunity by providing antigen presentation, cytokine stimulation and costimulation to T-cells. Hence, DCs are certainly players with the potential to contribute to anti-tumor immunity [181].

Yet, DCs, in their immature state, have proven to possess tumor promoting abilities supporting cancer-related inflammation and progression of established tumors [182]. In cancer patients, circulating immature DCs (iDCs) are found at increased levels [183-184], and tumor tissues contain preferentially iDCs [185]. The impaired differentiation of DCs has been attributed mainly to the presence of tumor derived VEGF [186], IL-6 [187], macrophage colony-stimulating factor (M-CSF) [187], IL-10 [188] and gangliosides [189]. Immature DCs are multi-faceted actors in the course of events leading to enhanced cancer-related inflammation and tumor growth [182]. Immunologically, iDCs are poor inducers of adaptive immune responses, due to their low levels of MHC class II, CD40, CD80 and CD86 [182], and DCs isolated from cancer patients or tumor bearing mice have indeed demonstrated a reduced capacity of inducing T-cell responses, instead generating anergic T-cells [184, 190-192]. Attenuation of T-cell responses by iDCs is also achieved via their ability to induce Tregs [193] and via the expression of IDO, which depletes the environment from tryptophan and hampers T-cell responses [194]. Infiltrating DCs have also been shown to promote tumor growth by instructing $CD4^+$ T-cells to secrete IL-13, which in turn drives tumor growth [195]. Additionally, recent reports have disclosed the ability of iDCs present within tumors to stimulate angiogenesis [196-197].

3.4 TARGETING CANCER-RELATED INFLAMMATION

Apparently, the inflammatory pathways being exploited during tumor initiation and progression are by several means potent confederates assisting the tumor by subverting anti-tumor responses and by directly stimulating tumor growth. Hence, the ability to target cancer-related inflammation as an epiphenomenon in cancer patients would potentially bring about beneficial clinical effects, and upcoming evidence indeed validates this hypothesis.

3.4.1 Targeting cytokine and chemokine pathways

Various attempts have been pursued to target cytokines, chemokines and/or their receptors, which promote cancer-related inflammation. Potentially, one could anticipate

a resolution of the immunosuppressive pressure within the microenvironment, rendering it more permissive for immunotherapeutic intervention.
The strong evidence for TNF-α as a tumor promoter prompted a number of clinical trials using anti-TNF-α treatment, showing clinical benefits for patients with metastatic breast cancer [198], ovarian cancer [199] and renal cell carcinoma [200-201]. Another cytokine which has successfully been targeted is IL-6, with clinical benefits for multiple myeloma and renal cell carcinoma patients [202-203].

Turning to chemokines, the major efforts to target these pathways have been by interrupting the interactions between CXCR4/CCL12, CCR2/CCL2, CXCL8/CXCR1/2 and CCL22/CCR4. Antagonists to CXCR4 have induced anti-tumoral and anti-metastatic responses in mouse models of melanoma, osteosarcoma, breast and prostate cancer [204-206], whereas targeting of CCL2 reduced metastatic as well as overall tumor burden in models of prostate cancer [99, 207]. A monoclonal antibody directed against CCR4 not only reduced lymphoma tumor growth, but also reduced infiltrating Tregs and enhanced infiltrating NK-cells [208] and similarly, anti-CXCL8 therapy reduced tumor growth in a model of bladder cancer [209].

3.4.2 Cyclooxygenase inhibitors

Using NSAIDs to target the inflammatory COX/PGE_2 pathway, by selective COX-2 inhibitors (such as celecoxib), or dual COX-1/COX-2 inhibitors (such as aspirin) [210] has, as already mentioned, proven to reduce cancer incidence [66, 68-71, 211-212] and affect tumor outgrowth in several animal models of cancer [111, 213-215]. The complete mechanisms underlying this correlation are still not fully unraveled, but are putatively attributed to direct pro-apoptotic effects on tumor cells [216-217], or reduced levels of PGE_2, and an ensuing abrogation of PGE_2-induced immunosuppression [218-219], tumor proliferation [220-221] and angiogenesis [222].

In clinical practice, however, the regular administration of NSAIDs is limited by their tendency to generate peptic ulcers among frequent users [223], and interfere with hemostasis, an effect brought upon by inhibition of tromboxane A_2 formation in platelets, which leads to a defect in platelet aggregation [210, 224]. Intriguingly, these anti-platelet effects mediated by NSAIDs or other anti-coagulants have been proposed to reduce the number of metastatic tumors [225]. To minimize the risk of gastrointestinal side-effects, the selective COX-2 inhibitors were designed, but instead conferred an increased risk for cardiovascular events due to their inhibition of PGI_2 synthesis which has restricted their clinical impact in certain risk groups [226]. However, quite recently, emerging evidence validates the daily usage of low doses (approximately 75mg) of aspirin, minimizing the risk for cardiovascular events and still retaining the cancer-protective effects [227-229]. Lower doses of aspirin are known to acetylate COX-2, and redirect the conversion of arachidonic acid towards the synthesis of anti-inflammatory lipoxins and resolvins [230-232], and herein resides a potential of exploiting this pathway to diminish cancer-related inflammation [233] with minimized side-effects.

3.4.3 Targeting inflammatory cells

In light of the devastating outcome of TAMs and MDSCs present in the tumor microenvironment, or systemically, the search for strategies to eliminate these subpopulations is warranted, and ongoing [234-235]. Targeting of MDSCs has been pursued using four major strategies [234]; *i*) forcing MDSCs to differentiate into mature cells using all-*trans*-retinoic-acid [236] or vitamin D_3 [237]; *ii*) inhibiting the maturation of MDSCs from precursor cells by targeting of the signal transducer and activator of transcription (STAT) 3 [238]; *iii*) reducing the accumulation of MDSCs by interrupting CXCR2 or CXCR4 signaling [239]; and *iv*) disrupting the inhibiting functions of MDSCs, such as attenuating Arg-1 and iNOS activity using nitroaspirin [240] or COX-2 inhibitors [241]. The COX-2 inhibitor celecoxib was shown to reduce the total number of MDSCs when combined with the cytostatic drug gemcitabine in tumor bearing animals [241-242].

The diversity of TAMs offers several therapeutic targets [235]. The most straight-forward attempts to interfere with TAM-related pathways include the administration of clodronate-encapsulated liposomes or bisphosphonates such as zoledronic acid, which completely depletes macrophages and have proven to inhibit tumor growth *in vivo* using animal models [142, 243]. An alternative approach aims at intervening in the M1 to M2 transition of TAMs, which has been achieved using zoledronic acid and the combined administration of an anti-IL-10 receptor antibody with CpG. Both settings clearly reduced tumor growth *in vivo* using mouse models [154, 244]. A recent publication also demonstrated that an M2 to M1 transition can be achieved using a glycoprotein, histidine-rich glycoprotein (HRG), with subsequent inhibition of tumor growth [245].

4 IMMUNOSURVEILLANCE

The idea that the immune system can scan its host for and eliminate arising neoplastic cells is collectively termed *"immunosurveillance"* [246]. Below, the history of this concept as well as the evidence for its existence and executing functions are reviewed.

4.1 IMMUNOSURVEILLANCE: A WALK THROUGH THE 20TH CENTURY

The very initial suggestion that the immune system would be searching for and eliminate continuously arising cancers was postulated by the immunologist Paul Ehrlich as early as in 1909 [247]. By then, the level of understanding of the immune system was however not sufficient to grasp the theory behind this statement. Hence, it was not until the middle of the 20th century when the experimental possibility arose to immunize mice against a subsequent tumor challenge [248], that the theory of the immune system as a gatekeeper in cancer development was revived. Although the underlying mechanisms of tumor rejection in early experiments were most likely due to allograft reactions, subsequent studies in the 1960´s using inbred mouse strains supported the existence of tumor-associated antigens (TAAs) and a specific immunological mechanism responsible for tumor rejection [249-250]. The following statement postulated by Sir Macfarlane Burnet in 1957 launched the new era of the cancer immunosurveillance theory [251].

"It is by no means inconceivable that small accumulations of tumor cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumor and no clinical hint of its existence."

This statement, together with studies performed at this time, and similar conclusions drawn by Lewis Thomas, increased the belief in immunosurveillance during this epoch [251-254]. Yet, in the 1970´s, the theory was disputed. In experimental models, athymic mice did not display any increased incidence of spontaneous cancers [255-256], and likewise no increased susceptibility to chemically induced tumors. Since at this time, these mice were believed to be more or less totally immunocompromised [257], the theory of immunosurveillance was as a consequence virtually abandoned.

The reply, however, was to come. In the late 1970´s the field was revived upon the discovery of the NK-cells [258], and that these cells could possibly mediate immunosurveillance. At the same time, it became clear that the athymic mice used in above mentioned experiments indeed harbored some functional T-cells, NK-cells and γδ T-cells, which could still exert immunosurveillance [259]. The next step towards the understanding of immunosurveillance came in the 1990´s and in the early 21st century, when studies revealed an increased incidence of spontaneous as well as induced tumors in IFN-$\gamma^{-/-}$, IFN-γ-R1$^{-/-}$ [260-262], perforin$^{-/-}$ [262-264] and recombination activating gene (RAG) 1/2$^{-/-}$ [265] mice, which all have immunological defects of varying degree.

4.2 EVIDENCE FOR IMMUNOSURVEILLANCE IN HUMANS

Even though it is still being questioned by some, the theory of cancer immunosurveillance is in line with several epidemiological as well as experimental observations correlating the immunological status of an individual to the risk of developing cancer. *First*, it has been shown that transplanted patients who are subjected to immunosuppressive agents have a greater risk of developing cancer, including virally as well as non-virally induced tumors such as melanoma, lung and head and neck cancer [266-268]. *Second*, a prospective study showed that a high level of cytotoxic capacity of lymphocytes in the peripheral blood correlates to a decreased risk of developing cancer, whereas a low cytotoxic capacity puts the individual at greater risk [269]. *Third,* genetic deficiencies disarming the immune system, such as perforin deficiency, are proposed to impair immunosurveillance, leading to an increased incidence of lymphoma [270]. *Fourth,* the presence of tumor-infiltrating lymphocytes (TILs) in tumor samples has proven to predict patient survival in several cancer types, including melanoma [271], glioblastoma [272], colon [273-274], ovarian [275-276], breast [277] and stage T3/T4 bladder cancer [278]. *Fifth,* there are more than thirteen case-reports describing the re-appearance of donor-derived melanoma in organ transplanted patients, originating in a long-time "cured" donor (a disease-free interval of the donor of up to 32 years has been described), which is suggested to occur due to a lack of immunosurveillance in the immunosuppressed transplant recipient [279-280].

4.3 TUMOR-ASSOCIATED ANTIGENS

The ultimate event taking place as a result of immunosurveillance is the elimination of a cancer cell by an effector cell of the immune system [246]. For this to occur, the effector cells need to perceive the neoplastic cell as foreign. As the concept of immunosurveillance settled, researchers started to understand the events enabling tumors to induce immune responses. In the 1980´s, it became clear how peptide fragments derived from viral proteins were presented to the immune system via HLA class I molecules on the cell surface [281], and ensuing reports followed in the early 1990´s which described the first HLA class I-restricted epitopes from melanoma antigens which could evoke anti-tumor responses [282-285]. Similarly, HLA class II-restricted epitopes derived from TAAs were identified during that decade [286-287]. Ever since, a great number of antigens present on tumor cells and capable of eliciting immune responses have been identified in various cancers [288].

In general, TAAs must differ from antigens present in non-transformed tissues in order not to induce tolerance. Either TAAs may be modified (through mutations and/or post-translational modifications) and will appear as an altered, non-self peptide on the cell surface, or they may be expressed at higher levels than in normal tissues (overexpression) [289]. In melanoma patients, immune responses are elicited against the cancer/testis antigens MAGE-1 and NY-ESO-1, normally only expressed in the immune-privileged testis, and against the differentiation antigens MART-1 and gp100, which are normally restricted to melanocytes in certain stages of differentiation. Similarly, HER2/neu is often overexpressed above the threshold needed to generate anti-tumor responses in breast and ovarian cancer [290]. As an example of post-translational modifications, aberrantly glycosylated MUC-1 is a TAA present in

pancreatic, breast and ovarian cancer [291]. Taken together, the presence of these antigen-specific anti-tumor responses demonstrates the ability of the host to apprehend a growing cancer as a danger signal.

4.4 EFFECTOR CELLS MEDIATING IMMUNOSURVEILLANCE

The major delegates of the immune system mediating immunosurveillance are T-cells and NK-cells, and these cells are described below.

4.4.1 Cytotoxic T lymphocytes

The importance of having $CD8^+$ cytotoxic T lymphocytes (CTLs) present within the tumor to control its growth has already been touched upon, and below, the underlying mechanisms for CTL-mediated immunosurveillance are reviewed.

4.4.1.1 The generation of an anti-tumor CTL response

Circulating naïve $CD8^+$ T-cells in the blood require assistance to develop into armed CTLs. The most efficient cell at delivering the necessary signals to activate naïve CD8s is the DC, since it is outstanding at engulfing external antigens, such as antigens derived from apoptotic tumor cells, and providing the proper signals for activation [292-293]. Upon encountering a mature DC in the T-cell areas of a peripheral lymphoid organ, or as was recently shown, within the tumor milieu itself [294], the CTL might receive all the signals needed to commence the maturation into an armed effector cell. The CTL will need three signals to get activated [51]. *First*, it must recognize the peptide for which it is specific for in the context of HLA class I at the cell surface of the DC. *Second*, the CTL needs to receive proper costimulation, or else it is at risk of becoming anergic, or even apoptotic [293, 295]. Costimulation is achieved by the interaction between CD28 on the CTL and the B7 molecules CD80 or CD86 on the DC. Depending on the maturation status of the DC, assistance might be needed from a $CD4^+$ T-helper cell to upregulate the expression of CD80/CD86 on the cell surface of the DC via interaction between the CD40 ligand (CD40L) and CD40 [296-299]. By receiving costimulation, the threshold for the actual number of TCR-MHC interactions needed is lowered [300]. Then, *third,* upon ligation of CD28 and CD80/CD86, the synthesis of IL-2 is initiated by the CTL [301], which favors the generation of a complete effector response [302].

Upon receiving appropriate signals, naïve $CD8^+$ T-cells turn into effector cells, and undergo a number of changes preparing them for their mission. The generated effector cells are re-distributed within the body [303-304] and their extravasation is facilitated by an altered expression pattern of adhesion molecules, such as an increased expression of, among others, lymphocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4), as well as a decrease in CD62L (L-selectin) [305-306]. Following the initial expansion, 90-95% of $CD8^+$ T-cells will be eliminated in a contraction phase, with the remaining cells forming the T-cell memory pool. These memory cells are then sustained in the body, and categorized into central memory (T_{CM}), effector memory (T_{EM}) and T_{EMRA} cells, based on their expression pattern of, among others, CD45RA and CCR7 [307]. The memory cells provide the basis for the ability of the immune

system to rapidly re-generate effector responses against previously encountered antigens; T_{EM} cells home to sites of inflammation, carry high contents of perforin, display rapid effector functions and confer immediate protection, whereas the T_{CM} cells prevail in lymphoid tissues and are able to clonally expand and re-direct into T_{EM} cells upon antigen encounter, hence providing the long-term protection. T_{EMRA} cells, which constitute a part of the T_{EM} pool, are not as well characterized, but known to carry the highest content of perforin [307].

Below, the major pathways utilized by activated CTLs to eliminate a target cell will be discussed.

