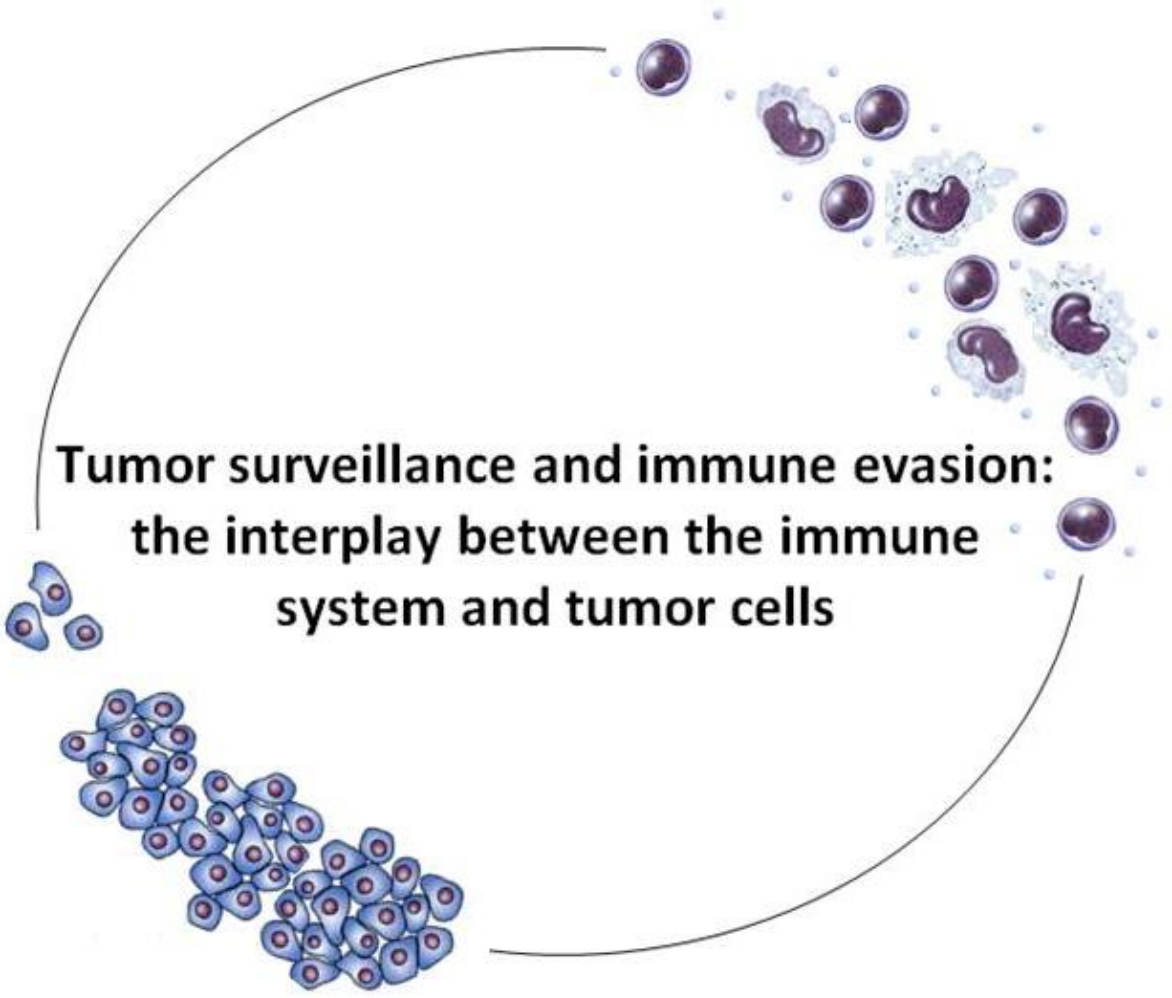

Master thesis

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Biology of Disease

Tumor surveillance and immune evasion: the interplay between the immune system and tumor cells

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Master thesis
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List of abbreviations

ADAM	A Disintegrin And Metalloproteinase
ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
Apaf-1	Apoptosis protease-activating factor-1
APC	Antigen Presenting Cell
ARF	Alternative Reading Frame
BAD	Bcl-2-Associated Death promoter
BAK	Bcl-2 Antagonist / Killer
BAX	Bcl-2-Associated X protein
Bcl-2	B-cell CCL/Lymphoma 2
BIM	Bcl-2 Interacting Mediator of cell death
CAT	Cationic Amino-acid Transporters
CD	Cluster of Differentiation
Cdk	Cyclin-dependent kinase
cFLIP	cellular FLICE-Inhibitory Protein
CGK	cGMP-dependent protein Kinase
COX-2	Cyclooxygenase-2
CTL	Cytotoxic T-Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DC	Dendritic Cell
DcR3	Decoy Receptor 3
DISC	Death-Inducing Signaling Complex
DNAM-1	DNAX Accessory Molecule-1
EP	E-prostanoid
EIF2α	Eukaryotic translation Initiation Factor 2α
FADD	Fas-Associated Death Domain
FAS	Factor-related Apoptosis
$Foxp3^+$	Forkhead / winged helix transcription factor
GC	Guanylate Cyclase
GCN2	General Control Non-repressible 2
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor

HIF-1α	Hypoxia-Induced Factor 1α
HLA	Human Leukocyte Antigen
HSP	Heat Shock Protein
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon γ
IL	Interleukin
iNOS	induced Nitric-Oxide Synthase
IRS	Inhibitory Receptor Superfamily
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
Jak-STAT	Janus kinase / Signal Transducer and Activator of Transcription
KIR	Killer cell Immunoglobulin-like Receptor
LPS	Lipopolysaccharide
MDSC	Myeloid-Derived Suppressor Cell
MDM2	Mouse Double Minute 2
MHC	Major Histocompatibility Complex
MIC	MHC class I Chain-related protein
MO	mononuclear
mTOR	mammalian Target Of Rapamycin
NCAM	Neural Cell Adhesion Molecule
NCR	Natural Cytotoxicity Receptors
NK-cell	Natural Killer cell
NKT-cell	Natural Killer T cell
NO	Nitric Oxide
NOX2	NADPH oxidase
$\cdot O_2^-$	Superoxide anion
ONOO-	Peroxynitrite
PD	Programmed cell Death
PGE2	Prostaglandin E2
PMAIP1 (NOXA)	Phorbol-12-Myristate-13-Acetate-Induced Protein 1
PMN	polymorphonuclear

PUMA	p53-Upregulated Modulator of Apoptosis
PVR	Poliovirus Receptor
Rb	Retinoblastoma
ROS	Reactive Oxygen Species
RNOS	Reactive Nitrogen and Oxygen Species
TAA	Tumor-Associated Antigens
TAM	Tumor-Associated Macrophages
TAP	Transporters associated with Antigen Processing
TCR	T-cell Receptors
TGF-β	Transforming Growth Factor β
Th3	T helper type-3
TLR	Toll-Like Receptor
TME	Tumor Microenvironment
TNF	Tumor Necrosis Factor
Tr1	T-Regulatory lymphocytes type-1
TRAIL	Tumor necrosis factor-Related Apoptosis-Inducing Ligand
Treg	Regulatory T-cells
TSA	Tumor-Specific Antigens
ULBP	Unique Long 16-Binding Protein
VEGF	Vascular Endothelial Growth Factor

Abstract

This thesis encloses the interplay between the immune system and tumor cells. The immune system is, next to defending the body against pathogens, specialized in tumor cell recognition and elimination in various ways. NK-cells, NKT-cells, $CD8^+$ T-cells, and γδ T-cells are important players in tumor surveillance. Tumor cells are distinguished from healthy cells by the expression of tumor-specific antigens (TSA) and tumor-associated antigens (TAA). Diverse receptors on the before mentioned immune cells recognize these antigens, leading to immune cell activation and it provides a license to kill. The main killing mechanisms are the perforin / granzyme pathway, the death-receptor pathways, and antibody-dependent cell-mediated cytotoxicity (ADCC). Attacking tumor cells is accompanied by the production of cytokines, primarily interferon gamma (IFN-γ). IFN-γ steers into an anti-tumor, Th1 direction.

Tumor surveillance is counteracted by immune evasion performed by tumor cells and cells in tumor microenvironment (TME), like myeloid-derived suppressor cells (MDSC). Tumor cells (and associated cells) developed many possibilities to evade the immune system; they prevent recognition by immune cells, become apoptosis resistant, deceive the immune system, inhibit immune cell development, proliferation, and maturation or induce immune tolerance. MDSCs are important players in tumor immune evasion and are discussed more thoroughly.

Introduction

Tumors and the immune system are strongly associated. Although, cancer is defined as a disease caused by genomic changes [1], the immune system is essential in tumor modulation and elimination [2]. Immune cells, from both the innate and adaptive immune systems, are able to recognize, attack, and kill tumor cells in various ways. They collaborate and stimulate each other (e.g. with IFN-γ) to induce a potent immune response against the tumor cells. However, cancer still occurs. Tumor cells are able to fight back or hide from the immune system. These events are called tumor surveillance and tumor immune evasion and are correlated in cancer research. Nowadays, these processes together are also defined as cancer immuno-editing, indicating the important interplay [2]. You cannot study one without having knowledge of the other. The effectiveness of cancer immunotherapy, for example, is often impeded by tumor immune evasion due to immune suppression.

Cancer research is a very broad field and a hot topic for decades. In this thesis, I combined this intriguing subject with my major interest: the immune system. They meet each other in an unnoticed battlefield, where the winner determines if cancer becomes clinically visible or not.

This thesis gives a compact view of tumor surveillance by the immune system and tumor immune evasion with a focus on myeloid-derived suppressor cell functioning.

