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Immunological Checkpoint Blockade and TLR Stimulation for Improved Cancer Therapy

SARA MANGSBO

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Abstract
Mangsbo, S. 2009. Immunological Checkpoint Blockade and TLR Stimulation for Improved Cancer Therapy. (TLR-stimulering och CTLA-4 samt PD-1 blockad för förbättrad cancerterapi). Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 506. 84 pp. Uppsala. ISBN 978-91-554-7675-5.

This thesis concerns the investigation of novel immunotherapies for cancer eradication. CpG therapy was used in order to target antigen-presenting cells (APCs), facilitating antigen presentation and activation of T cells. Blockade of the two major immune checkpoint regulators (CTLA-4 and PD-1) was also studied to ensure proper and sustained T cell activation. The therapies were investigated alone and compared to BCG, the standard immunotherapy in the clinic today for bladder cancer. In addition, CpG as well as BCG was combined with CTLA-4 or PD-1 blockade to examine if the combination could improve therapy.

Single and combination strategies were assessed in an experimental bladder cancer model. In addition, one of the therapies (local aCTLA-4 administration) was evaluated in an experimental pancreatic cancer model. To be able to study the effects of CpG in humans, a human whole blood loop system has been used. This allowed us to dissect the potential interplay between CpG and complement.

CpG was found to be superior to the conventional therapy, BCG, in our experimental model and T cells were required in order for effective therapy to occur. Used as a monotherapy, CTLA-4 blockade but not PD-1 blockade, prolonged survival of mice. When CTLA-4 or PD-1 blockade was combined with CpG, survival was enhanced and elevated levels of activated T cells were found in treated mice. In addition, Treg levels were decreased in the tumor area compared to tumors in control treated mice. CTLA-4 blockade was also effective when administrated locally, in proximity to the tumor. Compared to systemic CTLA-4 blockade, local administration gave less adverse events and sustained therapeutic success.

When CpG was investigated in a human whole blood loop system it was found to tightly interact with complement proteins. This is an interesting finding which warrants further investigation into the role of TLRs in complement biology. Tumor therapy could be affected either negatively or positively by this interaction.

The results presented herein are a foundation for incorporating these combination therapies into the clinic, specifically for bladder cancer but in a broader perspective, also for other solid tumors such as pancreatic cancer.

Keywords: CpG ODNs, TLR-9, CTLA-4, PD-1, PD-L1, B7. H1, immunotherapy, checkpoint blockade, bladder cancer, cancer, complement, TLRs, Compstatin, properdin, C3, experimental animal model, whole blood loop system, combination therapies

Sara Mangsbo, Clinical Immunology, Rudbecklaboratoriet, Uppsala University, SE-75185 Uppsala, Sweden

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“I have not failed. I’ve just found 10 000 ways that won’t work.”
Thomas Edison

Till min familj och mina vänner

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Mangsbo, S.M.*, Ninalga, C.*, Essand, M., Loskog, A., Tötterman, T.H. CpG therapy is superior to BCG in an orthotopic bladder cancer model and generates CD4+ T cell immunity. J Immunotherapy 2008, 31(1):34-42.

II Mangsbo, S.M., Sandin, L., Anger, K., Korman, A., Loskog, A., Tötterman, T.H. Enhanced tumor eradication by combining CTLA-4 or PD-1 blockade with CpG therapy. Accepted J Immunotherapy.

III Sandin, L.*, Mangsbo, S.M.*, Feinstein, R., Loskog, A., Tötterman, T.H. *In situ* CTLA-4 blockade mediates regression of local and distant tumors with minimal side effects. Manuscript.

IV Mangsbo, S.M., Sanchez, J., Anger, K., Lambris, J.D., Nilsson-Ekdahl, K., Loskog, A., Nilsson, B., Tötterman, T.H. (2009). Complement activation by CpG in a human whole blood loop system: Mechanisms and immunomodulatory effects. J Immunology 2009, 183(10):6724-32.