4.4.1.2 CTL cytotoxicity: The granule exocytosis pathway

The importance of the granule exocytosis pathway (the granule-mediated / the perforin/granzyme pathway) in mediating CTL cytotoxicity has been demonstrated in several studies [308-311]. The model, which was first proposed in 1985 [312], suggests that cytolytic granules are released by activated CTLs into the immunological synapse between the CTL and the target cell expressing the correct peptide-MHC class I complex, and hereupon the content induces cell damage within the target cell [310]. This ensures a rapid destruction only of target cells presenting the peptide for which the CTL is specific for.

The model supports the significance of two major components within the granules; the pore forming protein perforin, and the serine protease granzyme B (GrB). It was early demonstrated that perforin alone could not induce the complete death-cascade [313], and that assistance by GrB was required to induce target cell apoptosis [314-315]. Today, although GrB and perforin are acknowledged as important mediators of the granule exocytosis pathway, their exact mode of action is still a matter of debate [316].

One topic which has been extensively debated is the role of perforin in mediating cytotoxicity. The fact that perforin, upon release into the immunological synapse, could polymerize and create a pore in the target cell membrane [310, 317] generated the hypothesis that perforin would be responsible for the subsequent entry of GrB through this pore. However, the pores generated by perforin were soon described as too narrow for this to occur [318], and the primary importance of perforin was instead attributed to its assistance in releasing GrB from the endosomes after uptake in the target cell [319-321].

In addition, the underlying mechanisms for GrB entry into the target cell have been debated. Eventually, evidence argued that GrB enters the target via route(s) circumventing the need of perforin-generated pores, and several other plausible mechanisms have been discussed [316]. Following the release of GrB in complex with serglycin into the immunological synapse [322], the subsequent uptake into the target cell has been suggested to involve receptor-mediated endocytosis, through the mannose-6-phosphate receptor [323-324]. However, later it was shown that entry still occurred via micropinocytosis in the absence of this receptor [325-326]. Other proposed mechanisms for the entry of GrB into the target cell include binding to heat shock protein 70 (Hsp 70) [327], and ion-exchange of serglycin to heparin sulfate

glycosaminoglycans on the target cell membrane [328]. Furthermore, others claimed that GrB was internalized during reparative endocytosis as a consequence of membrane pores generated by perforin [329-330].

Reaching the interior of the target cell, GrB can initiate the apoptotic machinery directly by activating pro-caspase-3, or indirectly via activation of caspase-8 [331-332]. Additionally, GrB can induce caspase-independent cell death through an alternative pathway involving direct mitochondrial damage and cytochrome c release [333]. Furthermore, caspase-independent DNA fragmentation can occur by inactivation of ICAD, the inhibitor of caspase-activated DNAse (CAD), which in its free form initiates a DNA fragmenting cascade [334] (Figure 3).

In addition to GrB, cytotoxic granules also contain other granzymes such as GrA, GrM and granulysin, with somewhat modified modes of action (reviewed in [310] and [321]), but these are beyond the scope of this thesis. Finally, it deserves to be stressed that an activated CTL, as compared to a naïve CD8, is indeed a carrier of increased amounts of the cytolytic components described above [335-337].

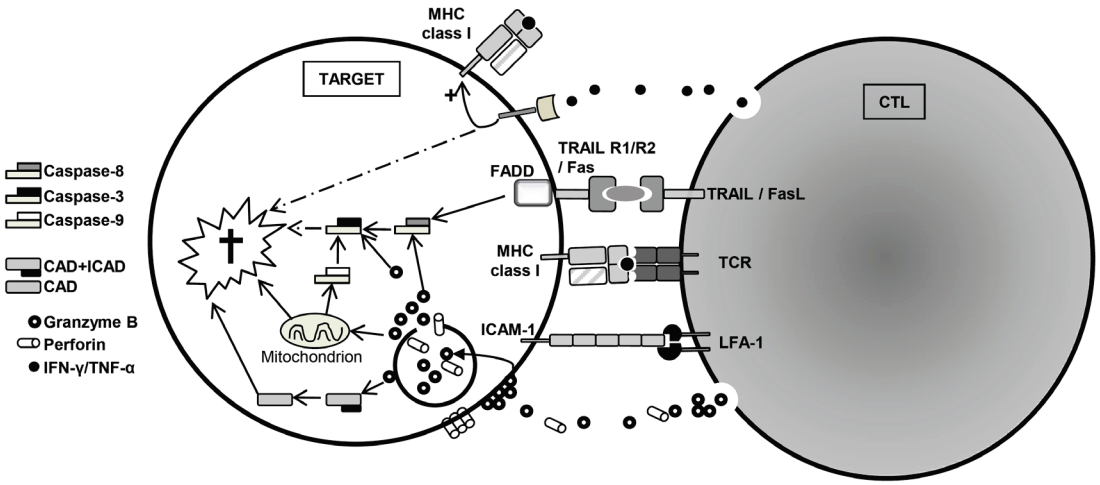

Figure 3. Mechanisms of CTL-mediated killing of target cells. CTLs utilize GrB and perforin in the granule-mediated pathway to induce target cell death via activation of caspases, mitochondrial damage and ICAD cleavage, the latter leading to DNA fragmentation by CAD. In the DR pathway, interactions between Fas/FasL and/or TRAIL/TRAIL-R1/2 induce the FADD complex, which leads to caspase activation. Secreted cytokines such as IFN-γ and TNF-α can both enhance MHC class I expression and induce target cell death on their own.

4.4.1.3 CTL cytotoxicity: The death receptor pathway

Targeting a tumor cell in an HLA-restricted fashion requires an intact HLA class I expression, which in the light of HLA downregulation as a tumor escape mechanism (discussed in section 5.2) may render CTL-mediated responses insufficient. However, this HLA-dependent pathway can be circumvented by employing the death receptor (DR)-mediated pathway, taking advantage of DRs on the target cell membrane and the expression of their respective ligands on activated CTLs.

The most well characterized DR ligands include Fas ligand (FasL, CD95L), TNF-α and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, Apo-2L). The corresponding receptors involved in target cell death are Fas/CD95, TNF-R1 and TRAIL-R1 (DR4) and -R2 (DR5), respectively [338]. These DRs belong to the TNF receptor superfamily and bear resemblance to one another by their intracellular DDs, capable of transmitting death-inducing signals into the target cell [339]. Bearing in mind that signaling through DRs does not always lead to cell death, as exemplified by Fas-induced proliferation of fibroblasts [340], the DR ligands are here discussed in the context of their pro-apoptotic abilities conveying T-cell and/or NK-cell cytotoxicity.

As a prototype for death inducing ligands, FasL was first cloned in the early 1990´s [341], and is now well recognized as a DR ligand present on CTLs, NK-cells and T-helper cells. The Fas/FasL system has been shown to regulate the peripheral T-cell repertoire via activation-induced cell death (AICD) [342], and defective Fas/FasL interactions have been shown to result in lymphoproliferative disorders [343-344]. Both *in vitro* and *in vivo* studies suggest a role for Fas/FasL in tumor immunosurveillance, as it was shown that mAbs directed towards Fas could induce cell death in tumor cell lines and that a deficient Fas/FasL pathway conferred metastatic capabilities to osteosarcoma and melanoma cells [345-347]. The intracellular signaling through the Fas/FasL system initiates the death-inducing signaling complex (DISC) through the Fas-associated DD (FADD) protein. Through a series of events, this leads to the activation of caspase-8 and caspase-10, with ensuing apoptosis in the target cell [348-350] (Figure 3).

The function and pathways of TRAIL resemble those of FasL in many aspects. TRAIL also participates in regulating the peripheral T-cell compartment by AICD [351], and has been shown to suppress metastatic spread *in vivo* [352-353]. In a similar manner to FasL, TRAIL induces the DISC when interacting with TRAIL-R1 or -R2, which in turn leads to caspase-8 and/or caspase-10 induction [338, 349, 354]. In addition to TRAIL-R1 and -R2, TRAIL can bind to osteoprotegerin, TRAIL-R3 and -R4, which are decoy receptors lacking or exhibiting a truncated intracellular domain [354-356]. Initially, it was suggested that the decoy receptors inhibited TRAIL-mediated apoptosis, but subsequent studies have shown no correlation between TRAIL-R3 and -R4 expression and sensitivity to TRAIL-mediated lysis [357]. However, TRAIL-R4 has been shown to sequester TRAIL-R2 and form an unresponsive complex, hence abrogating TRAIL-R2 signaling [358]. Uncertainty regarding the possible *in vivo* regulation of TRAIL activity by decoy receptors still remains [354]. When administering recombinant TRAIL *in vivo*, tumor cells were more sensitive to TRAIL than non-malignant cells [359], a finding that has encouraged a number of ongoing studies and argues in favor of employing TRAIL in cancer therapy [354].

4.4.1.4 CTL cytotoxicity: Pro-inflammatory cytokines

Although granule exocytosis and the death receptor pathway are undisputed as the most prominent mechanisms whereby CTLs induce target cell death, the release of pro-inflammatory cytokines, including IFN-γ and TNF-α, is also of importance. Indirectly, IFN-γ and TNF-α can promote CTL cytotoxicity by enhancing the immunogenicity of the target cell. TNF-α as well as IFN-γ have been shown to increase the expression of HLA molecules on the cell surface [360-361], and IFN-γ can efficiently enhance

antigen presentation by inducing the immunoproteasome, which will result in an altered set of antigenic peptides [362-363].

The presence of TNF-R1 on a target cell also enables TNF-α to directly induce cell death, since TNF-R1, under the premise that NF-κB is inactive, mediates apoptosis [364]. Using adoptive transfer of perforin/IFN-γ-deficient CTLs into mice, tumor rejection has been reported and was attributed to the secretion of TNF-α [365]. Likewise, IFN-γ can also abrogate tumor growth *in vivo* via indirect mechanisms [366], and tumor cells expressing truncated IFN-γ receptors may be resistant to immune-mediated rejection and display an enhanced growth rate in mice [260].

4.4.2 $CD4^+$ T-helper cells: Foes in disguise?

The current view of $CD4^+$ T-helper cells in tumor immunity is paradoxical, and contentious evidence supports a protective role as well as a role in tumor-promotion [367]. The classical T_H1/T_H2 paradigm of $CD4^+$ T-cells was first proposed in 1986 [132], following the recognition that the generation of both B-cell responses [368] and CTL responses [369] were dependent on the assistance by T-helper cells. The direction of $CD4^+$ T-cells into the T_H1 lineage is highly dependent on the presence of IL-12 and results in the ensuing production of IFN-γ, IL-2, IL-12 and TNF-α, whereas a T_H2 response is mounted after exposure to IL-4 and tips the balance towards a production of IL-4, IL-5, IL-6 and IL-13. The T_H1 lineage fosters cell mediated immunity while T_H2 cells promote B-cells and humoral immunity [370]. Below, the current view, which underpins the anti-tumor properties of T_H1 cells and at the same time positions T_H2 cells in liaison with the tumor, will be discussed.

4.4.2.1 Immunosurveillance by T-helper cells

T-cell based immunosurveillance of tumors was for a long time mainly attributed to HLA class I-restricted CTL responses. Yet, during the last years, the existence of HLA class II-restricted peptides from TAAs has been acknowledged, and there are now a number of known class II peptides from common tumor antigens, such as MART-1 [371], NY-ESO-1 [372] and MUC-1 [373], which possess the capability to elicit tumor-specific $CD4^+$ T-cell responses. Clear-cut proof for a protective role of T-helper cells in the tumor microenvironment resides in the correlation between tumor-infiltrating CD4s and a favorable outcome in head and neck, cervical and non-small cell lung cancer [374-376]. Furthermore, several *in vivo* models have continuously demonstrated the absolute dependence on the presence of $CD4^+$ T-cells for immunological tumor rejection [377-379].

However, most tumors do not express HLA class II, and hence fail to directly provoke a TCR response in $CD4^+$ T-cells. Alternative pathways are hence likely to be more pertinent to the anti-tumor effects exerted by T-helper cells [367]. One proposed mechanism involves the activation of tumoricidal M1 macrophages by CD4-derived IFN-γ [380], and T_H1 CD4s have also been shown to co-operate with NK-cells and to render tumors more susceptible to NK-cell-mediated killing by modulating the expression of ligands recognized by NK-cell receptors, such as NKG2D [381-382]. The anti-tumor effects exerted by IFN-γ have also been attributed to modulation of non-hematopoietic cells within the stroma, resulting in inhibition of angiogenesis [383]. As

a major lineage-specific effect, T_H1 cells promote CTL responses by assuring the efficient generation of secondary memory responses and by providing costimulation and IL-2 [384-385]. Of particular relevance for tumor immunity, T-helper cells have been shown to positively regulate CD8 infiltration into the tumor milieu [386-387], to promote CTL expansion on site [388] and to enhance the expression of GrB and the cytolytic activity of CTLs within the tumor [386, 389]. Indirect effects are thus likely to be the most important whereby CD4s suppress tumor growth, yet direct targeting of tumor cells utilizing DRs and IFN-γ might also be of importance in some models [366, 390].

4.4.2.2 Tumor promotion by T-helper cells

The T_H2 lineage of $CD4^+$ T-cells opposes the anti-tumor ability of T_H1 cells. The encounter between a CTL and a T_H2 $CD4^+$ cell can induce CTL anergy in response to cytokines released by the T_H2 cell, and thereby abrogate the expected proliferation [370, 391]. Furthermore, the cytokine milieu orchestrated by T_H2 $CD4^+$ cells can directly stimulate tumor growth, as has been shown for IL-13 in breast cancer [195] and IL-6 in NB [86-87].

T_H2 cytokines also promote the innate immune system to enhance cancer-related inflammation. For example, the activity of MDSCs is highly promoted by T-cell derived IL-4 and IL-13 [392] and their accumulation is enhanced by IL-6 [164]. In a recent publication by De Nardo *et al.* $CD4^+$ T-cells were shown to promote pulmonary metastasis of breast cancer, and the effects were attributed to a CD4-dependent M1 to M2 transition of macrophages, which was significantly inhibited by blocking of IL-4 [393]. But, as true and perplexing as it is for many mediators and cells within the field of tumor immunology, there is no law without exceptions. Anti-tumor activity by T_H2 cells has indeed been demonstrated, and was mainly attributed to the induction of cytolytic eosinophils [394-395].

In line with this, a third lineage of T-helper cells, T_H17, defined by its TGF-β dependent development and ability to produce IL-17, has been ascribed a dual role in immunosurveillance [396]. T_H17 cells have shown remarkable anti-tumor potential by completely eradicating established melanoma tumors in experimental animal models [397], yet their presence correlates to poor prognosis in various cancers and contributes to the onset of inflammation-induced colonic tumors [381, 398-399].

Of note, it has been shown that an impaired response to mitogenic stimuli of lymphocytes in NB patients with disseminated disease correlates to favorable prognosis, suggesting that circulating lymphocytes, including $CD4^+$ cells, may promote tumor growth at this stage of disease [400].

4.4.3 Natural Killer Cells

Turning to the innate immune system, a cell type with potent anti-tumor activities is found in the NK-cell [401]. The existence of NK-cells was demonstrated in 1975 by Kiessling *et al.* and Herberman *et al.* [258, 402-403]. The original observations described the NK-cell as a cell able to lyse tumor cell lines without the need of prior stimulation, and this finding reinforced the theory of immunosurveillance at that time.

The molecular pathways governing NK-cell activation versus inhibition have ever since been intensively studied, and today, it is well established that NK-cell killing depends on the net effects of signaling through inhibitory as well as activating receptors [404] (Figure 4).