1. Tumor surveillance

Ehrlich was the first to hypothesize that the immune system is able to eliminate tumor cells by tumor immune surveillance [2]. Tumor cells were suggested to express 'tumor-specific antigens', which are recognized by immune cells. The hypothesis was more specified by Thomas (1959) and Burnet (1970), implying that lymphocytes are the main effector cells. Nevertheless, the existence of tumor immune surveillance was doubtful. Meanwhile, it is shown that immunodeficient individuals are more vulnerable to develop tumors from viral and non-viral origin than immunocompetent individuals, implying that the immune system inhibits tumor development [2, 3].

Extensive research during the last decade has shown that tumor immune surveillance is mainly mediated by natural killer cells (NK-cells), natural killer T-cells (NKT-cells), cytotoxic T-cells ($CD8^+$ T-cells), and γδ T-cells. These cell types and their roles in protecting the body against tumors are discussed in this chapter.

1.1 NK-cells

NK cells are involved in the innate and adaptive immune system, where they play an important role in the elimination of infected and tumor cells. There are two different types of NK-cells; the $CD56^{bright}CD16^-KIR^-$ NK-cells, which are proliferative and produce cytokines like IFN-γ, and the $CD56^{dim}CD16^+KIR^+$ NK-cells, which are more specialized in the killing of infected and transformed cells [4]. (CD56 is also called Neural Cell Adhesion Molecule (NCAM) and is a NK-cell marker; CD16 is a synonym for the FcγRIII, which is involved in ADCC.) $CD56^{dim}CD16^+KIR^+$ NK-cells originate from $CD56^{bright}CD16^-KIR^-$ NK-cells [5]. NK cells can kill cells in three different manners; via the perforin / granzyme pathway, death-receptor-dependent pathways and antibody-dependent cell-mediated cytotoxicity (ADCC) [6].

1.1.1 Tumor surveillance by NK-cells: recognition of tumor cells by NK-cell receptors

Cytokine production and killing of affected cells are the main tasks of NK-cells and especially the elimination of cells is performed is several ways [5]. Natural killers cells

can, as the name says, eliminate other cells without being activated by DCs or T-cells, but their ability to kill or induce apoptosis depends on the net effect of activating and inhibiting receptors [7, 8] (Figure 1). The inhibiting receptors are dominant over the activating receptors to prevent autoimmunity. Most receptors on NK-cells contain an immunoreceptor tyrosine-based activation / inhibitory motif (ITAM / ITIM), a cytoplasmic domain responsible for inducing an activating or inhibiting signal [9]. Although the names presume so, ITAMs and ITIMs are not exclusively activating or inhibiting [9]. NK-cells have two categories of receptors, the lectin-like and immunoglobulin-like receptors, both consisting different activating and inhibiting receptors. Still, not all ligands of these receptors are known [10]. Tumor cells are recognized by NK-cells in different ways based on 'missing-self' and 'induced-self' signals on cells. In tumor and virus-infected cells, MHC-I and HLA-E are frequently downregulated [7]. HLA-E expression is an indicator for total HLA-I expression on a cell [10]. MHC-I and HLA-E are ligands for the killer cell immunoglobulin-like (KIR) and CD94/NKG2A (lectin-like) receptors [4, 11]. In normal cells, ligand binding inhibits NK-cell activation via these receptors. Missing this 'self' signal combined with stimulatory signals from activating receptors induces the NK-cell to kill its target cell [7]. Stimulating signals can be offered by stress-induced molecules on tumor and infected cells. NKG2D is a NK stimulatory receptor and recognizes the stress-induced MHC class I chain-related protein A / B (MICA / MICB)

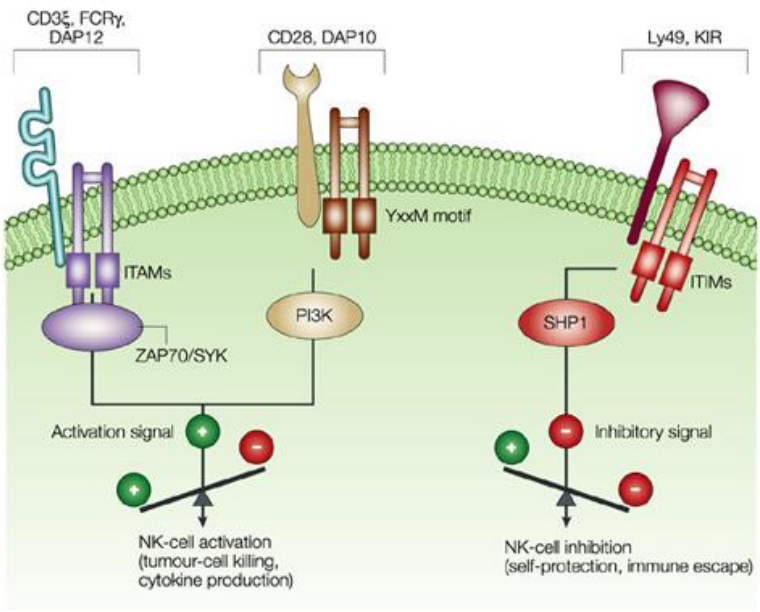

Figure 1: NK-cell activation
NK-cell activation depends on the net effect of inhibiting and activating signals. Most NK-cell receptors have an ITAM or ITIM domain providing a stimulatory or inhibitory signal for NK-cell activation. On the left hand and in the middle: NK activating receptors signaling via ITAMs and the YxxM motif. (The NKG2D receptor acts in the same way as CD28.) On the right hand: NK inhibiting receptor signaling via ITIMs.
Figure derived from Smyth et al. (2002)

and the glycoproteins unique long 16-binding protein 1 / 2 (ULBP1 / ULBP2) [4, 11]. DNA damage in tumors stimulates the expression of these NKG2D ligands and binding of NKG2D results in an activating signal [7]. Other stimulating signals are provided by the natural cytotoxicity receptors (NCR) and DNAX accessory molecule-1 (DNAM-1). The natural cytotoxicity receptors NKp30, NKp44, and NKp46 were shown to be important in the NK-cell-induced killing, however, their ligands are still unknown [10]. They are thought to bind to self-ligands that are upregulated on virus-infected or transformed tumor cells [7, 10]. DNAM-1 induces cytokine production and target cell killing upon binding of its ligands: the nectins, nectin-2 and poliovirus receptor (PVR) [10]. These ligands are strongly expressed on some tumor cells. Normal cells do also express nectins, but NK-cell activation is suppressed by MHC-I engagement.

1.1.2 Tumor surveillance by NK-cells: elimination of tumor cells

After recognition of molecules like MICA, ULBP, and nectins, or missing of specific ligands (e.g. MHC-I and HLA-E), NK-cells become activated to produce cytokines and eliminate a target cell. Killing is performed via the perforin / granzyme pathway, death-receptor-dependent pathways and antibody-dependent cell-mediated cytotoxicity (ADCC) [6]. The two first pathways to eradicate cells are also performed by cytotoxic T-lymphocytes ($CD8^+$ T-cells), which form together with NK-cells the cytotoxic lymphocytes.

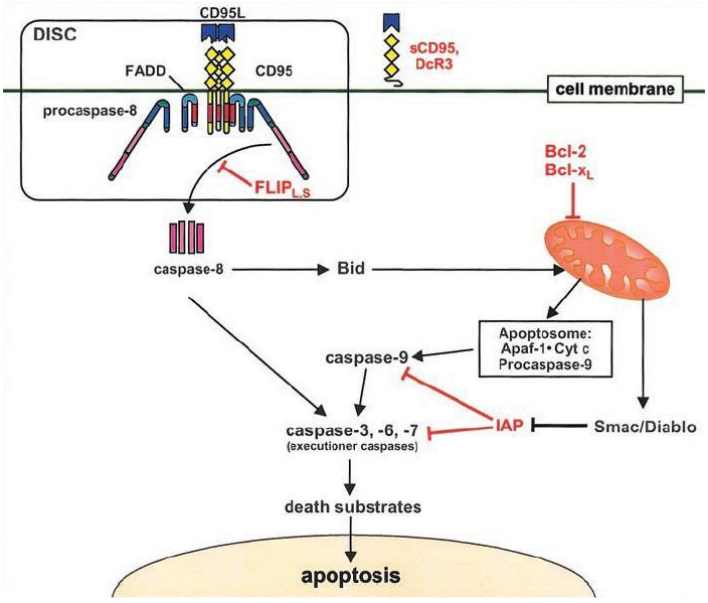

Figure 2: Death receptor signaling
The interaction of Fas (CD95), TRAIL-R, and TNF-R with their ligands induces apoptosis by cleaving procaspase-8. Caspase-8 initiates the caspase cascade.
Figure derived from Igney et al. (2002)

Upon activation of a cytotoxic lymphocyte, granules containing perforin and granzymes are released in the synaptic cleft between the killer and target cell. The exact mechanisms are not known; either granzymes enter the target cell via pores in the cell membrane made by perforin or granzymes and perforin are endocytosed by the target cell. Perforin makes pores in the endosomes, giving granzymes the opportunity to enter the cytoplasm of the target cell. In the cytoplasm, granzymes induce apoptosis by activating caspases, attacking the mitochondria or by other caspase-independent pathways [6].

There are different death-receptor-dependent pathways involved in the killing of tumor cells [12]. The most important death ligands are FasL (Factor-related apoptosis ligand or CD95L), TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), and TNF (tumor necrosis factor). They belong to the tumor necrosis factor (TNF) receptor superfamily, which induce apoptosis via the extrinsic pathway [13] (Figure 2). Cytotoxic lymphocytes induce apoptosis via FasL, TRAIL, and TNF-α by binding to respectively Fas, TRAIL receptor and TNF receptor on tumor cells. The engagement induces the intracellular recruitment of FADD (Fas-associated death domain) by FasL and TRAIL, or TRADD (TNF receptor death domain) by TNF-α. These death domain-containing adaptor molecules start the proteolytic caspase cascade by activating caspase-8 and -3 in the tumor cell, leading to apoptosis.