*The authors contributed equally

Preface

Why tumor immunology and immunotherapy? As an undergraduate we learn about cancer progression, the multiple steps that are required for tumor development and how we can treat these malignancies. We learn that tumors do not arise only after one mutation, but that several steps are required for tumor formation and that blood supply and nutrition requirements are essential for tumor cells to survive. We learn that there are different molecular pathways that prevent tumor formation by inducing apoptosis or repairing DNA strands correctly. However, theories regarding tumor immunosurveillance and the relevant checkpoint pathways involved in fine-tuning the immune response are not frequently discussed, at least not during my three first years as a biomedical student. That immune cells survey our body to defend us against pathogens is a concept that is broadly accepted. That immune surveillance protects us against pre-malignant cells is less accepted, at least in areas outside immunology; however it may soon become as an appreciated tool in controlling and perhaps even eradicating tumors, as vaccination is for the controlling viral infections. Today, many oncologists use immunotherapy, but not out of awareness that immune activation will occur, but rather as a means to shrink the tumor by cytotoxic drugs. Radiation and chemotherapy induce apoptosis as well as antigen release in the tumor vicinity allowing for danger signals to arise, activating APCs and subsequently T cells that can control tumor growth. By combining the knowledge and know-how of clinically applied cancer therapies with the newly developed immunotherapies we should witness improved therapeutic success providing that the obstacles of autoimmunity can be controlled. Bladder cancer has been treated with immunotherapy since the early 1970s. Yet, there is room for improvement of the standard immunotherapy for bladder cancer, and this thesis work was performed in order to set the foundation for new clinical methods that should be implemented in the clinic. The work can be regarded as a general tool to target various solid tumors, although the focus has been mainly on bladder and pancreatic cancer. As vaccination can be applied to different infections, with certain limitations, immunotherapy can target various cancer forms. The goal is to achieve an immunogenic environment allowing for specific targeting of the tumor. Behold the next generation of cancer drugs; it may be help from your own cells that might do the trick for you!

Contents

Abbreviations

ADM	AIDS-defining malignancies
AP	Alternative pathway
APC	Antigen presenting cell
BCG	Bacillus Calmette-Guérin
BCR	B cell receptor
cART	Combination antiretroviral therapy
C3	Complement component 3
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CpG	Cytosine-guanosine nucleotide
CR	Complete response
CRP	C-reactive protein
CT	Computer tomography
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
DAF	Decay-accelerating factor
DCs	Dendritic cells
ER	Endoplasmic reticulum
FLT3	FMS-related tyrosine kinase 3 ligand
GM-CSF	Granulocyte monocyte colony stimulating factor
HSC	Hematopoietic stem cells
HY	Male Y antigen
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
IP	Immunoproteasome
IFN	Interferon
IFNR	Interferon receptor
KO	Knockout
LLR	Leucin-rich repeat
LMP	Low molecular weight protein
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MB49	Mouse Bladder-49
MBL	Mannose binding lectin
MDSC	Myeloid-derived suppressor cell

MHC	Major histocompatibility complex
NK	Natural killer
NKT	Natural killer T
nADM	Non-AIDS-defining malignancies
PAMP	Pathogen-associated molecular pattern
PD	Progressive disease
PD-1/L1	Programmed death receptor-1/ ligand-1
PPR	Pattern-recognition receptor
PR	Partial response
ROS	Reactive oxygen species
SD	Stable disease
TAP	Transporter associated with antigen processing
TCR	T cell receptor
Tg	Transgenic
Th	T helper
TILs	Tumor infiltrating lymphocytes
TLR	Toll-like receptor
TGF	Transforming growth factor
Treg	T regulatory cell
TRP	Tyrosinase related protein
WBC	White blood cells