The negative regulation of NK-cell activity was first to be discovered, with the initial proposal of the "missing-self" hypothesis by Kärre *et al.*, and subsequent observations that MHC expression interfered with *in vivo* NK-mediated immunosurveillance of a murine lymphoma [405-406]. The evidence for a negative effect of MHC expression on NK-cell-mediated killing was substantiated when studies revealed that NK-cells expressed receptors which, upon recognition of HLA molecules, delivered inhibitory signals into the NK-cell [407-409]. These killer-cell immunoglobulin (Ig)-like receptors (KIRs), together with the receptor CD94/NKG2A, are considered the major negative regulators of NK-cell activity [404], and bind to HLA-A,-B, -C and -G molecules, or HLA-E, respectively. The ability to sense a lack of MHC expression as a danger signal enables the NK-cell to target virally infected cells, or tumor cells, since these often downregulate MHC expression to avoid CTL recognition [410-411].

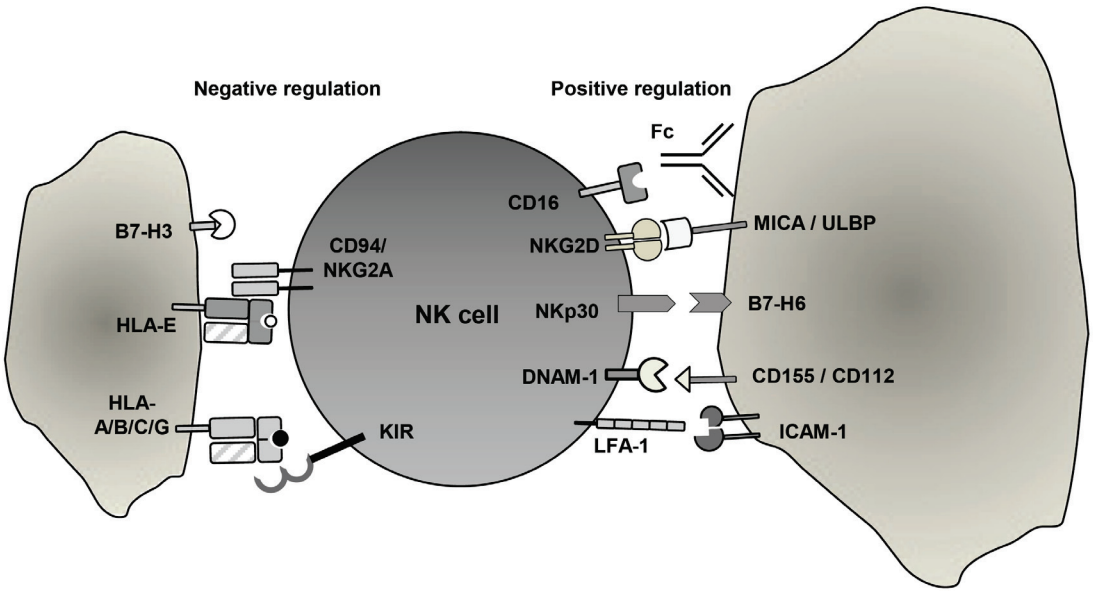

Figure 4. Regulation of NK-cell activity. NK-cell activity is negatively regulated by inhibiting receptors (left side) as well as positively by activating receptors (right side). The corresponding known ligands on target cells are also displayed in the figure. NKp30 is shown as an example of NCRs.

The expression of MHC on the cell surface is hence a potent disruptor of NK-cell cytotoxicity. But still, killing of tumor cells with detectable MHC levels by NK-cells can still occur [412], arguing for other mechanisms overriding the inhibitory signals. Indeed, NK-cell activity is not only regulated by inhibiting receptors, but also through activating receptors [404]. These include the natural cytotoxicity receptors (NCRs) NKp30, NKp46 and NKp44, with so far two identified ligands, the human leukocyte antigen-B-associated transcript 3 (BAT3) and B7-H6 [413-414]. Another well-studied activating receptor is NKG2D, the ligands of which are known to be the stress-inducible MHC class I-related chain (MIC) A and -B as well as the UL-16 binding proteins (ULBPs) [415-416]. NKG2D has been attributed a prominent role in NK-cell-

mediated immunosurveillance, since its ligands are expressed by, and can induce NK-cell-mediated *in vitro* killing of cell lines of various origins [417]. Not to be left out is DNAX accessory molecule-1 (DNAM-1), an activating receptor with CD155 (the poliovirus receptor (PVR)) and CD112 (PVR- related 2) as identified ligands [418]. DNAM-1 has been attributed a pivotal role in mediating anti-tumor immunity of NB *in vitro* [419] and of fibrosarcoma *in vivo* [420]. Of a different type is the activating signal rendered upon binding of the Fc region of IgG to CD16 (Fc-receptor (FcR) γIII) expressed on NK-cells. This interaction induces antibody-dependent cellular cytotoxicity (ADCC) [421-422] and might be of importance when targeting tumors with antibodies, as has been suggested for the responses seen in NB to anti-GD2 therapy [423-424].

For an NK-cell to eliminate its target it needs to efficiently form an immunological synapse with the other cell. This initial interaction has been demonstrated to be highly dependent on the interactions between LFA-I on the NK-cell and intercellular adhesion molecule-1 (ICAM-1) on the target cell, which not only facilitate physical contact but also polarize the distribution of intracellular granules in the NK-cell towards the synapse [425]. This interaction has been suggested to modify NB sensitivity to NK-cell mediated killing [426].

Once the activating signals have surmounted the threshold needed to initiate NK-cell effector functions, the NK-cell has the option to utilize the three major effector mechanisms that are also utilized by CTLs; *i)* NK-cells carry preformed granules containing perforin and GrB, which are utilized in the granule exocytosis pathway to rapidly kill a target cell [335]; *ii)* DRs including FasL and TRAIL can be expressed and employed by an activated NK-cell [352, 427-428]; and *iii)* NK-cells produce high amounts of cytokines participating in tumor immunity, such as IFN-γ and TNF-α [429].

4.5 IMMUNOSURVEILLANCE IN NEUROBLASTOMA?

The question regarding whether or not immunosurveillance actually exists in NB patients was raised already in the 1960´s and 1970´s when it was suggested that the spontaneous regression seen in stage 4s NB patients could be due to anti-tumor immunity [19, 430]. Furthermore, observations showed that lymphocytes preferentially infiltrated more differentiated NB tumors and correlated to good prognosis [431]. Already in 1968, it was discovered that autologous lymphocytes suppressed NB colony formation, and similar effects were seen with plasma obtained from NB patients, also suggesting the existence of humoral anti-tumor immunity [432].

The idea that the immune system would play a role in the spontaneous regression of NB is strengthened by observations that the expression of MHC class I is more prominent in stage 4s tumors [433]. Furthermore, there is one case report describing a transient increase in serum levels of granulysin, an effector molecule released through the granule exocytosis pathway by CTLs [434], during the spontaneous regression of NB [435].

Subsequent studies have revealed that NB indeed expresses several putative TAAs [436], such as survivin [437], MAGE-1 [438], NY-ESO-1 [439], MYCN [440] and tyrosine hydroxylase (TH) [441]. Indeed, a recent study by Coughlin *et al*. showed that 8 of 9 HLA-A2 positive high-risk NB patients harbored circulating survivin specific CTLs, and that the majority of these could mount functional IFN-γ responses towards survivin *in vitro* [442]. Furthermore, it has been shown that NB patients display both humoral and cellular immune responses towards NY-ESO-1 [443]. Although Coughlin *et al*. noted that intratumoral T-cells were "strikingly rare", another study demonstrated intratumoral expansion of T-cell clones not seen in peripheral blood of NB patients, possibly indicative of an on-site immune response [444]. Functional clones have been established from TILs residing in NB tumors, further stressing that immunosurveillance within the tumor might actually occur [445]. Moreover, NB cells were able to establish tumors in T-cell deficient mice, but not in T-cell competent mice, which argues in favor of T-cell mediated immunosurveillance of NB [446].

In NB patients presenting with localized or regional disease, a striking correlation between low lymphocyte counts at the time of diagnosis and poor prognosis was seen, which indicates that the absence of circulating lymphocytes predisposes the patient to a more aggressive disease [400]. Although NB is often described as a tumor lacking the necessities for an efficient CTL response, (as discussed in section 5.5), the above mentioned studies foretell that immunosurveillance in NB patients indeed exists.

5 IMMUNE ESCAPE

Despite the given evidence for the existence of immunosurveillance, immunocompetent individuals still develop cancer, which uncovers an incapability of the immune system to completely control the outgrowth of tumors. Today, tumor immunologists claim that the escape from immunosurveillance should be incorporated into the hallmarks of cancer [65, 246, 447], since the ability to avoid recognition by the immune system is a necessity for any tumor to sprout in its host.

5.1 CANCER IMMUNOEDITING – THE CONCEPT

Arising as a complementary theory, or a refinement of cancer immunosurveillance, *cancer immunoediting* is nowadays recognized as a concept incorporating the whole aspect of the interactions between the immune system and tumors [246, 448]. The theory encompasses the three E´s of cancer immunoediting; the elimination phase, the equilibrium phase and the escape phase [449] (Figure 5). During the elimination phase, tumor growth is suppressed due to successful immunosurveillance, whereas the equilibrium phase symbolizes a state of balance in which the tumor can be immunologically sculpted by the pressure exerted by present immune cells. The equilibrium phase most often occurs prior to the clinical detection of tumors and will in the end lead to survival of the fittest in accordance with Darwinian selection- the tumor cell which can avoid immunological recognition will survive. Such a cell will carry a phenotype conditioned by immunological pressure, and has been edited to be of low immunogenicity. In the escape phase, the selected clones evade and eventually outwit the invading effector cells [450].

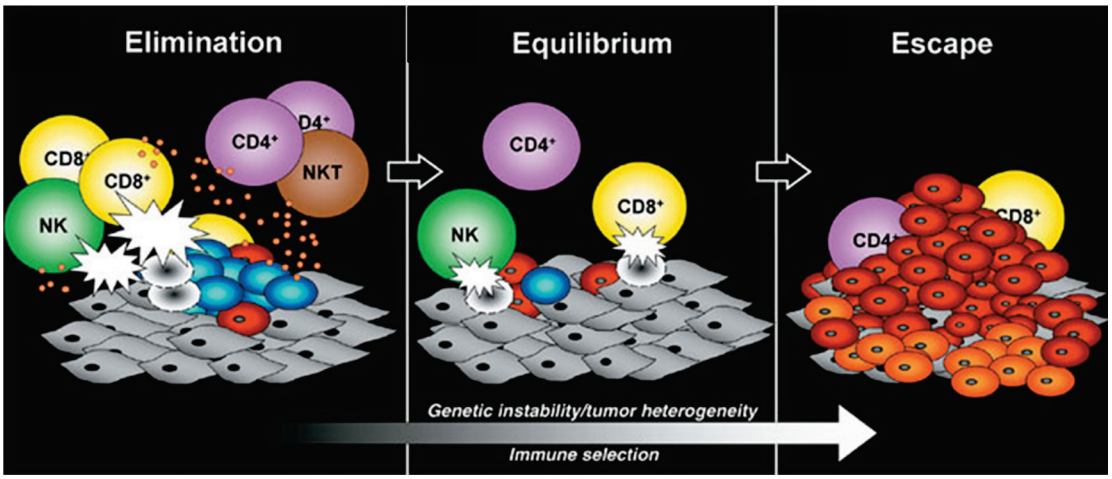

Figure 5. The three E´s of cancer immunoediting. The theory of cancer immunoediting encompasses three phases. In the elimination phase, immunosurveillance prevails and tumor cells are eradicated. In the equilibrium phase, the tumor is sculpted by immunological pressure and immune escape variants are selected. In the escape phase, these selected clones expand and escape from immune recognition. Reprinted with permission from Macmillan Publishers, Nature Publishing Group. Dunn *et al*., Cancer immunoediting: from immunosurveillance to tumor escape. Nature Immunology, 2002.

Indirect evidence for the existence of immunological sculpting originates in studies revealing that tumors growing in immunodeficient hosts are more immunogenic and rejectable when transplanted into immunocompetent hosts, suggesting that the lack of immunoediting rendered the tumors more susceptible to immune-mediated clearance [265, 451-452]. Furthermore, transplantable tumors that are passed through immunocompetent hosts exhibit a subsequent lack of immunological recognition when transplanted into another host, arguing for immunoselection of clones with low immunogenicity [453]. Below, certain phenomena occurring in parallel and/or due to cancer immunoediting, and their importance for the failure of immunosurveillance, will be discussed.

5.2 ALTERED ANTIGEN PRESENTATION AND RECOGNITION

The recognition of a tumor cell as foreign by the immune system confers an evolutionary drawback to the tumor, since it prevents its sustenance and further generation of off-spring. Manipulation of the pathways leading to immune recognition is one of several frequent phenomena contributing to tumor escape [450].

5.2.1 Downregulation of antigen presentation

Downregulation of MHC class I antigens is a well-known mechanism by which tumors escape from immune recognition [454]. In 1976, the first report demonstrated downregulation of an H-2K antigen in a murine lymphoma [455], and today, substantial evidence reveals a similar pattern of defective HLA expression in various human cancers, including among others lung, breast, cervical and colon cancer [456-459]. An attenuated HLA class I expression has also been shown to correlate to disease progression [460-461], and metastatic lesions have a propensity to further downregulate HLA expression in comparison to primary tumors [462-464]. In patients receiving immunotherapy, an altered HLA expression pattern has been observed for partial responders or upon tumor recurrence. For instance, melanoma patients with partial responses to T-cell-based immunotherapy frequently display a subsequent loss of β2m and/or downregulation of HLA expression [462, 465]. This argues for an *in vivo* immune selection process, sculpting the tumor towards a silent immune phenotype.

The antigen processing machinery (APM), which is responsible for the complete assembly of HLA class I molecules with their peptides, is frequently defective in cancer cells, which *per se* leads to a diminished antigen presentation [454]. “Soft” as well as “hard” lesions exist, of which the soft lesions (e.g. transcriptional downregulation) can be corrected by cytokine therapy, whereas hard lesions (e.g. gene deletion) are irreversible and render the tumor refractory to HLA-restricted T-cell-based therapies [466]. As an example, the immunoproteasomal subunits low molecular weight protein (LMP) -2 and -7 are often subjected to mutations, leading to a deficiency of the immunoproteasome and a decreased antigen presentation [467-468]. Likewise, peptide transportation into the ER can be prevented due to a decrease in the transporter associated with antigen processing (TAP)- 1 or -2 [467, 469-471]. These defects are however in some cases restored upon IFN-γ treatment, arguing for a regulatory “soft” defect [361, 472]. In ovarian carcinoma, defects in the APM are independent prognostic markers for poor survival [473].

Cancer cells that downregulate their surface HLA expression should turn into suitable targets for NK-cells, and this has indeed been demonstrated following β2m downregulation during T-cell therapy [474]. Yet, evasion from NK-cell killing can still occur, putatively due to a lack of activating signals or due to the local immunosuppressive microenvironment [450, 475].

5.2.2 Loss of tumor-associated antigens

An alternative route to alter the surface immunogenicity is to selectively downregulate TAAs dispensable to the tumor but recognized by the immune system [448]. In melanoma, representing the most well-defined and illustrative model for the occurrence of TAA loss, gp100 and MART-1 expression decrease during tumor development, and metastatic melanomas downregulate MART-1 in comparison to localized stage I tumors [462, 476-477].