NK-cells, but not cytotoxic T-lymphocytes, can kill tumor cells by antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC can occur when tumor antigens are recognized and bound by specific IgG molecules. NK-cells attach with their CD16 (FcγRIII) receptor to the Fc region on IgG antibodies, which are bound to a target cell. This engagement induces the release of lytic enzymes and TNF-α by the NK-cell, resulting in the death of the target cell. Unless the target cell is directly recognized by the NK-cell, specificity is provided by the IgG molecules attached to tumor antigens.

1.2 NKT cells

NKT cells are involved in both the innate and adaptive immune system serving as a bridge between the two systems [14]. They are rapid responders and belong to the first cells entering an inflammatory environment, where they release lots of cytokines e.g. IFN-γ and IL-4. NKT cells are able to regulate DCs, NK-cells, and myeloid-derived suppressor cells (MDSC) of the innate immune system, but also activate the adaptive immune system by their cytokines or receptor-mediated interactions. Just like normal T-cells, NKT cells have T-cell receptors (TCR) and can distinguish self and foreign peptides [14]. However, NKT recognize only a limited collection of specific antigens (mainly lipids) presented on CD1d. Normal T-cells use the classical MHC-I and II receptors and recognize peptides in the groove of these molecules [15]. Though, NKT cells belong to the group of T-cells with their TCR and CD3, they also have features of NK-cells, like specific markers (e.g. NK1.1), NK-receptors, and the way they can kill other cells. NKT development is, equally to NK-cells, dependent on IL-15, Irf-1 and other specific cytokines and transcription factors [16].

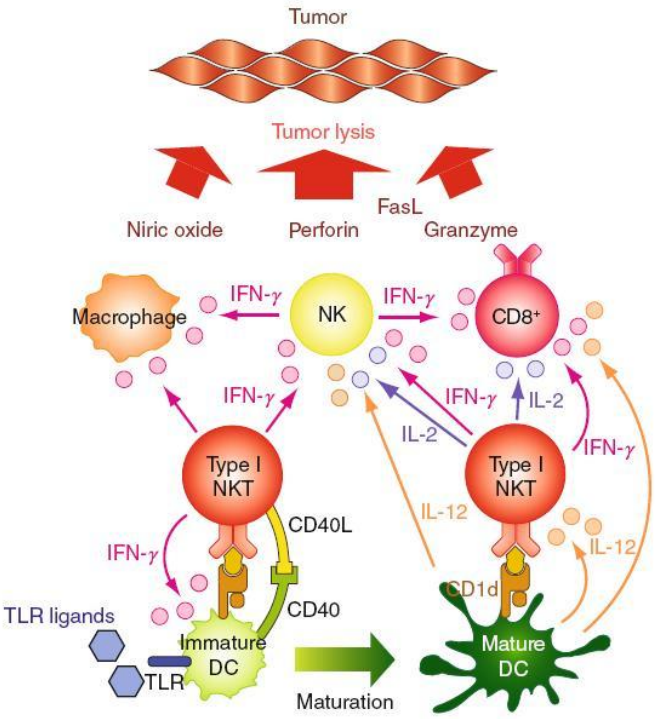

Figure 3: Tumor surveillance by type I NKT cells
Type I NKT cells mainly attack tumor cells indirectly by regulating other immune cells like macrophages, NK-cells, and cytotoxic lymphocytes ($CD8^+$ T-cells). Their actions are depend on the developmental stage of the dendritic cell (DC), which activates the NKT cell.
Figure derived from Terabe et al. (2008)

1.2.1 Tumor surveillance by NKT cells

There are two categories of NKT cells, the type I and type II NKT cells, which form an immunoregulatory axis together [15]. Type I NKT cells, also called classical or invariant

(iNKT) NKT cells, are able to defend against tumors by inducing DCs to produce IL-12, IL-15 and other cytokines and activating NK cells and cytotoxic T-lymphocytes with IFN-γ and IL-2 [14, 17]. Characteristic of these NKT cells is the invariant TCR using Vα24Jα18 (human). Type II NKT cells suppress tumor immunity and have different TCRs [14]. With these TCRs, they recognize lipids presented by CD1d on target cells. Both types NKT cells can be subdivided in $CD4^+$ or $CD4^-CD8^-$ NKT cells and $NK1.1^+$ or $NK1.1^-$ NKT cells [15]. These subsets differ in their capability to protect against tumors [18].

NKT cells play a central role in tumor surveillance. It was shown that NKT cell levels in the peripheral blood are significantly lower in patients with solid tumors [14]. Unless they are able to kill tumor cells directly e.g. by perforin [19], their major task in tumor surveillance is to regulate other effector cells like cytotoxic lymphocytes and NK cells [14, 17] (Figure 3). NKT cells are one of the first responders and steer the Th1 or Th2 polarization of the immune response [20]. Production of IFN-γ by NKT cells directs into a Th1 polarization, IL-4 release supports the Th2 response [14]. The Th1 or Th2 polarization by NKT cells is dependent on the circumstances during NKT cell activation e.g. the affinity between the TCR and the antigen presented by a target cell, the presence of co-stimulatory receptors or the interaction with other immune cells [20]. Strong binding between the NKT cell and the antigen presented by the target cell directs into a Th1 response, mediating tumor surveillance.

1.3 $CD8^+$ T-cells (cytotoxic T-lymphocytes)

$CD8^+$ T-cells belong to the αβ T-cells and are involved in the adaptive immune system. In contrast to NK-cells, $CD8^+$ T-cells need to be activated by DCs before they become cytotoxic T lymphocytes (CTLs) that can execute their killing properties. $CD8^+$ T-cells recognize peptides presented on the MHC class I receptor on cells and establish a MHC-I / TCR-CD3 complex interaction. Only the interaction between MHC-I and the TCR-CD3 complex is not enough to activate the $CD8^+$ T-cell, but also co-stimulatory signals are needed [21]. One of these co-stimulatory signals is the binding of CD80 or CD86 (B7 family) on the DC (or another APC) with CD28 on the $CD8^+$ T-cell and is required for an effective production of cytokines like IL-2. Activation of the $CD8^+$ T-

cell leads to proliferation and differentiation into effector and memory CTLs. Also the CTLs need co-stimulatory signals before they can kill a tumor. This can be provided by e.g. the NKG2D receptor, which binds to MIC expressed by the tumor cell [3]. The NKG2D receptor is also used by NK- and γδ T-cells, showing the importance of this receptor in tumor surveillance (Figure 4).

DCs (APCs) can only activate $CD8^+$ T-cells when they have received a licensing signal, or 'danger signal'. This signal is provided through the stimulation of TLRs (Toll-like receptors) by pathogens or the activation of MHC-II and CD40 (integral membrane protein and member of the TNF-receptor superfamily) by activated $CD4^+$ T-cells [22].

1.3.1 Tumor surveillance by $CD8^+$ T-cells

In the case of cancer, dying tumor cells or debris are taken up by DCs and tumor antigens are cross-presented on MHC class I to $CD8^+$ T-cells. When the DC received a licensing signal, the DC is able to activate the $CD8^+$ T-cell that will then develop into a CTL. These CTLs can kill (tumor) cells in two different ways; via the perforin – granzyme and the Fas – FasL pathways. (Previously described in 1.1.2) As shown in figure 3, cytotoxic T lymphocytes are important effector cells involving tumor cell elimination. They are steered by e.g. NKT cells.

1.4 γδ T-cells

The γδ T-cells, also called innate-like lymphocytes, are involved in combating infectious diseases and have non-redundant capacities in the inhibition of tumor development and progression [23, 24]. Equally to NK and NKT cells, forming a bridge between the innate and adaptive immune systems. They belong to the first responders in an immune reaction and regulate other immune cells by the production of IFN-γ. IFN-γ is an essential cytokine in establishing immunity against tumors [2]. γδ T-cells are the primary source of IFN-γ and are able to enhance the responses and IFN-γ production of $CD4^+$ and $CD8^+$ cells, thereby influencing the Th1 / Th2 balance to protect against tumors [24]. Furthermore, some γδ T-cells act as antigen

presenting cell (APC) and activate $CD4^+$ and $CD8^+$ T-cells [25]. The γδ T-cells are able to take up opsonized cells by CD16 and present the peptides on their MHC-II receptor [26]. There are several categories of γδ T-cells based on different markers. In mice, γδ T-cells are divided by CD27 expression [27]. (CD27 is involved in co-stimulation during- and T-cell activation.) γδ T-cells expressing CD27 are effector cells mainly producing IFN-γ, CD27 negative cells have regulatory features and primarily produce IL-17. In human, γδ T-cells are subdivided by different δ chains; Vδ1 and Vδ2 [23]. Vδ1 expressing cells are mainly present in mucosal tissues and are probably involved in the protection of the epithelia. γδ T-cells containing the Vδ2 chain are found in the circulation. A small fraction of these cells express IL-17 upon stimulation and have a regulatory phenotype.

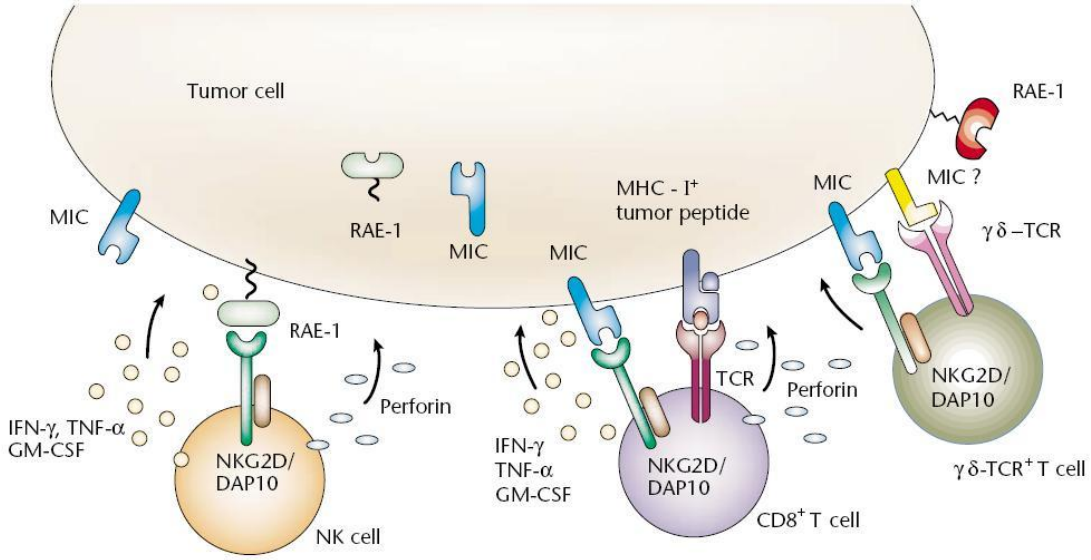

Figure 4: Tumor recognition mediated by the NKG2D receptor
The NKG2D receptor has an important role in tumor recognition. It is expressed by several immune cells like NK-cells, $CD8^+$, and γδ T-cells. The NKG2D ligands (murine) Rae-1 and (human) MIC are often overexpressed by tumor cells. T-cells require NKG2D as a co-stimulatory receptor. For NK-cells, it is the direct trigger for tumor killing.
Figure derived from Lanier et al. (2001)

1.4.1 Tumor surveillance by γδ T-cells

The response of γδ T-cells to tumors is species-specific [23]. γδ T-cells protect against cancer by killing tumor cells directly or by supporting other immune effector cells e.g. by providing IFN-γ [24]. Although cytotoxicity is achieved in the same way as for $CD4^+$ and $CD8^+$ T-cells, the actions of γδ T-cells are largely MHC-independent [23]. γδ T-cells

recognize tumor cells directly by the expression of self-antigens on the membrane, instead of non-self peptides displayed by MHC receptors. Activation of γδ T-cells is mainly mediated by the γδ TCR and NKG2D [3, 28] (Figure 4). Cytotoxicity is often performed with the same receptors as NK-cells, but they kill different tumor cells implying that more receptors or mechanisms are involved. Both γδ T- and NK-cells have the NKG2A, NKG2D, and KIR receptors, recognizing among others Rae-1 and the stress-induced molecules MICA and MICB expressed by tumors [23]. The γδ T-cells discriminate transformed tumor cells from healthy cells by the upregulation of self-antigens like heat shock proteins (HSP) and metabolic intermediates. These proteins can be increased in tumor cells due to a higher metabolism and serve as endogenous danger signals [23, 29].

The γδ TCR is also responsible for initiating immune responses against tumor cells, they recognize phospho-antigens and stimulate proliferation upon antigen engagement [23]. Although, phospho-antigens are recognized without being processed and displayed on MHC receptors, activation of γδ T-cells is APC-dependent. Activated γδ T-cells can exert as APCs and perform self-activation, but this results in limited proliferation [30]. The final elimination of tumor cells by γδ T-cells is done via the Fas – FasL and perforin – granzyme pathway as previously described in 1.1.2.

2. Immune evasion

Tumor development and shaping are influenced by the actions of the immune system, also called cancer immuno-editing [2]. Cancer immuno-editing consist of three phases; elimination, equilibrium, and escape. The elimination phase is also called immunosurveillance and is described in the previous chapters. In the equilibrium phase, the immune system still attacks the tumor cells, however, some tumor cells are not recognized and survive. The escape phase is marked by the expansion of these 'surviving' tumor cells, which are actually selected by the immune system. Tumor development is not only dependent on the limited capacities of the immune system, but is also due to the microenvironment created by a tumor. The way tumors influence their environment and the immune system will be discussed next.

2.1 Lack of recognition

A simple way of immune evasion is the lack of recognition. Tumor cells are not regarded as non-self or dangerous, because they develop from autologous cells. Or tumor cells avoid being recognized by modulating their expression patterns of MHC-I and inhibitory receptors. When tumor cells are not considered to be harmful, they are not attacked by the immune system and are able to expand.

2.1.1 Tumor cells are autologous cells

One important reason for tumor evasion from the immune system is the lack of recognition, which has differential causes. Cells of the immune system are trained to discriminate 'self' from 'non-self', but tumor cells develop from normal cells and are often classified by the immune system as 'self'. In the thymus, immature T-cells only survive and develop into mature T-cells when they sustain the positive and negative selection, meaning they should recognize MHC class I and / or II molecules, but their affinity for the MHC molecules loaded with a 'self antigen' should not be too strong. This selection prevents the reaction to inherent cells (autoimmunity), but also to tumor cells. T-cells in tumor-bearing hosts are frequently anergic due to the expression of 'self' antigens by tumors [31]. This phenomenon is called tumor-induced anergy.

Before initiation of an immune reaction against tumor cells, tumor cells need to be distinguished from normal, healthy cells [32]. Reactions to tumor cells are mediated in two different ways. The immune system reacts to tumor-specific antigens (TSA) or tumor-associated antigens (TAA); molecules exclusively expressed by tumor cells or molecules expressed by tumor cells in another way than normal cells, respectively [32]. Distinct tumor antigens are not always present on tumor cells or their expression is very heterogeneous [33]. Most antigens displayed by tumors are not unique to tumors, but are differentiation markers, which are also present on normal cells [31]. Furthermore, tumor cells show antigenic drift, just like viruses, hindering the recognition by immune effector cells [32, 33]. Tumor antigens differ in quality or immunogenicity; the more immunogenic the tumor antigen, the more efficient the immune reaction [34].

DCs are key players in initiating immune responses; they decide if an adaptive immune reaction is started or not [29, 31]. DCs take up cells and proteins, among others also dying tumor cells and cell debris, break them down and present the peptides on MHC molecules. Adaptive immune reactions, e.g. antigen-specific $CD4^+$ and $CD8^+$ T-cells, are only induced when the T-cells receive the right signals from the DC. They have to recognize the tumor antigen presented by MHC molecules on the DC and require co-stimulation by CD80 or CD86. However, the DC can only activate immune cells, when the DC is licensed to do so. As formerly depicted, the DC is licensed by a danger signal. These danger signals are endogenous signals derived from tissues suffering from abnormal death, stress, and damage or exogenously derived signals from pathogens [29]. Sometimes, this danger signal is not obtained. Additionally, the humoral and cellular immune responses induced by pathogens are much stronger than the responses induced by tumors [35]. Probably because tumor cells are considered to be 'self' and do not provide a danger signal as strong as foreign proteins do.

2.1.2 Recognition prevented by receptor modulation

Another mechanism impairing the recognition of tumor cells is the alteration in MHC expression. Frequently, MHC class I molecules are down-regulated or lost in tumors [33, 36-38]. MHC-I is essential in the $CD8^+$ T-cell mediated recognition and killing of tumor

cells. This escape mechanism is often used by viruses too, but the underlying mechanisms are different. Viruses produce specific proteins to down-regulate MHC-I expression of the host cell, but the immune escape of tumor cells by variations in MHC expression depends on genetic instability [37]. Alterations in MHC expression can be caused by different mutations leading to a lowered expression or even a total loss of the MHC molecules. In some cancers, the frequency of mutations in the HLA-I gene was shown to be almost 100% [37]. Although, loss of MHC-I is not only dependent on mutations in HLA, but may also be caused by defects in proteins involved in antigen processing, like TAP (transporters associated with antigen processing). The occurrence of specific mutations can be tissue-dependent, but some mutations are more universal, e.g. mutations leading to HLA haplotype loss.

Down-regulation or loss of MHC-I impairs $CD8^+$ T-cell functioning, but triggers killing by NK-cells and γδ T-cells. The presence of MHC-I on the cell surface inhibits NK-cell activation upon engagement. When MHC-I is absent, the inhibitory signal is lacking and stimulates, in combination with other signals, NK- and γδ T-cell activation. Activation depends on the net effect of inhibitory and stimulatory signals. When receiving a positive (stimulating) and a negative (inhibiting) signal in a short time, the negative signal overrules the positive signal and prevents killing [38]. Tumors cells use this mechanism to escape from NK- and γδ T-cells by upregulating other, non-classical HLA molecules (HLA-E, HLA-F, and HLA-G), providing inhibitory signals to the NK- and γδ T-cells [37, 38].

2.2 Apoptosis resistance

The recognition of tumor cells by the immune system is not per definition a death signal for the tumor. Tumor cells are still able to survive the attacks, for instance by evolving apoptosis resistance at receptor and intracellular levels. Furthermore, they may kill immune effector cells prior to being eliminated in the same way.

2.2.1 Apoptosis resistance at receptor level

Apoptosis resistance can be performed at different levels. At receptor level, this is mediated by the up- or down-regulation of death receptors e.g. Fas (CD95). Tumor cells often down-regulate their Fas expression, preventing the engagement with FasL

on immune effector cells [39, 40]. The establishment of this binding, leads to the apoptotic cell death of the Fas-expressing cell, in this case the tumor cell. So, down-regulation of Fas reduces the chance of being killed, however, this trick can also be performed the other way around. Tumor cells are able to upregulate their FasL or TRAIL expression and induce apoptosis in activated T-cells by binding to Fas. This mechanism is called 'tumor counterattack', but is quiet controversial [31, 40, 41].