Another illustrative proof of antigen loss due to *in vivo* immune selection is the loss of targeted antigens during immunotherapy. In melanoma patients receiving antigen-specific adoptive T-cell therapy, recurrent tumors exhibited downregulation of all three targeted antigens (gp100, MART-1 and tyrosinase) post therapy [478]. Similarly, melanoma patients with partial responses to peptide-based vaccines had recurrent disease with diminished antigen expression [479-481].

5.2.3 Expression of non-classical HLA molecules

The expression of non-classical HLA molecules, such as HLA-G and -E, provides the tumor with the ability to modulate host immune responses [59-60]. Particularly well studied is the expression of HLA-G in malignant cells. HLA-G expression has been detected and shown to be upregulated in several tumors, including melanoma, lung, renal and colon cancer [482-485]. The membrane-bound form of HLA-G interacts with the inhibitory receptors KIR2DL4, ILT2 and ILT4, of which KIR2D4 is restricted to NK-cells and some T-cells, and the most well recognized outcome of signaling via membrane bound HLA-G is the inhibition of NK-cell activity [486-488]. In addition, HLA-G may be shed by tumor cells, and in its soluble form it may induce apoptosis of activated CTLs [489], further inhibit NK-cell activity [490] and suppress the proliferative response within the CD4 and the CD8 compartment [491-492]. Intriguingly, high levels of HLA-G have been detected on immature myeloid cells infiltrating lung cancer [493], and transfer of HLA-G from APCs to T-cells confers a regulatory phenotype to the T-cells [494]. HLA-G may also indirectly contribute to immunosuppression by stabilizing HLA-E at the cell surface [495].

HLA-E, in turn, is yet another non-classical HLA molecule with prominent immunosuppressive abilities [59]. In normal tissue, HLA-E often fails to reach the cell surface [496], but if so, it preferentially binds HLA class I leader sequences including that of HLA-G [497]. However, in various malignancies, cell surface expression of HLA-E is frequently reported and upregulated as compared to the normal tissue counterpart [498-501]. The dominant pathway by which HLA-E inhibits immune responses is via interactions with the CD94/NKG2A inhibitory receptor on NK-cells and T-cells, leading to a disruption of effector cell function [500, 502-504]. Indeed, a relative increase in expression of HLA-E following downregulation of classical HLA

molecules is a putative mechanism whereby tumor cells escape from NK-cell mediated killing in this scenario [498, 501].

5.3 INHIBITION OF ANTI-TUMOR IMMUNITY

Suppressive cells within the immune system, as well as immunosuppressive molecules derived from tumors and/or immune cells with regulatory abilities, may also contribute to immune escape. Below, such immune escape phenomena pertinent to the studies included in this thesis will be addressed.

5.3.1 Regulatory T-cells

The putative presence of a suppressive subpopulation within the lymphocyte compartment was postulated in the 1970´s [505], but skepticism prevailed for several years, and the field of Tregs experienced its renaissance in the 1990´s, when the first evidence delineated a role for regulatory $CD4^+$ T-cells in controlling autoimmunity [506-507]. In 2003, it was discovered that the development of Tregs was under control of the transcription factor forkhead box P3 (Foxp3) [508]. Ample evidence for the crucial role of Tregs in maintaining immunological homeostasis originates in animal studies, where a lack of Tregs leads to the onset of various autoimmune disorders, mainly due to the unleashing of self-reactive effector T-cells [509-510]. Likewise, in humans, Foxp3 deficiency is linked to severe multi-organ autoimmunity [511].

Circulating Tregs either originate from the thymus as "natural Tregs" or are generated in the periphery as "adaptive Tregs", a derivative from naïve $CD4^+$ T-cells under certain circumstances, such as antigen stimulation in the presence of TGF-β [512]. A number of markers have been attributed to distinguish Tregs from other $CD4^+$ T-cell subsets, such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) [513], CD39 [514], CD25 (the IL-2R α-chain) [515], GITR [516] and $CD127^{low}$ [517]. Yet the single most validated marker to define Tregs remains in Foxp3, which is frequently used in combination with CD25 [518]. Tregs hence express the high-affinity receptor for IL-2 and are likewise highly dependent on IL-2 for their maintenance [519]. Tregs also need an initial antigen stimulation and TCR activation, but in their activated state exert immunosuppression in an antigen-independent manner [518].

Accumulating evidence derived from both *in vivo* and *in vitro* studies demonstrates a role for Tregs in cancer development and progression [515]. High numbers of circulating Tregs in the blood, as well as within the tumor itself, are reported in several human cancers, including lung [520], breast [520-521] ovarian [98, 275], pancreatic [521-522] and gastric cancer [523]. Strikingly, in ovarian cancer, a high ratio between tumor-infiltrating $CD8^+$ T-cells and Tregs is associated with favorable clinical outcome [275], and in breast cancer, the presence of intratumoral Foxp3-expressing Tregs can predict patients at risk of relapse after five years [524].

Several explanations are provided as to why Tregs selectively accumulate in the tumor microenvironment. One notion is that the chemokine CCL22, expressed by tumor cells and/or M2 macrophages, promotes the trafficking of CCR4-expressing Tregs into the

tumor [98, 525]. Alternatively, Tregs can be induced on-site in response to TGF-β derived from the tumor itself or from iDCs [193, 526-527].

Animal studies have demonstrated the relevance of Tregs for tumor immunity *in vivo*. Solely by depleting Tregs in tumor-bearing hosts, tumor regression has been observed [528-529], and on the contrary, the adoptive transfer of $CD4^+CD25^+$ Tregs enhances the growth of chemically induced tumors [530]. Furthermore, both human and animal studies demonstrate that Treg depletion enhances vaccine-mediated antigen specific T-cell responses [531-532], and that Treg depletion unmasks the existence NY-ESO-1 specific T-cells in the blood of both healthy individuals and cancer patients [533-534].

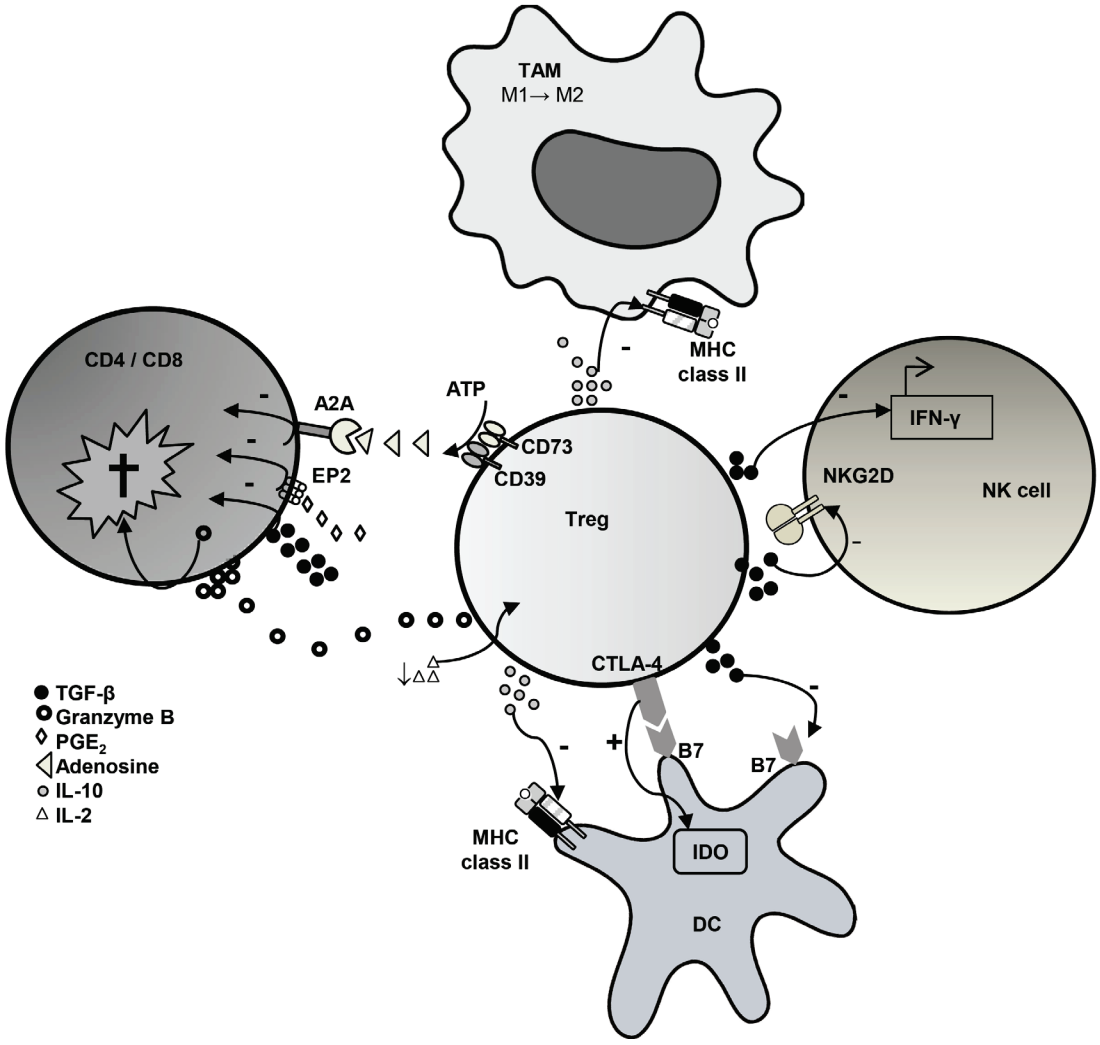

Figure 6. Immunosuppressive mechanisms exerted by Tregs. Tregs suppress T-cells and NK-cells by inhibiting their effector functions and by eliminating these effector cells. TAMs are subverted from an M1 to an M2 phenotype and downregulate MHC class II in response to IL-10 secreted by Tregs. Likewise, DCs downregulate MHC class II and the co-stimulatory B7 molecules and increase their expression of IDO in the presence of Tregs. Furthermore, IL-2 is also depleted from the microenvironment, which in turn abrogates T-cell proliferation.

Tregs are indeed versatile in their abilities to suppress immune responses (Figure 6). Suppression may be exerted on the level of the adaptive as well as the innate immune system, employing both contact-dependent and contact-independent mechanisms.

As such, the suppression of T-cell responses can occur both through contact-dependent inhibition of IL-2 production and proliferation [535-536], as well as by conversion of ATP to adenosine which is delivered to the T-cells via the A2A receptor, and renders the T-cell anergic [514, 537]. Indirectly, Tregs may hamper CTL responses by depriving the microenvironment of IL-2 [538], and furthermore negatively regulate the granule exocytosis pathway in CTLs [539]. The production of PGE_2 by Tregs also suppresses effector T-cells via signaling through the EP2 receptor [540]. Tregs may also induce the expression of IDO in DCs [541], which in turn inhibits T-cells by blocking their proliferation [542]. A number of studies demonstrate that Tregs utilize immunosuppressive cytokines, such as TGF-β and IL-10, to suppress innate as well as adaptive immunity, but whether or not Tregs depend on these cytokines to exert their suppressive effects remains controversial [518]. Nevertheless, Treg inhibition of NK-cell activity *in vivo* was in fact shown to be dependent on membrane-bound TGF-β, which downregulated NKG2D expression on NK-cells [543]. Furthermore, Tregs can suppress innate immunity, and subvert M1 macrophages towards an M2 phenotype *in vitro*, mainly via IL-10 dependent mechanisms [544-545]. Tregs also interact with DCs, and perturb their functions by downregulating co-stimulatory molecules [546-547]. Ultimately, Tregs utilize the Fas/FasL pathway, as well as granzymes and perforin, to exert direct cytotoxicity and kill off other activated effector cells, including CTLs, $CD4^+$ T-cells, DCs and macrophages [548-550].

Indeed, the given evidence demonstrates a wide array of tumor promoting abilities harbored within the Treg population. Still, it deserves to be mentioned that this view is currently being challenged [551]. In head and neck cancer, tumor-infiltrating Tregs correlate to good prognosis [374], and a similar correlation was recently reported in colorectal cancer [552]. Potentially, Tregs could have a beneficial effect in some tumors, by dampening locoregional inflammation, and furthermore, adoptively transferred Tregs have been shown to induce apoptosis in intestinal tumors, concomitant to downregulation of COX-2 expression in tumor cells [553].

5.3.2 Cytokines in immune escape

As has already been touched upon, certain cytokines present during tumor development may promote immune escape and favor tumor progression. In particular, TGF-β and IL-10 are both pleiotropic contributors to tumor escape [554-555].

5.3.2.1 TGF-β

TGF-β may contribute to immune escape mechanisms by downregulating MHC class I molecules on the cell surface of tumor cells [556-557]. On the level of effector cells, TGF-β displays a wide array of suppressive behaviors. Tumor-specific CTL cytotoxicity has been shown to be curtailed *in vivo* in the presence of TGF-β [558], and similarly, TGF-β may directly control genes involved in the cytolytic cascade, including those of GrB, IFN-γ, FasL and perforin [559-560]. Furthermore, membrane-bound TGF-β on Tregs represses the proliferation of $CD4^+CD25^-$ T-cells [561]. NK-cells represent another target for TGF-β, which downregulates the expression of NKG2D on the cell surface of NK-cells [562-564], and perturbs their release of IFN-γ [565].

5.3.2.2 IL-10

Similarly to TGF-β, IL-10 attenuates the expression of classical MHC molecules on the cell surface by downregulating genes associated with antigen presentation, such as TAP1, TAP2 and LMP-2 [566-567]. Furthermore, IL-10 has proven to increase HLA-G at the cell surface, which may contribute to escape from NK-cell-mediated killing [568]. IL-10 exerts divergent effects on DCs. Although some reports indicate that IL-10 can promote antigen uptake by DCs [569], most studies point out a negative regulation of DC activity by IL-10, via reduced levels of MHC class II and a hampered IL-12 production [569-570]. In turn, this translates into the induction of anergic T-cells with regulatory capacities [570-572]. IL-10 has also been shown to protect tumor cells from lysis by TILs [573], and blocking of IL-10 can enhance cellular anti-tumor immunity [574]. As has been mentioned, IL-10 is one of the major effector molecules utilized by Tregs to suppress immune responses, and it is also suggested to participate in Treg generation in the periphery [518, 575].

Intriguingly, IL-10 derived from Tregs was shown to restrain locoregional inflammation and reduce tumor growth in colonic as well as extra-intestinal tumors [576-577]. Indeed, tumor suppressive abilities of IL-10 are being proposed [555], and in some models an increased NK-cell activity was detected in the presence of IL-10 and translated into a reduced tumor growth [578-580]. Possibly, IL-10-mediated downregulation of the COX-2/PGE_2 axis could also alleviate PGE_2-mediated immunosuppression [581].

5.3.3 PGE_2 in immune escape

PGE_2 not only contributes to enhanced cancer-related inflammation, but also participates in mediating immune escape. As such, PGE_2 prevents the innate immune system from promoting and exerting efficient anti-tumor responses, by inhibiting the maturation of DCs [104, 582-584], perturbing macrophage cytolytic activity and IL-12 production [585-587], inhibiting NK-cells [588-589] and by recruiting MDSCs [163]. PGE_2 also contributes to the deviation of adaptive immune responses towards a pro-tumorigenic state, by subverting $CD4^+$ T-helper cells from a T_H1 to a T_H2 phenotype [104, 590-591], inducing regulatory T-cells [540, 592-593] and by attenuating effector T-cell responses [540, 594-596]. Furthermore, COX-2 inhibitors have proven to restore IL-12 production, enhance anti-tumor immunity and potentiate responses seen to tumor vaccines [219, 597].