No cancer cells were found with a total loss of Fas, probably because Fas has also some proliferating and growth-stimulating properties [39]. Binding of Fas by low amounts of tumor cell-derived FasL enhances tumor growth. This engagement seems to be essential for tumorigenesis in some cancers.

Another receptor involved in tumor evasion mediated via apoptosis is programmed cell death 1 (PD-1) [31, 42]. PD-1 belongs to the CD28 family and its ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC), belong to the B7 co-stimulatory molecule family and are expressed on some immune cells [42]. The interaction between PD-1 and PD-L1 or PD-L2 inhibits cellular and humoral immunity. PD-L1 binding to PD-1 on T-cells inhibits T-cell development and cytokine production or even induces T-cell apoptosis [42, 43]. PD-1 is upregulated on exhausted T-cells, which are often found in the tumor microenvironment [42]. Tumor cells are able to induce apoptosis in T-cells via PD-L1 / PD-1. In addition, PD-L1 is frequently upregulated by tumor cells [43]. PD-L1-induced apoptosis is mediated by tumor cells itself, but also by APCs in the tumor microenvironment.

2.2.2 Apoptosis resistance at intracellular level

Apoptosis resistance in tumor cells is not only mediated by the expression or down-regulation of specific receptors, but is also caused by changes in intracellular pathways. Tumor cell escape from apoptosis is mediated by upregulation of anti-apoptotic molecules or down-regulation or loss of pro-apoptotic molecules [41]. Cellular FLICE-inhibitory protein (cFLIP) is an anti-apoptotic molecule, which inhibits the initiator of the caspase cascade pro-caspase-8 [33] (Figure 2). There are two types of cFLIP, a long and a short form, which are structurally quite similar to pro-caspase-8, but lack the catalytic subunit [41]. Normally, pro-caspase-8 is recruited (intracellular) to the death-inducing signaling complex (DISC) upon activation of the death

receptors Fas, TNF-R or TRAIL-R. Pro-caspase-8 performs auto-activation and starts the caspase cascade to induce apoptosis. Many tumors have increased levels of cFLIP, which competes with pro-caspase-8 for the interaction with DISC. Binding of cFLIP prevents the activation of the caspase cascade, thereby resisting the T-cell attack via death receptors [41].

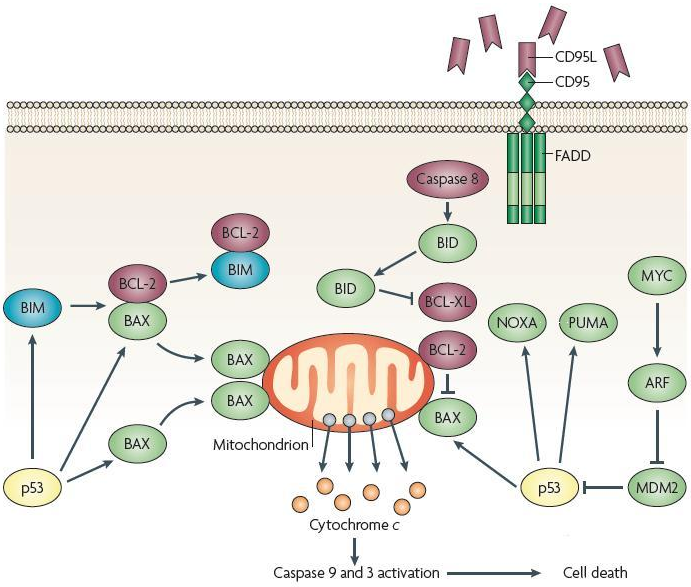

Figure 5: Bcl-2 family
The Bcl-2 family is involved in apoptosis. Bcl-2 inhibits BAX and BAK, which are both inducers of apoptosis. Upregulation of Bcl-2 in tumor cells prevents cell death. P53 induces apoptosis among others by repressing Bcl-2 via BIM and stimulating BAX. Pro-apoptotic mediators: p53, BIM (Bcl-2 interacting mediator of cell death), BAX, BAK, caspases, NOXA (or PMAIP1; phorbol-12-myristate-13-acetate-induced protein 1), PUMA (p53-upregulated modulator of apoptosis), MYC, and ARF (alternative reading frame). Anti-apoptotic mediators: Bcl-2 and MDM2 (mouse double minute 2).
Figure derived from Cotter et al. (2010)

The signaling pathway underlying Fas is also interrupted in tumor cells by the upregulation of anti-apoptotic Bcl-2 (Figure 5). Bcl-2 belongs to the Bcl-2 family consisting of both pro- and anti-apoptotic molecules [40]. This family of proteins is involved in the initiation, but also the maintenance of many tumors due to its anti-apoptotic properties [44]. Bcl-2 avoids apoptosis by repressing BAX (Bcl-2 associated X protein). Normally, apoptosis is induced by BAK (Bcl-2 antagonist / killer) and BAX by permeabilizing the mitochondrial outer membrane, which results in the release of cytochrome c in the cytoplasm. Cytoplasmic cytochrome c activates Apaf-1 (apoptosis protease-activating factor-1), triggering the caspase cascade [44]. Higher

levels of Bcl-2 protects the tumor cell against an immune attack and gives the opportunity to proliferate.

2.3 Deceiving the immune system

Tumor cells utilize the capacities of the immune system to deceive immune effector cells. Soluble molecules acquainted by immune cells are shed by tumor cells and systemically distributed. Elevated levels of these molecules in serum are frequently found in cancer patients. Shedding is used to shift the focus and mask the tumor and / or to eradicate immune cells.

2.3.1 Antigen shedding

Tumor cells deceive the immune system by the shedding of soluble molecules, which are involved in the recognition of tumor cells e.g. HLA, FasL, MIC, and ULBPs. Soluble molecules are produced in two ways; by alternative splicing or by the cleavage of these molecules from tumor cells by metalloproteases (MMPs) [11]. The metalloproteases ADAM10 and ADAM17 (a disintegrin and metalloproteinase) were shown to be involved in the shedding of MICA and ULBP2, ligands for NKG2D [45]. High levels of sMICA (soluble MICA) are found in the sera from cancer patients, indicating that shedding of molecules by tumor cells is a common event. MICA, but also the other NKG2D ligands MICB and ULBPs, are cleaved from the tumor cell membrane, thereby harming the immune actions of NK-cells, $CD8^+$, and γδ T-cells. The soluble ligands can bind to the NKG2D receptor on immune effector cells and impair NKG2D expression and functioning [38]. Furthermore, the expression of these ligands on tumor cells is decreased by shedding [45].

Shedding of molecules avoid the recognition of tumor cells by immune effector cells, but some of these molecules also induce apoptosis in immune cells. FasL is cleaved from tumor cells and trigger immune cells to go into apoptosis by binding to the death receptor Fas [31, 33]. In addition, the death receptor signaling is prevented by the engagement of soluble DcR3 (decoy receptor 3) with FasL. DcR3 competes with Fas for ligand binding and prevents apoptosis in tumor cells [41].

Another soluble receptor which provokes apoptosis in NK-cells and $CD8^+$ T-cells is HLA-I [38]. Usually, HLA-I expression on cells inhibits the activation of NK-cells, $CD8^+$-

and γδ T-cells by engagement with NKG2D. sHLA-I interacts with CD8 on NK-cells and $CD8^+$ T-cells [46]. This interaction stimulates the NK-cell to produce FasL and TGF-β, leading to apoptosis of the NK-cell itself [46, 47]. Although, the co-engagement with other receptors can influence the outcome. The interaction of sHLA-I with CD8 and inhibitory IRS (inhibitory receptor superfamily) leads to the suppression of NK-cell apoptosis and is a survival mechanism for the NK-cell [47]. sHLA-I and CD8 binding in combination with stimulatory IRS induces NK-cell death mediated by FasL and TGF-β.

2.4 Inhibition of immune cell development, proliferation, and maturation

As previously depicted, tumor cells escape from the immune system by avoiding their recognition, being apoptosis resistant, and deceiving the immune system, but tumor cells can also attack the immune system by interfering in the development and proliferation of immune cells. This is performed by the release of indoleamine 2, 3-dioxygenase (IDO) and / or prostaglandin E2 (PGE2) by tumor cells and associated stromal cells or by down-regulation of co-stimulatory signals circumventing the induction of an efficient immune response.

2.4.1 IDO and PGE2

Most human tumors have a continuous expression of indoleamine 2, 3-dioxygenase (IDO) induced by inflammatory cytokines like IFN-γ [48, 49]. IDO is an enzyme involved in the tryptophan catabolism converting tryptophan into *N*-formylkynurenine and is universally present in normal, healthy tissues and different cell types. This key-enzyme is responsible for the first as well as the rate-limiting step in the tryptophan catabolism [33]. Tryptophan is essential for T-cells; low levels of tryptophan thwarts clonal expansion. Increasing the breakdown of tryptophan in the cytosol of tumor cells reduces the transport of tryptophan into the tumor environment and draining lymph nodes [49]. In addition, tumor cells take up the tryptophan in the environment that is left. T-cells are tryptophan-dependent, so their functioning is impaired in a tryptophan-depleted area and forces T-cells to stay in the G1 phase of the cell cycle [49]. Furthermore, intermediates of the tryptophan catabolism induce apoptosis in T-cells [48]. Tumor cells themselves are still able to synthesize proteins and to proliferate.

IDO is also produced by DCs to inhibit T-cell responses and induce Tregs creating a tolerant environment [48].

Cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE2) are upregulated in many tumors [50]. PGE2 is the enzymatic product of COX-2 and plays an important role in immune suppression. It is produced by tumor- and associated stroma cells in the tumor microenvironment and influences several types of immune cells directly [38]. PGE2 inhibits $CD4^+$ and $CD8^+$ T-cell effector functions and proliferation, but stimulates their differentiation into Tr1 cells (regulatory T-cells)[51] [51, 52]. Furthermore, PGE2 influences NK- and γδ T-cell functioning; cytokine production and cytotoxicity is diminished in a PGE2-rich environment [50]. PGE2 binds to the E-prostanoid receptors (EP2 and EP4) on NK- and γδ T-cells resulting in elevated levels of cAMP and PKA activation, which inhibits the major receptors involved by anti-tumor immunity [51]. For NK-cells, this involves CD16 (ADCC), NKG2D, and the natural cytotoxicity receptors (NCR) NKp30, NKp44, and NKp46. In γδ T cells, these are the CD16, NKG2D, and Vγ9Vδ2 TCR receptors.

2.5 Induction of tolerance

Tumor cells create a protective environment to support their own growth and survival. This tumor microenvironment is primarily established by the production of specific immunosuppressive cytokines (e.g. IL-10, TGF-β, and VEGF) and the induction and attraction of suppressor cells like regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs). These inhibitory cytokines and cells form a relatively safe area for tumor cells by the repression of immune responses.

2.5.1 Secretion of immunosuppressive cytokines; IL-10 and TGF-β

Cytokines have a broad range of functions depending on the target cell and environment. Some cytokines both have a promoting as well an inhibiting role in cancer initiation and development. It is difficult to determine their exact functions [38]. One of the cytokines produced by tumor cells and other cells in the tumor microenvironment (mainly MDSCs) is transforming growth factor β (TGF-β). TGF-β is involved in ample homeostatic processes in the whole body, but is also important in creating an immunosuppressive environment for the tumor [53]. In first instance, TGF-β

protect against cancer development, but switches into a cancer promoting cytokine thereby attracting MDSCs which produce lots of pro-oncogenic TGF-β [54]. The underlying mechanisms of this switch are not exactly known. The immunosuppressive environment created by TGF-β is established in several ways. TGF-β inhibits DC migration and maturation, which is essential for inducing a potent immune reaction [53]. DCs are also stimulated to produce TGF-β, enforcing the effects of TGF-β among others by promoting the development of Tregs [53, 55]. Next to inducing immune suppression by Tregs, TGF-β inhibits the production of IFN-γ; the key cytokine in tumor surveillance. TGF-β signaling in NK- and $CD8^+$ T-cells represses T-bet, which is an essential transcription factor in the production of IFN-γ. NK-cells are impaired in their cytolytic activity and NKG2D expression as well, indicating that TGF-β works on different mechanisms involved in tumor killing. $CD8^+$ T-cells are affected in their functioning by attenuation of NKG2D expression and the direct repression of genes related to cytotoxicity like FasL, granzymes, and IFN-γ [53, 54]. Besides, the amount of $CD8^+$ T-cells is diminished by TGF-β due to the direct repression of Bcl-2, which forces the cells into apoptosis.

TGF-β stimulates the production of another cytokine, which is important in the tumor microenvironment: IL-10 [33]. Both are cytokines involved in Th2 directed (immunosuppressive) responses. IL-10 represses the maturation of monocyte-derived DCs, which impairs the development of Th1 responses and avoids in this way the induction of an efficient anti-tumor reaction [56]. Analogous to TGF-β, IL-10 advances the development of Tr1 regulatory T-cells [35, 56]. Furthermore, IL-10 inhibits the expression of co-stimulatory molecules CD80 and CD86, the production of chemokines and cytokines and the expression of MHC-I and MHC-II molecules. Down-regulation of MHC-I is unique for IL-10, other inhibitory cytokines do not affect MHC-I expression [56].

2.5.2 Induction of suppressor cells; Tregs and MDSCs

Immune cell infiltrates in tumors consist of substantial amounts of regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSC) [35]. These antigen-specific regulating cells are attracted by the tumor to create an immunosuppressive environment [55]. Tregs are expanded and guided by tumor-produced TGF-β, IL-10,

PGE2, and CCL22. There they perform immunosuppression by the secretion of TGF-β and IL-10 or via direct cell-cell contact [56, 57]. TGF-β can be membrane-bound as well. There are different types of regulatory T-cells. The natural occurring (n)Tregs ($CD4^+CD25^+Foxp3^+$) which form a distinctive T-cell lineage and the induced subtypes Th3 (T-helper type-3) and Tr1 (T-regulatory lymphocytes type-1), which are originated from peripheral $CD4^+CD25^-$ T-cells [56]. Natural occurring Tregs can be antigen-specific or non-specific. Tr1 cells are induced in the tumor microenvironment by PGE2, TGF-β, or IL-10 and are antigen-specific [35]. Upon activation Tr1 cells mainly produce IL-10, while Th3 cells secrete TGF-β. In general, regulatory T-cells are upregulated in cancer patients and are more potent in suppressing effector cells of the immune system than regulatory T-cells in healthy individuals. They have a high expression of CTLA-4, which is responsible for inhibiting T-cell activation and proliferation [57]. Furthermore, regulatory T-cells repress the functioning of DCs, αβ T-cells, NK- and NKT cells via cell-cell contact [57].

MDSCs consist of a heterogeneous population of immature myeloid cells and myeloid progenitor cells, influencing both innate and adaptive immune cells [31, 58]. They are mainly known as repressors of T-cell functions. Like regulatory T-cells, MDSC frequencies are strongly increased during cancer development [59]. Within the heterogeneous group of MDSCs two subsets can be distinguished, the mononuclear (MO) and polymorphonuclear (PMN) MDSCs. Both have distinct properties and killing mechanisms. It is difficult to determine the exact composition of the MDSC population, because this is highly dependent on, for example, the activating molecules and tumor subtype [60]. The activation and expansion of MDSCs is mediated by different factors [58]. Activation is mainly performed by factors produced by stromal cells of the tumor and activated T-cells e.g. IL-4, TGF-β, and IFN-γ. Expansion is primarily achieved by the tumor cells itself. They produce factors to enhance MDSC expansion and repress maturation of the immature myeloid cells, which normally develop into DCs, macrophages, and granulocytes. MDSC expansion is induced by various factors like IL-6, IL-10, TGF-β, and IFN-γ [58]. Both MDSC activation and expansion are required for their suppressing activities. Immune suppression is performed by direct cell-cell contact. MDSCs repress T-cell functioning by production of iNOS and arginase-1 resulting in impaired T-cell activation and proliferation or

even apoptosis [59]. Also MHC-II expression on T-cells is down-regulated by MDSCs [58]. DC maturation is inhibited, because immature DCs form part of the heterogeneous MDSC population. MDSCs are potent inhibitors of NK-cells. With membrane-bound TGF-β1, NK-cells are suppressed in NKG2D expression, IFN-γ production, and cytotoxicity in general [59]. IFN-γ production is repressed in $CD8^+$ T-cells as well [31]. MDSCs stimulate the development of regulatory T-cells.

3. Myeloid-derived suppressor cells (MDSCs)

As described in the previous chapter, the MDSC population comprises myeloid progenitor cells and immature myeloid cells. Normally, these cells are exclusively present in the bone marrow and lack the repressive functions [58]. Under pathological conditions, e.g. cancer, immature myeloid cells are recruited to secondary lymphoid tissues and tumors and acquire suppressive functions. The recruitment of this specific group of cells attracted my attention, because they do not belong to the ordinary, well-known immune cells, but they do have an essential role in tumor immunity. Several anti-tumor therapies failed due to the immune suppressive environment in tumors mediated by MDSCs, which are one of the most important suppressors in the tumor microenvironment (TME) [58]. This chapter will be focused on the inhibitory mechanisms performed by MDSCs.

3.1 Arginase-1 and iNOS

MDSCs have a high expression of arginase-1 and induced nitric-oxide synthase (iNOS or NOS2). Both enzymes use the same substrate L-arginine and convert it into respectively L-ornithine and urea, and nitric oxide (NO) and L-citrulline [61]. To compensate for the intracellular turnover of L-arginine, cationic amino-acid transporters (CAT) pump extracellular L-arginine into the cell. The CATs on MDSCs are the major transporters of L-arginine and deprive the extracellular environment from L-arginine [61]. The turnover of L-arginine by MDSCs affects T-cells in different ways. In the first place, environmental depletion of L-arginine represses the expression of the ζ-chain of the CD3 T-cell receptor. The ζ-chain is an essential compound in the formation of the T-cell receptor. T-cells internalize the TCR after antigen stimulation. Normally, the TCR is re-expressed and used to recognize and attack cells expressing the specific antigen. The lack of L-arginine prevents the re-expression of the TCR, leading to T-cell dysfunction. How this is established is not yet known.

Environmental deprivation of L-arginine induces a cell cycle arrest in T-cells as well, leading to T-cell anergy [62]. Cell cycle progression is dependent on the binding between cyclins and cyclin-dependent kinases (Cdk). During the cell cycle, cyclins are synthesized and degradated, and also the Cdk activity fluctuates. A lack of L-arginine makes the T-cell incapable of increasing the cyclin D3 and Cdk4 levels and impairs the phosphorylation of the retinoblastoma (Rb) protein. Unphosphorylated Rb arrests the transcription factor E2F, which normally translocates to the nucleus and is responsible for initiating the transcription of genes involved in the S-phase. Finally, the T-cell resides in the G_0-G_1 phase.