5.4 ESCAPE FROM T-CELL RESPONSES

Cancer patients often harbor circulating tumor-specific T-cells in the blood [598-599], yet the tumor often progresses, suggesting that T-cell responses fail to control tumor growth. The functional status of tumor-specific T-cells in the blood and tumor tissue of cancer patients is a matter of debate [599], but several reports claim that peripheral blood lymphocytes (PBLs) as well as TILs display poor proliferative response rates to mitogenic stimuli and often undergo spontaneous apoptosis [600-602].

In situ, TILs often demonstrate dysfunctional characteristics indicative of deregulated effector mechanisms. Commonly, the zeta-chain is downregulated in TILs, which abrogates antitumor responses [602-603]. Furthermore, tumors are suggested to utilize a number of mechanisms to induce apoptosis in the intruding lymphocytes. One such mechanism is the "tumor counter-attack" phenomenon, in which tumor cells outwit TILs by expressing DR ligands such as FasL and TRAIL, which in turn eliminate effector cells expressing the corresponding DRs [604-606]. However, the relevance of, and the methodological accuracy in the early publications in this field, have been disputed [607-608].

Yet another mechanism proposed to contribute to tumor-induced dysfunction of TILs is the expression of IDO by the tumor, which can abrogate T-cell proliferation and induce cell-cycle arrest, by depleting tryptophan *in situ* [153, 542]. Furthermore, cancer cells have been shown to express B7-H1 (programmed death-1 ligand, PD-1L), which may induce apoptosis in activated T-cells expressing the receptor PD-1 [609]. Of particular relevance to NB is the ability of tumor cells to shed gangliosides, which are also reported to contribute to apoptosis of TILs [610].

Presumable non-apoptotic TILs may still fail to clear the tumor. It has been shown that TILs are functionally impaired with a reduced ability to mobilize the lytic machinery [611]. If they succeed, tumor cells can however evade granule-mediated killing by expressing the proteinase inhibitor-9 (PI-9), an inhibitor of the GrB pathway [612]. Furthermore, tumors may circumvent DR-mediated killing by expressing decoy receptors or dysfunctional DRs, as well as by harboring defects in the downstream intracellular signaling cascades, such as silencing of caspase-8 [613-615].

5.5 IMMUNE ESCAPE BY NEUROBLASTOMA

Neuroblastoma is not an exception, and displays a battery of immune escape mechanisms which modulate the immunogenicity of NB and impede anti-tumor responses (Figure 7) [616]. On the level of tumor immunogenicity, NB cell lines as well as primary tumors are often described as devoid of surface HLA expression [617-619]. This was first suggested to be regulated by *MYCN* expression [620], but the interrelationship was however recently questioned [621]. Underlying defects in several components of the APM have however been observed in primary NB samples [619, 622]. The lack of HLA expression could render NB cells a suitable target for NK-cell-mediated killing. Yet, NB cells may secure escape from NK-cells by downregulating activating ligands for NK-cells, such as MICA [417, 623]. Furthermore, in a murine model of NB, it was shown that NB cells upregulated MHC molecules upon recurrence following an initial NK-cell dependent anti-tumor response [624]. This in fact demonstrates that NB can undergo immunological sculpting *in vivo*.

NB tumors can also be protected from DR-mediated killing by downregulating DRs and via defects in the intracellular signaling cascade conveying apoptotic signals. Decoy receptors for TRAIL were stated to be hypermethylated in NB [625], and some reports demonstrate expression primarily of TRAIL-R2 in NB cell lines [626-627], but others report an absence of TRAIL-R1 and -R2 in primary NB tumors and some cell

lines [628]. Furthermore, resistance to TRAIL has been linked to a loss of caspase-8 expression in NB [626-627, 629]. The inactivation of caspase-8 disrupts the apoptotic pathway in NB, and was initially suggested to occur in *MYCN*-amplified high-risk tumors and to correlate to the stage of the disease [626, 630-631], but this has however been questioned [632]. Expression of the other major death receptor, Fas, has been associated with low stage and a mature phenotype of NB cells [633]. Importantly, IFN-γ acts to restore the defects detected in the APM, as well as caspase-8 expression, and may hence override NB immune escape mechanisms [619, 622, 628, 634].

Besides alterations in immune recognition, potent immunosuppressive functions exerted by NB impose on tumor immunosurveillance. NB may interfere with NK- and T-cell-mediated killing of target cells, either directly or indirectly. Indirectly, NB cells may instruct monocytes to secrete soluble HLA-G molecules, which then obstruct CTL- and NK-cell-mediated lysis [635]. Directly, NB cells proved to secrete soluble MICA (sMICA), which in turn inhibited NK-cell-mediated killing of MICA expressing NB cells [623]. NK-cells were also shown to be negatively regulated by the expression of B7-H3 on NB-cells [636], a molecule for which the corresponding receptor on NK-cells is still unknown [637].

T-cells can be suppressed and enter the apoptotic pathway due to the expression of FasL on NB cells [633, 638]. In a murine model of NB, the expression of macrophage migration inhibitory factor (MIF) suppressed T-cell proliferation and MIF knockdown NB cells were superiorly rejected in a T-cell-dependent manner *in vivo* [639]. In addition, NB tumors secrete GD2, which further suppresses T-cell responses [640-641] and, as was recently demonstrated, promotes the induction of Tregs [642]. NB-derived gangliosides may also impair immune responses by inhibiting the maturation and function of DCs [189, 643]. Finally, the tumor microenvironment surrounding NB tumor cells holds the potential to suppress immune responses; primary NB tumor samples contain IL-10 as well as TGF-β [445, 644], and NB expresses COX-2, enabling the production of PGE_2 [111].

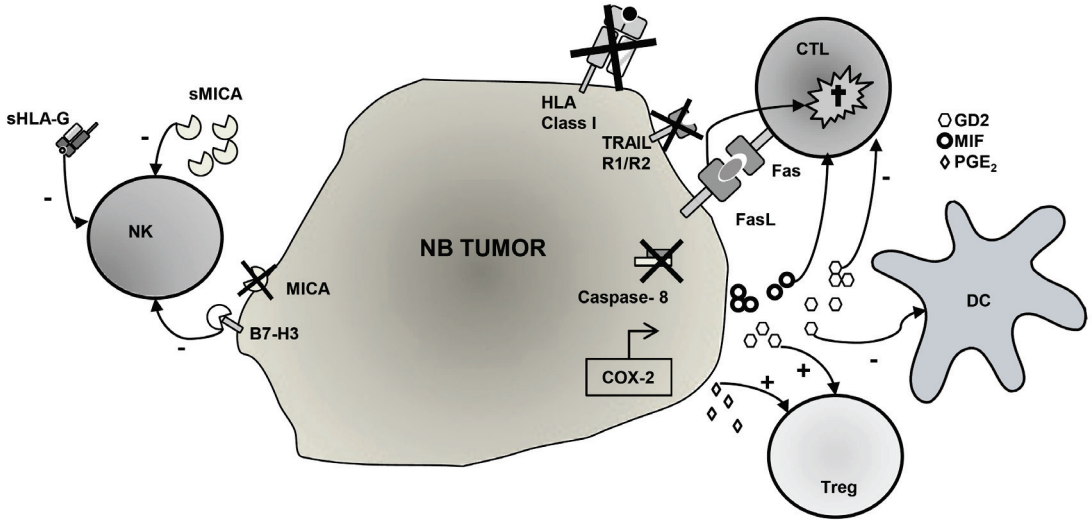

Figure 7. Immune escape by neuroblastoma. NB tumors may hamper NK-cell functions by shedding soluble MICA, by instructing monocytes to shed soluble HLA-G and by expressing the inhibitory B7-H3 molecule. T-cell responses may be abrogated by a lack of HLA class I expression, by a deficient caspase-8 expression or by the production of inhibitory molecules such as GD2, MIF and PGE_2. T-cells may furthermore be eliminated by NB tumors expressing FasL. NB-derived GD2 may also inhibit DCs and induce Tregs, the latter which may also be induced by NB-derived PGE_2.

6 CANCER IMMUNOTHERAPY

In the 1890´s, Dr William Coley observed that sarcomas could be cured by evoking immunological responses following administration of streptococci extract [645], and hereby, the first attempt to cure cancer by using the immune system had been pursued. Modern immunotherapy, however, evolved in the late 1980´s, and is currently experiencing an era of revival with several major break-troughs during the last decade, as will be reviewed below.

The following sections will give a brief overview on the current status of immunotherapy, with a focus on mechanisms and regimens of pertinence to the studies included in this thesis and to NB. Of note, it deserves to be stressed that most clinical trials involving immunotherapy have been pursued in patients with a fulminate, refractory disease not responding to conventional therapies, and given response rates should be viewed in light of these poor prerequisites.

6.1 PASSIVE IMMUNOTHERAPY

Passive immunotherapy implies the administration of preformed immunological mediators, including adoptive cell transfer (ACT) of effector cells such as T-cells and NK-cells, as well as mAb therapy.

6.1.1 T-cell-based therapies

In 1987, a landmark publication initiated the field of adoptive T-cell therapy, when TILs from a melanoma patient proved to possess the capacity to lyse autologous tumor cells *in vitro* after IL-2-based expansion [646]. Shortly thereafter, the first treatment of metastatic melanoma using *in vitro* expanded TILs was performed [647]. Ensuing studies using adoptive transfer of TILs in melanoma patients in the early 1990´s demonstrated objective response rates of around 30% [648], with one of the major obstacles to overcome being the low persistence of the transferred T-cells *in vivo* [649]. During the last 10 years, however, an improved understanding of T-cell biology has revolutionized this field, and current rates of persistence are now improved, with one study detecting that 75% of circulating $CD8^+$ T-cells 6 months after infusion were tumor-specific, originating from the transferred clone [650].

One of the milestones within the field was the introduction of lymphodepletion prior to ACT therapy (reviewed in [651]). Lymphodepletion renders the host in a state of non-myeloablative immunosuppression, either via cytostatic drugs such as cyclophosphamide and fludarabine, or via total body irradiation [649, 651], and several mechanisms underlying its additive effect on ACT therapy are suggested. Lymphodepletion eliminates circulating suppressive cells such as Tregs [652] as well as “cytokine sinks”, i.e. other cell types competing for the cytokines needed for T-cell proliferation [653], and furthermore enhances the function of APCs [654]. Following the first published study by Dudley *et al*. in 2002, with a response rate of 46% [650], subsequent studies employing improved protocols of lymphodepletion have demonstrated objective response rates corresponding to 50-70% [655-657].

Additional improvements of ACT therapy stem from gained insight into the importance of transferring $CD4^+$ T-cells together with CTLs, or as sole effector cells [652, 658]. It deserves to be mentioned that other sources than TILs can be used to isolate and expand tumor reactive T-cells, such as the tumor draining lymphnode, i.e. the sentinel node, where present T-cells may be of better quality [659].

Another upcoming strategy to potentiate T-cell-based immunotherapy is the generation and administration of genetically engineered T-cells [660]. In 2006, a first publication by Morgan *et al.* reported a successful attempt to treat melanoma patients with T-cells which had been transduced with TCRs specific for MART-1 [661]. Chimeric antigen receptors (CARs) represent yet a different way to modify T-cells for ACT. By engineering T-cells with antibodies linked to the intracellular signaling domains of the CD3 complex, the T-cells may bypass MHC class I restriction and target alternative surface structures such as glycolipids [662]. This paves the way for T-cell-based therapies for MHC class $I^{low/-}$ tumors, such as NB (section 6.4.1) [663]. Unfortunately, ACT therapy based on TCR specificity has resulted in immune escape variants of tumor cells with downregulated expression of the targeted TAA [664].

A different approach to enhance or initiate T-cell responses is based on the administration of mAbs targeting CTLA-4, which represents an immune checkpoint molecule halting the activation of T-cells [665]. Anti-CTLA-4 (ipilimumab) treatment has proven to augment lymphocyte infiltration into the tumor, increase CTL function and abrogate Treg function [666-669]. A study by Hodi *et al*. in 2010 demonstrated that although severe toxicities were seen, treatment with ipilimumab alone or in combination with the peptide gp100 improved the overall survival in metastatic melanoma patients in a phase III study [670].

6.1.2 Monoclonal antibodies

The administration of mAbs is an upcoming immunotherapeutic approach with particular relevance for NB. Several mAbs, including trastuzumab (Herceptin, anti-Her-2/neu) for breast cancer [671], rituximab (Rituxan, anti-CD20) for lymphoma [672] and cetuximab (Erbitux, anti-human EGF receptor (EGFR)) for colorectal cancer [673] have shown major clinical benefits with response rates of 8-10% as single agents and 20-30% when combined with radiation or chemotherapy [674].

Various mechanisms are proposed to underlie the effects exerted by mAbs [424]. One potential mechanism is the abrogation of downstream signaling pathways involved in proliferation and survival, as with the EGFR inhibitors cetuximab and trastuzumab [675]. Complement-dependent cytotoxicity (CDC) is suggested as an alternative mechanism of action and leads to the formation of cell membrane pores on the targeted cell [674]. Although suggested as an *in vivo* mechanism during rituximab treatment [676], CDC is a rapid process, and its relevance *in vivo* is questioned considering the time-window for mAb responses often being above one week [424].

The most well-studied mechanism of action of mAbs is the induction of ADCC. This occurs when the Fc domain of an antibody is recognized by FcRs (FcγRs in the case of IgG) present on NK-cells, neutrophils, monocytes and macrophages. Hereupon, these

effector cells are activated to induce target cell death [674, 677]. Indirect evidence for the crucial role of ADCC in mediating mAb responses stems from the fact that polymorphism in the FcγR region correlates to clinical responses to, among others, rituximab and trastuzumab [678-680].

As mentioned, mAb therapy in the clinic leads to tumor destruction over days, and not hours [424], which suggests that other mechanisms may underlie the effects behind mAb therapy than those described above. Accumulating evidence demonstrates that antibody therapy induces $CD4^+$ as well as $CD8^+$ T-cell responses to the target antigen, which would fit with the given time-window for clinical responses. The administered mAbs increase cross-presentation of target antigens to T-cells by APCs [681-682], and ensuing T-cell responses have indeed been detected following anti-CD20 and trastuzumab treatment in animal models as well as in humans [683-685].

6.2 ACTIVE IMMUNOTHERAPY

Active immunotherapy implies an attempt to vaccinate against cancer, and includes vaccines based on DNA, peptides, proteins, inactivated tumor cells and DCs [289]. Except for when the tumors are of infectious origin, such as human papillomavirus-induced cervical cancer, where vaccines efficiently target non-self antigens [686], the vaccines are often limited by the target being a self-antigen. In 2004, Rosenberg and colleagues reported the discouraging overall response rate of 2.6% to cancer vaccines [687]. However, an improved understanding of cancer immunology pushes the field forward. As an example, Sipuleucel-T, a DC-based vaccine for prostate cancer, became the first antigen-specific immunotherapy for humans to be approved by the US Food and Drug Administration (FDA) in 2010. Sipuleucel-T is believed to induce $CD4^+$ as well as $CD8^+$ T-cell responses against prostatic acid phosphatase [688-690].

DNA-based cancer vaccines are designed to elicit immunological responses *in vivo* to an encoded target protein/epitope. Following antigen synthesis and presentation, DNA vaccines can potentially induce a broad repertoire of CTLs and T-helper cells, as well as B-cell responses [691]. Clinical trials in melanoma and breast cancer patients have shown that DNA vaccines can elicit immunological responses in late-stage patients, yet clinical response rates in these settings are low [692-693]. In animal models, however, DNA vaccines have been potentiated by linkage to gene-encoded adjuvants, such as CD40L [694]. DNA vaccines may also target other structures than TAAs, such as molecules expressed on blood vessels, hence abrogating angiogenesis [695]. Considering the status of enrolled patients, results with peptide- and protein-based vaccines are encouraging. In metastatic melanoma patients, HLA-A2- restricted gp100 peptides have elicited both immunological and clinical responses [696-697]. In breast and ovarian cancer patients, immunization with Her-2/neu peptides or proteins has also proven to elicit T-cell responses and to decrease the risk of recurrent disease [698-699].

6.3 CHILDREN VERSUS ADULTS

Considering that most of the above mentioned trials are performed in adults, the question arises whether pediatric patients are suitable for immunotherapeutic approaches. A number of immunological observations and experimental proofs have demonstrated a deterioration of the immune system upon aging, altogether referred to as "immunosenescence" [700]. In an elderly population, the T-cell repertoire loses its diversity within both the CD4 and the CD8 compartment [701-702]. Moreover, $CD4^+$ as well as $CD8^+$ T-cells in the elderly downregulate their expression of CD28, hence becoming refractory to proper costimulation [703-704]. APCs in older mice also exhibit a decreased ability to present antigens to T-cells, and induce $CD8^+$ T-cells with lower cytotoxic ability [705]. Aging is also associated with a skewing of the cytokine pattern from a T_H1 towards a T_H2 pattern, which will further diminish the generation of $CD8^+$ responses [706]. Whereas contradictive reports exist describing altered as well as retained macrophage function [707], most reports demonstrate that NK-cells show impaired responses to IL-2 and functional alterations in older populations [708].

Low response rates to cancer vaccines in adult patients have been attributed to the above mentioned decline in immunological parameters [709]. Further strengthening this hypothesis, younger mice are superior in mounting anti-tumor responses to vaccines in experimental cancer models [710-711]. In humans, children furthermore display enhanced capacities to reconstitute their immunological competence following chemotherapy [712], which is a major advantage for subsequent immune-based therapies.

Taken together, the pediatric population has been suggested to be a highly suitable population for immunotherapy. Children possess a vigorous immune system with greater potential to co-operate during immunotherapy. Furthermore, tumors arising in pediatric patients have usually had a shorter time to subvert and push the immune system into the equilibrium phase [713].

6.4 IMMUNOTHERAPY OF NEUROBLASTOMA

Immunotherapy is gaining momentum as an auxiliary approach in NB treatment, and various passive as well as active strategies are being exploited with promising efficacy in NB models [714].

6.4.1 Adoptive cell therapy for NB

T-cell-based therapies for NB patients have been discouraged by the notion that NB is devoid of HLA class I expression. However, NB cells upregulate HLA class I in response to retinoids, and many tumors indeed display enhanced levels of HLA molecules upon conventional therapies such as radiation and chemotherapy [714-716]. Autologous CTLs have successfully been expanded from NB patients in various settings. Sarkar *et al*. demonstrated that CTLs could be generated from NB patients by restimulation either with irradiated autologous tumor [717] or with an HLA-A1-restricted MYCN-derived peptide [718], and the CTLs were able to kill autologous tumor and/or NB cell lines in an HLA-restricted fashion. Similar studies proved that

autologous DCs transfected with mRNA from NB cell lines could be used to restimulate CTLs from NB patients [719]. In 2008, an HLA-A2-restricted peptide derived from MYCN was identified and proved capable of inducing MYCN-specific CTLs from NB patients which could lyse autologous tumor [440]. Furthermore, previous work from our group demonstrated that NB cells can be killed in an MHC-non-restricted fashion by CTLs [720]. $CD8^+$ responses also mediate the anti-tumor effects of NB vaccines in animal models [721-723], which further argues for a role of CTLs in NB immunosurveillance.

NB tumors have recently been targeted by CTLs modified to express CARs targeting the L1 cell adhesion molecule CD171 [663, 724]. However, the *in vivo* persistence of administered CTLs was short. In 2008, Pule *et al*. published a beautiful example on how NB tumors can be targeted by CARs with longer *in vivo* persistence. By engineering Epstein-Barr virus (EBV)-specific CTLs from NB patients with a chimeric GD2 receptor, transferred CTLs could be sustained *in vivo* by receiving native TCR stimulation, and simultaneously target GD2 expressing NB cells. Tumor regression was seen in 50% of the patients [725].

A low expression of HLA class I molecules should render NB a favorable target for adoptive transfer of NK-cells. In mice bearing metastatic NB tumors, infusion of IL-2 activated NK-cells prolonged the survival time [726], and within an ongoing phase I/II trial it was shown that NK-cells infused into NB patients retained cytotoxic capacity in spite of the presence of sMICA [727]. Furthermore, a recent study evaluated the safety and feasibility to administer subcutaneous IL-2 to NB patients in an outpatient setting, and an increase in NK-cell activity was detected with tolerable side-effects [728]. This encourages further studies in NB based on ACT using NK-cells.

6.4.2 Monoclonal antibodies in NB therapy

The current cornerstone of immunotherapy for NB patients is based on passive immunotherapy with mAbs targeting GD2, which is expressed by virtually all NB tumors [729]. GD2 serves as an excellent target for immunotherapy since it is seldom lost following mAb therapy [730]. Pioneering clinical trials in the 1980´s and early 1990´s were based on the murine antibodies 3F8 and 14.G2a [731-732], and the chimeric human/mouse mAb ch14.18 was launched in clinical trials in 1995 [733]. Subsequent trials using 3F8 and 14.G2a were then modified by the separate addition of the adjuvant cytokines IL-2 or GM-CSF [734-736]. In 2010, Yu *et al*. could demonstrate a significantly improved outcome in high-risk NB patients receiving ch14.18 in combination with GM-CSF and IL-2 as an addition to standard therapy with isotretinoin. The EFS at two years was 66% in patients receiving immunotherapy compared to 46% upon standard therapy [737]. The mechanisms underlying the effects of ch14.18 were proposed to involve ADCC as well as CDC, with *in vivo* experiments arguing in favor of an NK-cell dependent ADCC mechanism [738-739].

GD2-specific antibodies have also been exploited as messengers in the delivery of targeted cytokines to the NB microenvironment. In experimental models of NB, a fusion protein of IL-2 and ch14.18 has demonstrated therapeutic benefits as well as prophylactic ability to evoke NK- and T-cell responses and to confer subsequent

protection against tumor challenge [740-742]. The administration of a humanized analogous fusion protein, hu14.18-IL-2, has recently demonstrated complete response rates of 22% in high-risk NB-patients in a phase II study [743]. Subsequent analyses indicated that NK-cells were mediating the clinical effect [423, 744].

6.4.3 Active immunotherapy for NB

Active immunotherapy for NB has been pursued using vaccines based on DCs, DNA and autologous tumor cells. Pilot clinical trials using DCs derived from peripheral blood monocytes of NB patients and pulsed with tumor lysates or RNA showed the ability to induce T-cell as well humoral responses, but limited clinical responses [745-746]. Vaccines based on modified tumor cells have however been more successful clinically. Autologous tumor cells engineered to express IL-2 showed clinical response in 5 of 10 high-risk NB patients [747], and allogeneic NB cells engineered to secrete lymphotactin and IL-2 mounted similar response rates [748]. Clinical trials using the autologous setting are underway and intermediate results report on initial immunological as well as clinical responses [749].

The administration of DNA vaccines represents another promising strategy and several TAAs expressed by NB have successfully been targeted in animal models. As such, a DNA vaccine encoding TH has efficiently been delivered using an attenuated strain of *Salmonella typhimurium* [750-751], and was furthermore potentiated by posttranscriptional modifications to enhance TH expression [722, 752-753]. In addition, GD2, although a glycolipid, was effectively targeted using DNA vaccines as well as peptides encoding decapeptides mimicking GD2, which induced cellular and humoral responses and reduced tumor growth in NB models [754-755]. Finally, a DNA vaccine encoding survivin-derived peptides was shown to be efficient in a prophylactic as well as a therapeutic setting in a mouse model of NB [721]. Taken together, active immunotherapy for NB shows promising efficacy and deserves further attention.

AIMS OF THE THESIS

The overall aim of this thesis was to elucidate the prerequisites for immune-mediated recognition of neuroblastoma.

The specific aims of this thesis were;

- To evaluate the sensitivity of NB to effector mechanisms of the immune system in the course of differentiation

- To evaluate how effector molecules released by CTLs affect the immune phenotype and sensitivity of NB to DR-mediated killing

- To gain further insight into the status of T-cell responses in human NB tumors

- To evaluate tumor-infiltrating cells during tumor progression in the transgenic TH-MYCN model, and to assess how anti-inflammatory treatment with low-dose aspirin affects tumor development and inflammatory parameters

RESULTS AND DISCUSSION

NEUROBLASTOMA AS A TARGET FOR EFFECTOR CELLS (PAPER I AND II)

In paper I and II, the sensitivity of NB to effector mechanisms of the immune system was investigated. Specifically, paper I aimed at investigating the effects exerted by differentiating agents on the recognition of NB by NK-cells as well as T-cells. Paper II aimed at investigating how effector molecules released by activated CTLs affect the immunogenicity of NB, including the sensitivity to DR-mediated killing.

Sensitivity of NB to effector cells in the course of differentiation

Spontaneous regression of NB has been suggested to involve immunological mechanisms [19], yet no study has addressed the ability of NB to be targeted by effector cells during physiological differentiation. Previous work in our group demonstrated that retinoic acid, known to induce differentiation in NB cells, sensitized NB cells to CTL-mediated killing [715]. Retinoids, however, exert their effects through nuclear receptors with several downstream target genes [756], and effects seen could potentially be mediated by various biological pathways other than differentiation.

Hence, in paper I, we performed a systematic analysis of the immune phenotype in NB cell lines as well as primary tumors upon treatment with NGF, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) or a combination of EGF and basic fibroblast growth factor (FGF). NGF and TPA are well known for their ability to induce differentiation of NB cells [757], and an FGF/EGF combination has been used to potentiate TPA induced differentiation [758], as well as to propagate neural stem cells [759].

During differentiation using these agents, the immune phenotype of NB cell lines as well as primary tumors was altered. We could detect an increase of the surface expression and the total pool of HLA molecules. Of note, the non-classical HLA molecules HLA-E and HLA-G were not induced, but rather downsized upon EGF/FGF treatment. Instead, differentiation triggered the expression of the classical alleles HLA-A2 and HLA-A11. In parallel, we monitored an increase in the levels of surface ICAM-1, which is known to facilitate the initial interaction between lymphocytes and their target cell [425, 760]. In order to investigate whether these changes would translate into an increased killing of NB by effector cells, we examined the ability of NB cells to form immune conjugates and their sensitivity to be lysed by NK-cells and T-cells. Indeed, differentiated NBs more readily formed conjugates with CTLs, and using a standard 4 hour ^{51}Chromium-release assay, we could demonstrate an increased lysis of differentiated NBs by HLA-A2-specific allogeneic T-cells as well as by 2 out of 3 NK-cell lines used. The increase in lysis was reflected by an enhanced propensity of NB cells to bind GrB at the cell surface.

Interestingly, although a prominent induction of HLA molecules at the cell surface was seen, NK-cells still lysed differentiated NB cells more efficiently than non-differentiated counterparts. Potentially, the increase in ICAM-1 could override the inhibitory effects of HLA class I expression and translate into a facilitated interaction between NB and NK-cells. It was previously shown that ICAM-1 is of importance in determining the sensitivity of NB to NK-cell-mediated killing [426]. Other activating ligands, such as MICA and CD155 (PVR), could also potentially be differently expressed upon differentiation. Of note, the cell line FL-2 displayed detectable basal levels of MICA (Paper II).

In general, allorecognition by CTLs may occur as a direct peptide-independent recognition of intact HLA molecules present on foreign cells. Alternatively, it may be dependent on the presence of peptides derived from allogeneic MHC molecules in the context of surface MHC molecules which succeed to provoke a TCR response in the allogeneic T-cells [761]. The increase in lysis of differentiated NBs by CTLs was observed both using allogeneic CTLs derived from an HLA-A2 negative donor and specific for HLA-A2, as well as by using HLA-A2-restricted CTLs specific for the EBV-derived peptide GLC (data not shown). In the first setting, an increased killing might reflect an altered peptide repertoire as well as enhanced surface levels of HLA molecules. Using GLC-specific CTLs, the killing reflected a peptide-specific interaction with pre-pulsed target cells.

The primary tumors used in paper I were a stage 2 and a stage 1 tumor (according to INSS staging [39]) and genetically classified as tumors with 17q gain and other structural aberrations, respectively [27, 762] (sample 4 and 6, see Table 1). Ultimately, all genetical subtypes should have been included, but due to the limited amount of material, this was not feasible. Our data, however, indicate that *ex vivo* differentiation is possible using all employed differentiating agents in the study, with observed changes in the immune phenotype that paralleled those seen in NB cell lines. Importantly, the one tumor expressing detectable levels of trkA was able to differentiate using NGF.

The fact that NGF treatment had a prominent effect on the immune recognition of NB cells sheds further light on the hypothesis of an immunological mechanism underlying the spontaneous regression of some NB tumors [19]. As noted, the expression of HLA has been observed to be higher in stage 4s tumors [433], and trkA signaling is suggested to participate in the regulation of spontaneous regression [18]. Taking our findings into account, it is tempting to argue in favor of immunological mechanisms as mediators in the spontaneous regression of NB.

Our observation in paper II that soluble factors released by activated CTLs increase the expression of trkA on NB cells invites a speculation on a loop where the immune system boosts the ability of NB to undergo differentiation. Indeed, it has been shown that IFN-γ can induce NB differentiation [763], and together with retinoic acid downregulate *MYCN* target genes [764]. Furthermore, it was shown that IFN-γ works synergistically with both NGF [765-766] and TPA [767] in the induction of NB differentiation. The fact that effector molecules released by CTLs may induce differentiation could then potentially reflect a circuit whereby CTLs facilitate recognition of their own target. Altogether, this supports a regimen combining

differentiation therapy and attraction of activated CTLs into the tumor microenvironment.

Bypassing HLA class I

Whether or not NB is a suitable target for T-cell-based therapies has been questioned, mainly owing to the reported low/absent expression of HLA class I [617-618, 620] and the lack of caspase-8 expression [626-627, 768]. During the last years however, several successful attempts to target NB using CTLs have been pursued [440, 725], and NB patients were shown to harbor circulating tumor reactive CTLs which could lyse autologous tumor cells in an HLA class I-dependent manner [442]. Previous work from our group also demonstrated that CTLs could target NB in an MHC-non-restricted fashion [720].

In paper II, we analysed the immunological profile of NB cell lines and primary tumors *ex vivo*. The immunological profile was defined by us as the expression of ICAM-1, HLA class I, Heavy chain, HLA-G, MICA, HLA class II, Fas, TNFR1/R2 and TRAIL-R1/R2/R3/R4. Furthermore, we monitored how effector molecules released by activated CTLs (activated supernatant, AS) modulated the immunological profile and affected the sensitivity of NB to DR-mediated killing. In this setting, we used CTLs specific for, and activated by, the EBV-derived peptide IVT.

All NB cell lines analysed by us expressed detectable amounts of HLA class I at the cell surface. However, this could potentially reflect an *in vitro* selection of certain subclones. Yet, strongly arguing in favor of CTL-based immunotherapy, we could detect expression of HLA class I in 5 of 8 primary NB tumors, and likewise, all tumors expressed at least one DR (Table 1).