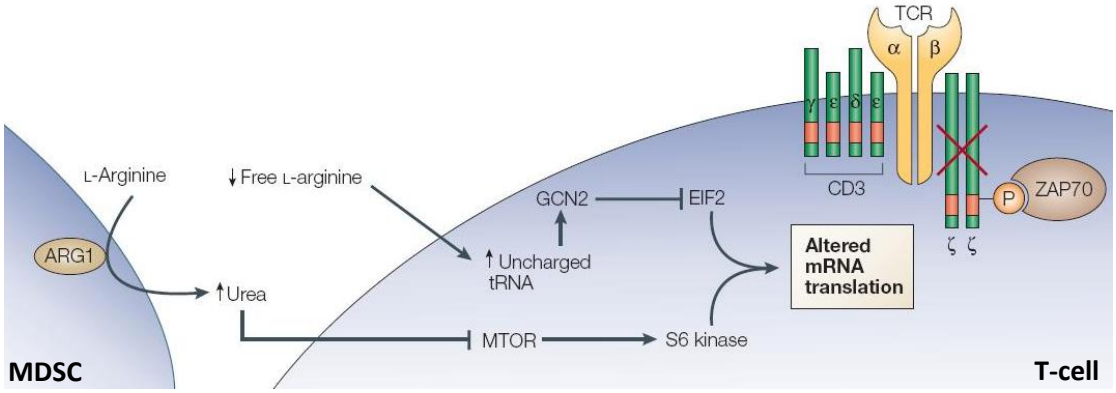

Figure 6: T-cell repression by MDSC is mediated by arginase
MDSCs repress T-cell via arginase in several ways. High arginase activity in the MDSC deprives the environment of L-arginine. Less arginine decrease the amount of loaded tRNAs, which activates GCN2. Amino acid shortage is an unfavorable condition for cell growth, so T-cell proliferation is halted via inhibition of EIF2; a protein involved in the initiation of translation. One of the end products obtained by L-ariginine conversion is urea. Urea represses mTOR signaling and S6 kinase to stop translation.
Figure derived from Bronte et al. (2005)

Another way of T-cell suppression by arginase-1 is mediated by both the deprivation of L-arginine and the production and release of urea in the environment [61] (Figure 6). These two actions have modifying effects on mRNA translation in adjacent cells, in this case T-cells. Less L-arginine in the environment leads to less L-arginine in the T-cell. Amino-acids in the cytosol are bound by tRNA and guided to the ribosomes for translation. L-arginine scarcity causes relatively more unloaded tRNA molecules, which induces the binding and activation of General Control Non-repressible 2 (GCN2). In this manner, amino-acid shortage is measured and cell proliferation needs to be halted. GCN2 phosphorylates eukaryotic translation initiation factor 2α (EIF2α)

inhibiting translation initiation. Another pathway involved in T-cell suppression is the mTOR (mammalian target of Rapamycin) pathway. The lack of L-arginine or the raised urea levels represses the mTOR pathway in T-cells preventing the activation of S6 kinase in ribosomes. This action also avoids the initiation of translation, which restrains T-cell proliferation.

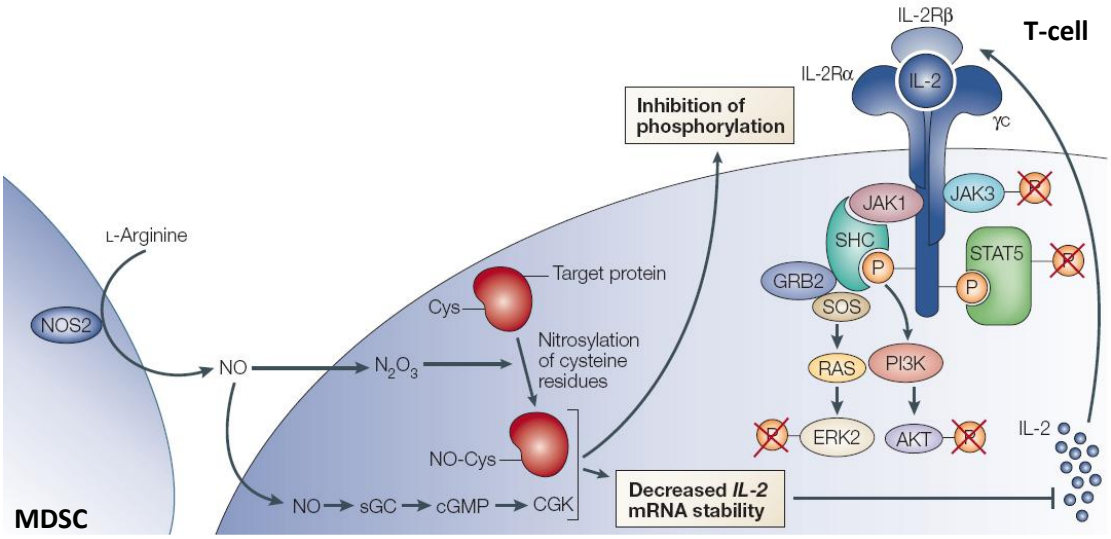

Figure 7: T-cell repression by MDSC is mediated by iNOS (or NOS2)
T-cell functioning is repressed by NO produced by the iNOS-induced conversion of L-arginine. NO nitrosylates proteins in the T-cell and activates CGK. Both inhibit the phosphorylation of mediators of the JAK/STAT signaling pathway underlying the IL-2 receptor. Furthermore, IL-2 release by the T-cell is impaired. Diminished IL-2 levels and intracellular signaling prevents T-cell proliferation.
Derived from Bronte et al. (2005)

Nitric oxide (NO), the end product of iNOS-mediated conversion of L-arginine, affects T-cell functioning by interruption of the signaling pathway underlying the IL-2 receptor [61] (Figure 7). Engagement of IL-2 with the IL-2 receptor is essential for T-cell growth and differentiation. NO performs S-nitrosylation of intracellular proteins. This post-translational modification impairs the functioning of the proteins involved in the signaling pathway following activation of the IL-2 receptor. NO also stimulates soluble guanylate cyclase (GC) leading to the activation of cGMP-dependent protein kinase (CGK). CGK inhibits IL-2 release and T-cell proliferation. Both the S-nitrosylation of proteins and CGK activation prevents the phosphorylation of proteins in the Jak/STAT signaling pathway downstream the IL-2 receptor. Furthermore, IL-2 mRNA is destabilized leading to less IL-2 release.

Tumor cells themselves are not severely affected by arginase-1 and iNOS produced by MDSCs. The enzymes interfere, directly and indirectly, with essential T-cell processes, preventing a potent immune response to tumor cells. Furthermore, tumor cells benefit from arginase-1 because of the L-ornithine production. L-ornithine can be converted to polyamines, which are used for growth and differentiation of tumor cells [61].

3.2 Reactive oxygen species (ROS)

Reactive oxygen species are shown to be upregulated in MDSCs of tumor-bearing hosts [63, 64]. They mediate some suppressing mechanisms and prevent the maturation of MDSCs. ROS are produced in several ways, for example by NADPH oxidase (NOX2), which converts oxygen (O_2) into superoxide anions ($\cdot O_2^-$), but also by arginase (see figure 8). In MDSCs, two important subunits of NOX2, $p47^{phox}$ and $gp91^{phox}$, are upregulated and increase the NOX2 activity. Tumor cells are thought to expand these MDSCs with more active NOX2 by tumor-derived factors triggering the Jak/STAT3 signaling pathway [63]. As mentioned before, arginase levels are upregulated in MDSCs, increasing the ROS levels as well. Arginase and iNOS compete for L-arginine. Conversion of L-arginine by iNOS can result in NO and L-citrulline or in (instable) superoxide anions, which are converted in H_2O_2. The end product produced by iNOS is dependent on the L-arginine levels and arginase activity [65]. High levels of arginase activity leads to low levels of L-arginine and results in superoxide anion and H_2O_2 production by iNOS. Saturation of iNOS by L-arginine results in NO production. Thus, arginase and iNOS have both direct and indirect (via ROS production) suppressing effects.

It was shown that MDSCs repression of $CD8^+$ T-cells is mediated by ROS [64]. MDSCs engage with $CD8^+$ T-cells via the MHC-I receptor expressing specific antigens and is supported by integrins. This close interaction is needed, because ROS act within a small distance due to their short half-life in which they are very reactive [66]. The binding of the integrins CD11b, CD18, and CD29 on MDSCs with their ligands on $CD8^+$ T-cells increases the ROS production in MDSCs. ROS are small molecules, which can cross the cell-membrane and can damage DNA and mitochondria leading to

apoptosis by the release of cytochrome c and the activation of the caspase cascade. ROS also induce apoptosis by down-regulation of the anti-apoptotic Bcl-2 and upregulation of the pro-apoptotic BAD (Bcl-2-associated death promoter) and BAX (Bcl-2-associated X protein) [67, 68]. In relation to $CD8^+$ T-cells, they primarily induce antigen-specific tolerance [64]. The inhibition of T-cell functioning by MDSCs via ROS occurs only in the peripheral lymphoid organs of tumor-bearing hosts [69]. In the tumor microenvironment, T-cell suppression by MDSCs is performed via arginase and iNOS, but does not require antigen-specificity. These MDSCs mature into tumor-associated macrophages (TAM) due to hypoxia-inducible factor 1α (HIF-1α).