Sample	Age[a]	Stage[b]	Genetics[c]	Pre-treatment[d]	Sex[e]	Survival[f]	HLA I[g]	Fas[h]	TRAIL R1/R2[i]	CD4/CD8[j]
1	13.5	1	Oth str		M	54+	+	-	++	0.4
2	35	4	11q-	x	M	6	-	+	-	ND
3	41.5	1	Num only		M	52+	++	-	+	0.3
4	14	2	17q+	x	M	10	++	-	+	1.2
5	4	1	Num only		M	50+	-	-	+	1.1
6	4.5	1	Oth str		F	49+	+	-	+	0.7
7	18	3	MNA	x	F	44+	-	+	+	0.4
8	66	3	Oth str	x	F	42+	+	-	+	1.7

Table 1. Characteristics of NB patient samples in paper I-III.
[a]Age in months at surgery
[b]Stage according to INSS
[c]Genetical subtype; Oth str = Other structural abnormalities, 11q- = Loss of chromosome 11q, Num only= Only numerical aberrations, 17q+ = Gain of chromosome 17q, MNA= *MYCN* amplification
[d]Pre-treatment prior to surgery
[e]Sex; M=male, F=female
[f]Months after diagnosis, + = still alive
[g]HLA class I positivity of tumor as defined in paper II
[h]Fas positivity of tumor as defined in paper II
[i]TRAIL-R1 and/or -R2 positivity of tumor as defined in paper II.
[j]CD4/CD8 ratio of tumor-infiltrating $CD3^+$ T-cells
ND= not done

Further analysis of the immunological profile revealed that most primary tumors as well as NB cell lines expressed either TRAIL-R1 and/or TRAIL-R2. This is in contrast with a previous report stating that NB tumor tissue and cell lines were devoid of these receptors, which in turn conferred resistance to TRAIL-induced target cell death [628]. Since TRAIL-R1 and -R2 are the functional TRAIL receptors conveying signals through the DISC complex, our results indicate that NB could indeed be targeted by the TRAIL pathway, as is being pursued for other cancers [354]. We could also detect a discrepancy in the expression of TNF-R1 in low-stage versus high-stage primary tumors (stage 1/2 versus 3/4, according to INSS [39]), with a reduced expression in stage 3/4 tumors. Considering that TNF-R1 may mediate death-inducing signals exerted by TNF-α [364], the downregulation of this receptor could potentially provide a mechanism of immune escape in high-stage NBs.

In the case of an HLA class $I^{low/-}$ tumor, CTLs in the tumor vicinity might exert other effects than HLA-restricted killing of the tumor. When exposing NB cells to soluble factors released by activated CTLs, we could detect a skewing of the surface immune phenotype. NB tumors exposed to AS displayed enhanced levels of surface HLA class I as well as ICAM-1. Again, this could indicate a route by which CTLs, not necessarily with specificity for the target, modulate the tumor to become more sensitive to lysis by other CTLs. AS also induced the expression of Fas and TNF-R2 in cell lines as well as in a primary NB sample. By performing blocking experiments, we could identify IFN-γ and TNF-α as the major responsible molecules for the observed changes in the immune phenotype. Indeed, an enhanced expression of Fas as well as TNF-receptors has previously been reported to occur in NB upon IFN-γ treatment [769-770]. As will be discussed below, we have demonstrated that autologous PBLs from NB patients secrete increased amounts of IFN-γ as well as TNF-α when encountering NB tumors (Paper III).

The lack of caspase-8 in NB [626-627] represents a challenge for the application of T-cell based therapies. We could monitor an increase in the expression and activity of caspase-8 in NB cells exposed to AS. This observation indicates that CTLs present in the vicinity of NB tumors may restore the apoptotic machinery as well as the sensitivity of NB to DR-mediated killing. Furthermore, in an *in vivo* model of NB, loss of caspase-8 correlated to the metastatic potential of NB cells, whereas its restoration suppressed cell dissemination [771]. The attraction of activated CTLs into NB tumors may hence limit metastatic spread of NB via indirect mechanisms on the level of the apoptotic machinery.

Of particular relevance to our studies, it was recently shown that caspase-8 signaling induces terminal differentiation of NB cells [772]. Hence, another connection appears, linking differentiation with the immunological responses in NB. IFN-γ, by inducing trkA and caspase-8 expression, may promote a transition of NB cells towards a mature phenotype, which renders NB a more suitable target for CTLs as well as NK-cells.

To determine whether the changes seen in NB immune phenotype would have functional consequences, we exposed NB cells that had been primed with AS to recombinant TRAIL and TNF-α, as well as to the Fas agonistic antibody CH-11. This could potentially mimic an *in vivo* setting where bystander CTLs prime an encountered

target by the release of effector molecules, whereupon a subsequent interaction between the tumor and a death-ligand expressing lymphocyte takes place. NB cell lines were more efficiently lysed by recombinant TRAIL after expose to AS, although TRAIL-R1/R2 were not upregulated. This argues that the restoration of caspase-8 expression is the major determinant of TRAIL-induced killing of NB cells. Similarly, an increase in FasL-mediated killing was observed, which could potentially be ascribed to the observed increase of surface Fas as well as to the restoration of caspase-8. Although TNF-α did not induce NB cell death even after exposure to AS, TNF-α released by CTLs could still promote cell death by sensitizing cells to Fas-mediated death [773].

Using EBV-specific T-cells for immunotherapeutic strategies is tempting, considering the high frequency of EBV-positive individuals (over 90% of the adult population) and the ability to restimulate EBV-specific T-cells *in vitro* using highly immunogenic peptides [774]. In the study by Pule *et al.*, EBV-specific CTLs were obtained from NB patients and were engineered to express GD2 coupled to the CD3 zeta chain [725]. While receiving native stimulation *in vivo* by their TCR specific for EBV, the CAR-CTLs are redirected to the tumor via their dual specificity for GD2.

Our results highlight other potential mechanisms whereby the attraction of these CTLs could have an anti-tumor effect. *First,* these activated CTLs could release effector molecules that upregulate HLA class I molecules and hence facilitate HLA-restricted killing of the tumor by TILs. *Second*, DR-mediated killing by death-ligand expressing TILs would be facilitated as a consequence of the presence of these effector molecules. *Third,* NK-cell-mediated killing could still be enhanced since the upregulation of HLA molecules does not always impair NK-cell responses (Paper I), and since ICAM-1 might be induced by the effector molecules. *Fourth,* the release of effector molecules by the CAR-CTLs could be an alternative pathway for the induction of target cell death, since these effector molecules are sufficient to induce NB cell death at varying degree (Paper II, [720]).

Based on our findings, the administration of CAR-CTLs to NB patients could potentially be even more beneficial if combined with NGF and/or TRAIL. Co-administration of IL-2 could be a strategy to support activation of TILs and a co-operative anti-tumor response by TILs and transferred CAR-CTLs.

Our results also demarcate IFN-γ as a versatile molecule, with the ability to modulate NB immunogenicity, induce differentiation and restore the apoptotic machinery. A recent study by Reid *et al.* also demonstrated that IFN-γ regulates T-cell infiltration into NB tumors in a model where immunodeficient mice received ACT with survivin-specific T-cells derived from NB patients [775]. Thus, the delivery of IFN-γ to the NB microenvironment appears as a promising approach to enhance intratumoral T-cell responses. Furthermore, as shown in paper III, only 2 of 8 human NB tumors produced IFN-γ *ex vivo*, arguing that an additional source of IFN-γ would be needed. Since systemic administration of cytokines can evoke toxicities, an alternative route for administration would be preferred. Possibly, IFN-γ could be linked to hu14.18, as has been performed with IL-2 [743]. This could be beneficial as a single treatment, or combined with ACT therapy and/or differentiating agents such as NGF.

TUMOR-INFILTRATING CELLS IN NEUROBLASTOMA (PAPER III AND IV)

In paper III and IV, the interactions between NB and tumor-infiltrating cells of the immune system have been investigated. Specifically, paper III aimed at monitoring the status of T-cell responses in the peripheral blood and within the tumors of NB patients. Paper IV aimed at investigating inflammatory patterns, including infiltrating cells of the adaptive as well as the innate immune system, and the effect of anti-inflammatory treatment on NB using the TH-MYCN mouse model.

T-cell responses in the tumor microenvironment of NB

Studies on T-cell responses in NB patients have been performed with varying outcomes. Coughlin *et al.* reported that tumor-infiltrating T-cells were rare or absent in 26 of 26 high-risk NB samples investigated by immunohistochemistry. In peripheral blood however, survivin-specific T-cells were detected, and T-cells were also present in the perivascular areas of tumor samples [442]. Reid *et al*. furthermore showed that T-cells can infiltrate NB tumors in an IFN-γ-dependent manner [775] and another study demonstrated that clonal expansion of T-cells took place in NB tumors [444]. In addition, $CD4^+$ T-cell clones isolated from NB tumors retained their ability to secrete T_H1 cytokines *in vitro* [445]. Hence, it appears that T-cell responses towards NB tumors may actually exist, but the functional status of these T-cells remains to be fully elucidated.

The tumor compartment may represent an immunosuppressive environment unfavorable for the generation of anti-tumor T-cell responses. In a melanoma vaccine trial, the absence of a clinical response was attributed to functional dissociation between systemic and local immune responses [480]. However, an *in vivo* model for melanoma demonstrated that naïve T-cells can be activated by APCs within the tumor and hence acquire an effector cell phenotype upon entering the tumor area [294].

To elucidate whether or not the tumor microenvironment of NB is suppressing T-cell responses, a systematic comparison with other compartments may lead to further insight. Hence, we evaluated T-cells in the peripheral blood as well as within the tumors of NB patients, with respect to their CD4/CD8 distribution, activation status and memory phenotype. In contrast to previous published reports, we could detect intratumoral T-cells in all screened primary NB samples. Furthermore, immunostaining revealed that T-cells were proliferating *in situ* and aggregated their TCRs towards the contact sites with NB tumor cells. When evaluating the prevalence of $CD4^+$ versus $CD8^+$ T-cells in the $CD3^+$ compartment, we could detect a redistribution in favor of $CD8^+$ cells in tumor-associated lymphocytes (TALs) in 5 of 7 evaluated tumors. In all but one tumor, a stage 3 *MYCN*-amplified NB, the proportion of $CD8^+$ T-cells was higher in the tumor than in peripheral blood. Further evaluation revealed a higher expression of the activation marker CD25, the IL-2 receptor α-chain, on T-cells within the tumor compartment in 5 of 7 patients.

T-cells can also be classified according to their memory phenotype. In general, T_{EM} and T_{EMRA} cells are considered to be the most differentiated subtypes in terms of effector function. Besides carrying high amounts of perforin and GrB, they also mount robust T_H1 cytokine responses upon simulation [307, 776]. Concomitant to the increase in

CD25 expression, TALs displayed a phenotype of memory cells to a higher extent, as compared to PBLs, where a naïve phenotype predominated. The most prominent difference was observed within the $CD3^+CD8^-$ compartment, where an increase in T_{EM} cells was detected in 6 of 6 tumors. $CD3^+CD8^+$ cells were mainly of the T_{EMRA} phenotype, which is known to carry the highest amounts of perforin [307]. Interestingly, Pages *et al.* demonstrated that the presence of memory T-cells correlated to an increased survival in colon cancer patients [274]. Although we have not been able to determine the specificity of the T-cells present within NB tumors, our findings in paper II support the notion that activated T-cells within NB tumors might exert anti-tumor effects even if they would not be specific for TAAs.

Considering the increase in CD25 expression, we also set out to monitor the presence of Tregs in human NB samples, by detecting Foxp3 expression in combination with CD4 and CD25. Intracellular stainings of whole tumors represent a challenge, since these often necessitate more cells due to extended handling and since the additional steps can affect the quality of the staining. In the two samples where enough cells were available, the expression of Foxp3, as detected directly *ex vivo*, was surprisingly lower in intratumoral $CD4^+CD25^+$ cells than in the corresponding population in autologous PBLs. Hence, in these samples, Tregs did not appear to accumulate within the tumors, which supports the picture of a prevailing anti-tumor T-cell response in NB tumors.

Differences in T-cell subsets detected in the tumor compartment and peripheral blood may reflect a preferential homing or a preferential on-site expansion of certain subsets of cells. To monitor this *in vivo* in NB patients would be logistically, practically and ethically hard to perform. We used an *in vitro* system where autologous PBLs from NB patients were subjected to an encounter with tumor cells in culture, which enabled us to monitor tumor-inflicted changes on the phenotype of PBLs. The overall changes seen upon this co-culture system skewed the phenotype of PBLs towards that seen in the intratumoral compartment on the day of tumor excision. In the majority of cases, the CD4/CD8 ratio was skewed towards CD8, and CD25 expression either increased or remained at similar levels. Similarly, the frequency of T-cells with a memory phenotype was increased, mainly T_{EM} in the $CD3^+CD8^+$ population, indicating an acquisition of effector functions. In other studies it was shown that soluble factors released by tumor cells could induce a regulatory phenotype in T-cells [526], or even trigger cell death in autologous PBLs [777].

Altogether, it appears likely that lymphocytes infiltrating NB tumors are not prevented from being activated on-site and obtain the functional characteristics of armed $CD8^+$ effector cells.

In paper IV, we evaluated intratumoral T-cells present in the transgenic TH-MYCN tumors of various stages of disease. Previously, this model was suggested to lack lymphocytic infiltration due to a "non-immunogenic" phenotype [778]. In our hands, TILs were detected in all screened samples using immunohistochemistry as well as flow cytometry. However, in contrast to the observed pattern in human NB samples, we could detect a preferential presence of $CD4^+$ T-cells compared to $CD8^+$ T-cells, with a gradual increase in the CD4/CD8 ratio in parallel to tumor progression. Surface expression of activation markers (FasL and CD25) was low, but the capability to

produce IFN-γ upon mitogenic stimulation *ex vivo* was retained. The IFN-γ levels were low within the tumors, as detected by quantitative real-time RT-PCR (qRT-PCR), but the intrinsic ability of the T-cells to mount T_H1 responses upon proper stimulation appears to be intact. It has been shown that $CD8^+$ responses gradually decline during tumor progression, with a reduced ability to induce target cell death at later stages of disease [779].

A note on the TH-MYCN model

The TH-MYCN model was first described by Weiss *et al*. in 1997. By inserting human *MYCN* cDNA under control of the rat tyrosine hydroxylase promoter, the expression of *MYCN* was successfully directed to migrating cells of the neural crest [780]. The mice presented with thoracic and/or abdominal tumors, and a subsequent study confirmed the origin of the tumors to be paravertebral ganglia [781]. Histologically, the TH-MYCN tumors resemble human NB. The tumors are highly vascularized and contain small, round blue cells. Varying degree of neuronal differentiation is detectable. Macroscopic metastases have been detected in the liver, lungs and ovaries, and microscopic metastases are found in several organs [780, 782].

The progression of heterozygous TH-MYCN tumors has been suggested to resemble that of human NB, with early, intermediate and late tumors corresponding to stage I-III NB, respectively [782]. Furthermore, the genetical aberrations detected in the TH-MYCN tumors also reflect those in human NB, exemplified by chromosome gains corresponding to 17q gain in human NB [783]. Previous studies have denoted 100% of homozygous mice as tumor bearing at the age of 6.5 or 7 weeks [781, 784]. Heterozygous mice have shown varying degree of disease penetrance, with two studies reporting 27% or 65% of heterozygous mice as tumor bearing at the age of 95 days [782, 784], and another study denoting 33% as tumor bearing at the age of 13 weeks [781].

Whereas the level of $CD4^+Foxp3^+$ Tregs as a function of infiltrating $CD4^+$ cells remained steady, the TH-MYCN tumors still appear as more suppressive to T-cell responses than human NB. Several explanations could be true for this difference. The TH-MYCN model is an extremely aggressive model for NB, with homozygous mice rapidly developing large intra-abdominal tumors. A systemic immunosuppression during such a process is likely to be of importance and dampen the quality of T-cell responses systemically as well as locally. In a tumor expanding as aggressively as the TH-MYCN tumor, rapid turnover of large amounts of cells will unequivocally trigger inflammatory pathways with an ensuing influx of cells with suppressive abilities, as will be discussed below.

Another factor defining the prerequisites for a successful intratumoral T-cell response is the prevailing balance between T_H1 and T_H2 cytokines. In our cohort of primary human NB samples, we monitored the levels of secreted IL-1β, IL-12, IFN-γ, TNF-α, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, TGF-β and GM-CSF *in vitro* (paper III). The dominant cytokine produced at high levels by all NB tumors, and possible remaining TALs, was IL-8. Previously, the mRNA for IL-8 has been detected in NB samples [445], and IL-8 is known for its pro-angiogenic properties [785]. The immunosuppressive cytokines IL-

10 and TGF-β were produced by 1 and 4 of 7 tumors, respectively. Of note, the highest amounts of TGF-β were detected in a *MYCN* amplified stage 3 NB, followed by a non-survivor with a stage 2 NB. The immune-stimulating cytokines IFN-γ and TNF-α were detectable in 2 and 3 of 7 tumors, respectively. Interestingly, when exposing autologous PBLs to tumors, their corresponding cytokine pattern was altered such as to downregulate TGF-β in 5 of 7 patients and increase the T_H1 cytokines TNF-α and IFN-γ in 4 and 5 of 7 patients, respectively. This finding further strengthens the notion that T-cells encountering NB tumors may be activated in the vicinity of the tumors. In the TH-MYCN model, we monitored the mRNA levels of T_H1 (IFN-γ and IL-2) versus T_H2 (IL-10, IL-6 and TGF-β) cytokines in homo- as well as heterozygous tumors of various sizes. We could detect a predominance of IL-10 and TGF-β above other cytokines, a pattern which persisted throughout tumor progression. This indeed mirrors the attenuated $CD8^+$ responses, but also raises the question how Tregs are sustained, considering their need for IL-2. One possible explanation would be a continuous influx of Tregs from peripheral blood, where IL-2 was detectable at low but increasing levels during tumor progression. Alternatively, Tregs might be short-lived but constantly replenished on-site by alternative activation pathways under the influence of TGF-β [512].

A note on flow cytometry on tumor samples

One common technique applied in paper III and IV is multi-color flow cytometry on whole tumor samples. This is a method which represents a technical challenge *per se*. A tumor harbors a cell population of extreme heterogeneity, not only with cells of divergent origin, but also of varying viability and propensity to bind antibodies non-specifically.

Factors of major importance that will affect this type of staining are *i*) the type of compensation applied, *ii*) the usage of isotype controls versus fluorescence minus one (FMO) controls and *iii*) Fc-blocking reagents as well as dead cell markers. In paper III, we have used isotype controls to define positivity. During the last years, isotype controls have been questioned and the alternative FMO has arisen as another way to validate the positivity of a staining [786]. An FMO control is represented by a tube where the fluorochrome-conjugated antibody of interest is omitted, but the remaining setup is intact. Since a tumor represents a tissue where one can expect extensive binding to and uptake of antibodies by dead cells, as well as differential autofluorescence and FcR binding of antibodies, an FMO might be a preferred control for certain stainings. With experience, one can learn how to interpret background noise and distinguish a true staining from false antibody uptake by apoptotic cells. This however requires a trained eye and variations between samples may cause technical problems. To circumvent or minimize these problems, one can use Fc-blocking reagents as well as dead cell markers to reduce background. Manual compensation during acquisition is also difficult to achieve with a satisfactory outcome. Instead, compensation using mathematical matrices applied by the software is preferred, either while running the samples or during the analysis (for example using the FACSDiva or Flow Jo software).

Tumor-associated inflammation versus immunosurveillance

Inflammation was recently suggested to be incorporated into the hallmarks of cancer [64], and anti-inflammatory treatment with aspirin has proven to reduce the risk of cancer in the adult population [227-229]. Suppressive populations such as Tregs have also been ascribed protective roles in some cancers, possibly by dampening loco-regional inflammation [374, 552]. Nevertheless, the immune system can control tumor growth, given the right circumstances and a proper target cell.

In paper III, human NB tumors are shown to be permissive for anti-tumor T-cell responses. In paper IV, we define how tumor-infiltrating cells in the TH-MYCN mouse model for NB are being subverted towards a tumor-promoting phenotype during tumor growth. In light of these results, the question arises how anti- and pro-tumorigenic properties of the immune system regulate NB tumor growth and direct the responses seen upon immunotherapy.

Figure 8 summarizes the tumoricidal and the tumor-promoting arms of the immune system in the tumor microenvironment and of relevance for this thesis.

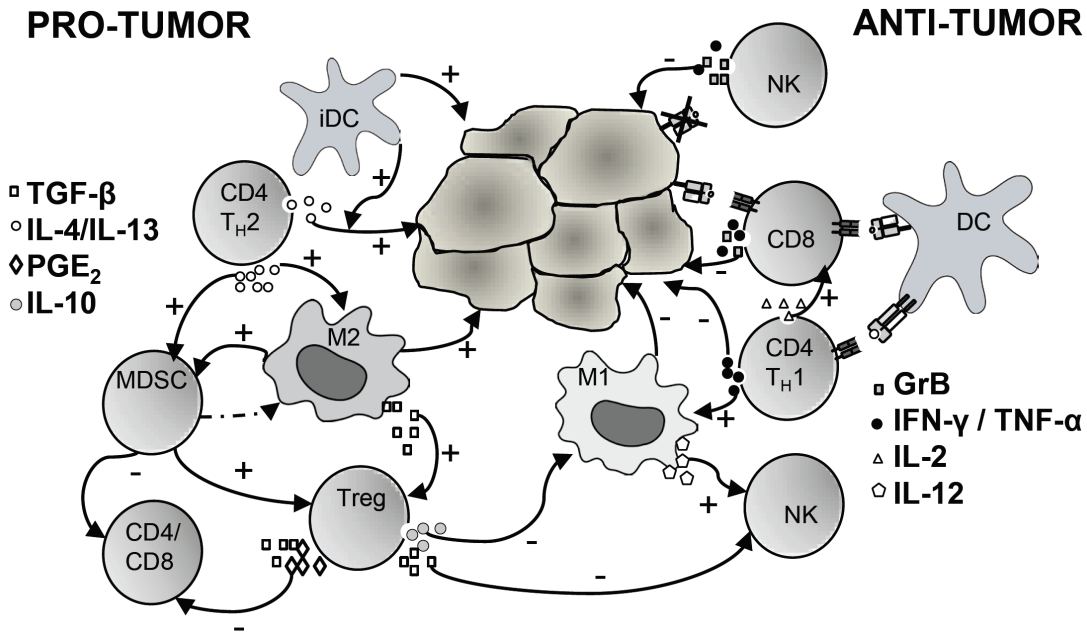

Figure 8. Immune responses in the microenvironment highlighting some of the pro-and anti-tumorigenic properties of the immune system. On the left side, tumor-promoting abilities of the immune system are shown, defined by a T_H2 response and an induction of M2 macrophages, Tregs, MDSCs and iDCs. On the right side, anti-tumor immunity prevails with a skewing towards a T_H1 response and an induction of $CD8^+$ T-cells, NK-cells, M1 macrophages and mature DCs. M1= TAM (M1 phenotype), M2= TAM (M2 phenotype)

Although the TH-MYCN tumors behave disparate to primary NB samples in respect of T-cell responses, it is still to be considered a preferential model above syngeneic or xenogenic models for studying interactions with the tumor microenvironment. Using the latter, an acute onset of inflammatory reactions upon introduction of foreign cells is inevitable. Any immune response seen upon tumor cell inoculation is less likely to mimic native interactions occurring without external stimuli. The targeted expression of

MYCN in the TH-MYCN model might of course be considered an artificial system *per se*, yet the stroma of the tumor consists solely of host-derived components and the tumors are assembled in the actual location for human NB.

Employing the TH-MYCN model, we could demonstrate a diminishment of intratumoral $CD3^+$ T-cell responses in favor of immature cells of the innate immune system in the course of tumor growth. A detailed analysis was performed on the phenotypical characteristics of TAMs in TH-MYCN tumors. In parallel to an increase in the total number of TAMs, the M1 phenotype detectable at early stages of tumor development was subverted towards a tumor-promoting M2 phenotype as the tumor progressed. In a study by Song *et al.,* it was shown that natural killer T (NKT) cells mediated NB anti-tumor immunity by killing CD1d expressing TAMs [86]. NKT cells have been linked to favorable prognosis in NB and their infiltration is abrogated upon *MYCN* expression [787]. Interestingly, in paper III, we demonstrate an increase in $CD3^+CD4^-CD8^-$ cells in TALs in 4 of 7 samples investigated, possibly representing infiltrating NKT cells. In an immunotherapeutic model for NB, macrophages were however shown to mediate the protective effects, again stressing the dual role for intratumoral macrophages [788]. Furthermore, observed clinical responses to the 3F8 anti-GD2 mAb were shown to correlate to an FcγRIIA polymorphism, and macrophages were proposed to mediate the ADCC [789].

In addition to TAMs, DCs and MDSCs also outnumbered infiltrating T-cells during TH-MYCN tumor progression. The infiltrating DCs exhibited low levels of co-stimulatory CD86 and a gradual decline in their MHC class II expression, consistent with an immature phenotype unable to initiate T-cell responses and with the potential to promote tumor growth [197]. MDSCs, well known for divergent mechanisms to suppress T-cell responses [158], constituted a small proportion (median 1%) of infiltrating cells in early tumors, but increased in advanced tumors (median 10%).

It appears that at some stage of tumor development, the on-site immune system is subverted and the recruitment of immune cells is skewed to favor tumor growth. Initially, in the early events of transformation, an acute inflammatory reaction may evolve and be successful in controlling tumor growth. However, many tumors will eventually turn into a tissue where chronic inflammation prevails and repeated activation of immune cells occurs in a suboptimal setting. In such a scenario, a polarization of the immunological profile will inevitably occur [63, 120]. In our model, the negative impact of a prolonged state of disease is clearly demonstrated by the higher expression of IL-10 and TGF-β in tumors from heterozygous mice, which have a slower onset of disease and longer time as tumor bearing animals compared to homozygous mice.

Our results indicate that tumor-associated inflammation contributes to shaping NB tumor growth in the TH-MYCN model. Furthermore, NB has previously been shown to express COX-2, and targeting of the COX-2 pathway reduced tumor growth in a murine model of NB [111]. In the TH-MYCN model, we could detect a prominent expression of COX-1 and a weak expression of COX-2 in tumors of early stages, which indicated the presence of functional inflammatory pathways. Consequently, we further evaluated how anti-inflammatory treatment with low-dose aspirin would affect the

tumor microenvironment and/or tumor outgrowth. Homozygous mice were randomized to receive 10 mg/kg of aspirin daily by oral gavage, for 10 consecutive days. At the day of sacrifice, treated animals presented with a tendency towards a lower tumor burden, and concomitantly, the pattern of infiltrating cells remained comparable to that of early tumor lesions. Whether or not the altered pattern of infiltration is brought upon by aspirin, or is a bystander phenomenon to a reduction in tumor growth, can at this time only be speculated upon.

Recent studies correlate a daily intake of low-dose aspirin to a reduced incidence of and mortality due to several adult cancers, but offered no mechanistic explanations [227-228]. Considering that PGE_2 has the potential to contribute to all of the hallmarks of cancer [119] and given its prominent role in immunosuppression [104], several mechanisms could explain this reduced incidence of cancer. Sparse information is however available regarding the role of inflammation as a contributor to tumor growth in pediatric cancers. Our results highlight inflammatory pathways as a potential therapeutic target in NB.

Considering that the local immunological balance within a tumor will affect or even determine the outcome of immunotherapy, it would be favorable to implement immunotherapy prior to reaching the state of chronic inflammation. In fact, prostate cancer vaccines, including the DC-based Sipuleucel-T and DNA-based vaccines were shown to be more effective in patients with less advanced stages of disease [689, 790]. Interestingly, in NB, the fusion protein hu14.18-IL-2 was effective only in patients with non-bulky disease, whereas patients with bulky disease did not respond [743]. In combination with our results outlined in paper IV, this argues for an early implementation of immunotherapy in clinical protocols for NB.

Alternatively, if the prevailing microenvironment is suppressive, it could be primed prior to the application of immunotherapy. The purpose of such a regimen would be to shift the local balance from chronic inflammation to immunosurveillance. A recent review by T. Whiteside highlights the potential of targeting cancer-induced immunosuppression [791]. Several possible ways to restore immunosurveillance exist, including mAbs directed at inhibitory molecules such as CTLA-4 and PD-1, or directed at suppressive cytokines such as TGF-β or IL-10. The tyrosine kinase inhibitor sunitinib is also promising, since it has proven to reduce the levels of Tregs and MDSCs and promote a shift towards T_H1 responses [792]. COX-inhibitors also constitute a possible auxiliary approach to be combined with immunotherapy. Reduced levels of PGE_2 would be beneficial for a subsequent introduction of immunotherapy, considering its immunosuppressive effects. Talmadge *et al.* could demonstrate reduced numbers of MDSCs upon COX-inhibition, which provides a potential link between COX-inhibitors and immunotherapy [241]. Recent publications have also demonstrated the ability to reverse the phenotype of TAMs from M2 to M1, with *in vivo* effects on tumor burden [244-245].

Taken together, the tumor might provide a non-favorable site for immune responses, but an improved understanding of the balance between chronic inflammation and immunosurveillance offers new potential strategies to improve the outcomes of immunotherapy.

GENERAL CONCLUSIONS

The work presented in this thesis addresses the fundamental prerequisites for the implication of immunotherapy in NB, by investigating how NB interacts with cells of the immune system.

Our results demonstrate that NB may be a suitable target for cellular immunotherapy, although the prevailing notion is that NB is a tumor of low immunogenicity. We show that the induction of differentiation in NB is accompanied by an enhanced ability of T-cells as well as NK-cells to eradicate NB tumor cells. This argues strongly in favor of a combined approach where adoptive transfer of tumor-reactive lymphocytes is performed concomitant to the administration of differentiating agents. Furthermore, we show that activated tumor-non-specific CTLs release effector molecules that modulate the immunogenicity of NB, such as to enhance the sensitivity to DR-mediated killing and to restore the apoptotic machinery. This pathway offers the ability to circumvent the need for HLA-restricted killing and emphasizes the importance of attracting activated CTLs into NB tumors.

This thesis also provides evidence that primary human NB samples harbor tumor-infiltrating T-cells which proliferate *in situ* and are of a memory phenotype. Again, this argues in favor of implementing T-cell based cell therapies for NB patients.

In addition, using the transgenic TH-MYCN model for NB we demonstrate how tumor progression is accompanied by a shift in the composition and phenotype of tumor-infiltrating cells, in favor of immature cells with tumor-promoting abilities. Concomitantly, early anti-inflammatory treatment of homozygous mice with low-dose aspirin showed a promising efficacy in delaying tumor outgrowth and the inflammatory switch. This uncovers tumor-associated inflammation as a possible contributor to, and target in, NB growth.

In conclusion, the work presented in this thesis demonstrates how NB interacts with the immune system, and depicts possible interventions on how to enhance the recognition of NB by the immune system. It argues in favor of an early implementation of cellular immunotherapy, which could preferentially be potentiated by the attraction of activated tumor-non-specific CTLs to the tumor, or by differentiating agents.

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