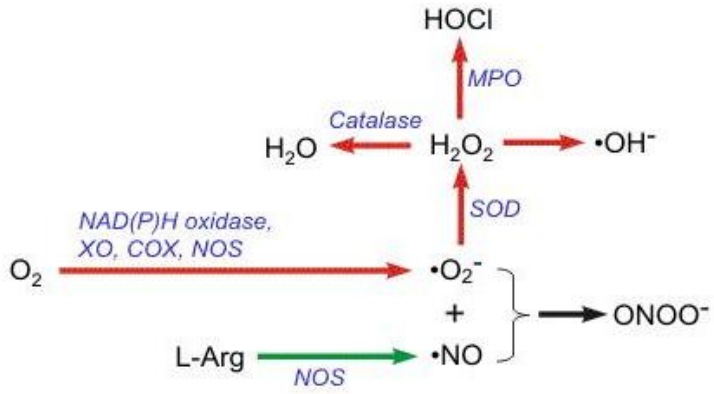

Figure 8: Reactive oxygen species (ROS)
ROS production is an important player in the suppressing mechanisms of MDSCs. NADPH oxidase and arginase can form superoxide anions ($\cdot O_2^-$). iNOS produces NO by converting L-arginine. A reaction between superoxide anions and NO results in peroxynitrite ($ONOO^-$). Figure derived from http://www.cvphysiology.com/Blood%20Flow/BP016%20ROS%20formation.gif

Peroxynitrites ($ONOO^-$) belong to the reactive nitrogen and oxygen species (RNOS) and are formed by the reaction between a superoxide anion and NO derived from e.g. arginine and iNOS [61] (Figure 8). Their effects are mediated via the nitration and nitrosylation of amino acids [58]. Peroxynitrites are released by MDSCs, repressing extracellularly and intracellularly the functions of the adjacent T-cell. MDSCs present specific antigens on MHC-I and interact with T-cells via the TCR and CD8. Upon this engagement, MDSCs produce peroxynitrate (and ROS), which nitrates / nitrosylates the T-cell receptors. Nitration makes the receptors more rigid and prevents the T-cell to respond to the specific antigen any longer [66]. Nitration of proteins also affects cell functioning, for example by depressing enzyme activity or cell proliferation, or even induces apoptosis [61].

3.3 Interaction of MDSCs with regulatory T-cells (Tregs)

MDSCs can provoke and maintain T-cell anergy via regulatory T-cells [70]. Tregs are produced by converting $CD4^+CD25^-$ T-cells into $CD4^+CD25^+$ T regulatory cells or by inducing the expansion of current tumor-specific Tregs. MDSCs act as antigen presenting cells by taking up and presenting tumor antigens to tumor-specific T-cells. The expansion of Tregs is arginase-dependent, but independent of iNOS. Furthermore, MDSCs require the IL-4Rα, which they obtain during their development into suppressive MDSCs. Huang *et al.* showed in mice that only monocytic ($Gr\text{-}1^+CD11b^+CD115^+$) MDSCs induce the development of tumor-specific forkhead / winged helix transcription factor ($Foxp3^+$) Tregs [71]. The induction of $Foxp3^+$ Tregs by MDSCs requires IL-10 and IFN-γ. These cytokines enhance the expression of MHC-II and co-stimulatory ligands, e.g. PD-L1 and CD86, favoring the interaction and activation of $Foxp3^+$ Tregs [71]. Recently, it was shown that the expansion of tumor-specific $Foxp3^+$ Tregs by MDSCs is dependent on the engagement between CD40L and CD40 [72]. It is suggested that $Foxp3^+$ Treg induction by MDSCs occurs in the following manner. First, MDSCs need to be stimulated with IFN-γ produced by activated T-cells. IFN-γ increases the production and expression of IL-10, TGF-β, and CD40 on MDSCs. CD40 (on MDSCs) interacts with CD40L on $Foxp3^+$ Tregs. In combination with Il-10 and TGF-β stimulation, this interaction results in the activation and expansion of $Foxp3^+$ Tregs [72]. MDSCs can also provoke the expansion of Tr1 regulatory T-cells [73]. In contrast to the induction of $Foxp3^+$ Tregs, Tr1 regulatory T-cell activation and expansion is arginase independent, but they require direct cell-cell contact as well.

Discussion and concluding remarks

Tumor surveillance and immune evasion are both complicated processes mediated by a variety of cells. Ample research is performed to elucidate the underlying mechanisms, however, single cell types have frequently more than one task in the whole process. They can do their job in different ways, like NK-cells who kill tumor cells by death-receptor-dependent pathways, the perforin / granzyme pathway, and by antibody-dependent cell-mediated cytotoxicity (ADCC). Cell behavior is highly dependent on the environmental circumstances. Myeloid cells attracted by tumor cells become highly immunosuppressive and arrested in their maturation, while in healthy individuals myeloid cells lack the immunosuppressive properties and mature into DCs, granulocytes, and macrophages.

Tumor surveillance is primarily achieved by NK-cells, NKT-cells, $CD8^+$ T-cells, and γδ T-cells. These cells, except $CD8^+$ T-cells, are members of the innate immune system and function without being activated by other cells. This makes them rapid responders and probably helps to remove potential tumor cells before a big and massive adaptive immune response has to be initiated. NK-cells, NKT-cells, and γδ T-cells serve as a bridge between the innate and adaptive immune systems implying the requirement of both systems to recognize, attack and kill tumor cells. DCs are not explicitly discussed in this thesis, but they are the best known connection among the innate and adaptive immune systems and do play important roles in the immunity as well as tolerance induction to tumors. They can initiate an adaptive immune response by presenting tumor-specific antigens and providing co-stimulation to $CD4^+$ and $CD8^+$ T-cells. An adaptive immune response is accompanied by the development of memory cells, which lack in the innate immune system.

The main cytokine in tumor surveillance is IFN-γ, which is produced or used by all before mentioned mediators of tumor surveillance. IFN-γ enforces immune responses into the Th1 direction. In the immune system it is all about the balances between antagonizing cells and / or cytokines. Also the equilibrium of Th1 / Th2 responses. Th1 is essential in tumor surveillance, Th2 reactions induce an immunosuppressive environment and favors tumor escape.

Obviously, MDSCs are also activated by IFN-γ. MDSCs are key players in tumor evasion, but are stimulated by the most important cytokine in tumor surveillance. Greifenberg *et al.* (2009) showed (*in vitro* and *in vivo*) that myeloid precursor cells only developed into MDSCs when stimulated by both IFN-γ and lipopolysaccharides (LPS). The presence of both IFN-γ (produced during an adaptive immune response) and LPS (immunogenic component of bacteria) is an indication for sepsis or an chronic infection [74]. In this context, MDSCs are needed to suppress the ongoing inflammatory reaction, preventing immune cells to cause damage to the host. It is not very likely that MDSCs, activated and recruited in tumor-bearing individuals, are stimulated by both IFN-γ and LPS. LPS is a component of the bacterial cell wall and not present in tumors, but the cooperation of IFN-γ with another cytokine to induce MDSCs is possible. To speculate; a potential cytokine is TGF-β. TGF-β has immunosuppressive properties and activates MDSCs, too [58].

Tumor immune evasion is, just like tumor surveillance, performed in several ways e.g. by the lack of recognition, apoptosis resistance, deceiving the immune system, inhibition of immune cell development and proliferation, and induction of tolerance. Tumor cells are potent manipulators of their environment, creating a 'safe' area to proliferate. The main mechanism responsible for tumor immune evasion, as well as the general ineffectiveness of immunotherapy, is immune suppression. Immune suppression is executed by tumor cells itself, but also via other cells induced by tumor cells. Important players are the regulatory T-cells and MDSCs in cooperation with the (Th2 directed) cytokines IL-10 and TGF-β. Regulatory T-cells, IL-10, and TGF-β are involved in immune suppression and prevention of autoimmunity in healthy individuals. However, MDSCs are specifically recruited in (immune-)pathological conditions like cancer and acquire an immunosuppressive phenotype [58]. They mainly restrain T-cell functioning in different manners; by arginase, iNOS, ROS, and the induction of regulatory T-cells. All are used to impair T-cell proliferation, induce T-cell anergy or apoptosis. Inhibition of T-cells impairs a considerable part of the anti-tumor immunity. For example, $CD4^+$ T-cells cannot activate and regulate other immune cells (e.g. B-cells) to attack tumor cells. $CD8^+$ T-cells, which are essential effector cells, cannot fulfill their killing properties.

Co-evolution of both immune and tumor cells for a long period of time is responsible for the complicated interplay between these cells nowadays. Each action from the immune system or tumor cells evokes a counterattack from the other. Both systems are able to adapt to changing circumstances; the immune system in a controlled manner and tumor cells in a more random way by mutations. However, the establishment of tumor microenvironment (TME) and the recruitment of immune suppressing cells seem to be well-developed safety-mechanisms engineered by the tumor. As depicted in this thesis, the immune system has multiple mechanisms to tackle tumor cells and the tumor has evolved many ways to evade from the immune system. Both systems work on several levels. I foresee that future cancer therapy requires a multi-target approach i.e. a therapy should not be focused on a single protein or cell type, but should be a combination of medications working on different levels. Think of combining anti-tumor therapies with the (local) support of immune effector cells with Th1-directed cytokines (e.g. IFN-γ). Furthermore, it would be useful to have therapies that disrupt the tumor microenvironment, which is dominated and manipulated by the tumor. The TME promotes tumor growth and protects against attacks from the immune system and probably against attacks mediated by medications. In primary, accessible tumors it would be beneficial to work locally and interrupt the TME e.g. by suppressing regulatory T-cells or MDSCs. When treating metastases, you have to work systemically. In that case, it is more difficult to infiltrate the TME. Additionally, systemic down-regulation of regulatory T-cells or MDSCs can have serious side effects. These immune suppressing cells are needed to stop immune reactions and to protect against autoimmunity. My recommendation for treating metastases would be to inhibit either regulatory T-cells or MDSCs. For example, by administering granulocyte macrophage colony stimulating factor (GM-CSF) which inhibits the development of MDSCs and stimulates the expansion of granulocytes and macrophages; two beneficial effects in anti-cancer therapy. GM-CSF is already used in immunotherapy and is promising.

Taken together; in cancer therapy, it is useful to know about the abilities of the immune system how to attack tumor cells and to have knowledge about the tumor evasion mechanisms. Both are required for developing a successful, multi-level and multi-target anti-cancer therapy.

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