INTRODUCTION

INTRODUCTION

1. Introduction

Around 500 million years ago the adaptive immune system, as we know it, with clonally expanding B and T cells appeared for the first time. An alternative branch of adaptive immunity also developed with an intrinsic capacity to rearrange genes. Leucine-rich repeats (LRRs) have been found to rearrange in jawless fish, allowing for an assembly of variable receptors that can recognize specific molecular patterns [1]. The innate immune system in vertebrates also contains pathogen-recognition receptors (PRRs) with the capacity to recognize a spectrum of pathogen-associated molecular patterns (PAMPs). These PAMPs are germ-line encoded and cannot rearrange as the LLRs in jawless fish, thus there is a limitation to the number of PAMPs recognized. Utilizing PAMPs as agonists for immune activation was initially applied without realizing which ingredients were actually immunostimulatory. Bacillus-Calmette Guérin (BCG) was developed as a vaccine, but was also used in cancer immunotherapy. When Tokunaga and coworkers discovered that the most immunogenic portion of the BCG vaccine was the DNA component and later Krieg and colleagues found that synthetic single-stranded unmethylated DNA sequences containing CG motifs were immunogenic, the refinement of BCG therapy began. This thesis work is in line with this theme, utilizing cytosine-guanosine nucleotide (CpG) oligos as a means to attract and activate tumor infiltrating immune cells in order to counteract tumor growth. However, recent advances in immunology also point out the importance of counteracting suppressor pathways that prevent activated cells from performing their function. Thus, in order to circumvent this immunosuppression, a set of antibodies that block immunoregulatory pathways have been investigated. The first section of this thesis will go through basic immunology, laying the foundation for a deeper introduction into the areas of immunostimulatory ligands, such as CpG, and complement biology in the context of toll-like receptor (TLR) signaling and cancer therapy. Further, two important pathways involved in immune regulation will be addressed. Since an experimental bladder cancer model was utilized, the topic of bladder cancer will also be presented. Too a lesser extent, a pancreatic model was studied but will not be discussed in this thesis.

1.1 Innate Immunity

Innate immunity is our first immunological shield against organisms and other foreign material that may cause us harm. The rapid response that is the trademark of innate immunity is also unspecific and serves as a bridge to the adaptive arm of our immune system. The anatomical barrier such as skin, mucosal layers, gut pH and our own body temperature must first be penetrated by the pathogen. Organisms that do break our anatomical barriers encounter chemical factors such as defensins, interferons (IFNs), complement serum proteins and chemokines such as anaphylatoxins formed after complement activation and that attract other cell types. Other substances released during an inflammation are leukotrienes and histamines. Foreign bodies, such as metal or stone dust, are also recognized by the coagulation and complement cascade systems. Binding of Factor XII to the surface leads to activation of coagulation via the intrinsic pathway and deposits of C3b initiate the alternative pathway (AP) of the complement cascade.

Innate immune cells attracted to the site of inflammation are leukocytes or white blood cells (WBC) and they are composed of: Phagocytes, consisting of macrophages, dendritic cells (DCs) and neutrophils that circulate in our body and will, upon pathogen encounter, take up the foreign substance/pathogen and release various substances such as reactive oxygen species (ROS) causing destruction of the intruder. Monocytes are present in the blood and have the capacity to differentiate into various types of macrophages depending on the organ/tissue site. Dendritic cells reside in the tissue and are of great importance in the antigen presentation process. Neutrophils circulate in the blood as the most abundant leukocyte and are the first to arrive at the site of inflammation.

Lymphocytes consist of B cells, T cells (part of the adaptive immune system), natural killer (NK) cells, γδT cells and natural killer T (NKT) cells. NK cells sense missing-self i.e. the lack of major histocompatibility complex I (MHC-I) on the cell surface, and destroy the cell by secretion of granzymes and perforin that cause apoptosis. MHC class I downregulation can be caused by increased viral load or tumor formation. γδT cells and NKT cells can bridge innate and adaptive immunity by producing cytokines that direct the adaptive responses toward either T helper 1 (Th1), Th2, T regulatory (Treg) or Th17 responses.

Other cells of the innate immune system are granulocytes consisting of neutrophils, basophils and eosinophils. The two latter combat parasites, basophils by releasing histamine and eosinophils by releasing toxic substances and free radicals both of which can cause asthma and tissue damage during an attack. Mast cells, which bare many similarities to basophils, reside in connective tissue or mucosal membranes and will upon activation via immunoglobulin (Ig)E crosslinking, degranulate and release histamine, heparin, hormones and chemokines causing vasodilation and cell migration. Although

mast cells are necessary to combat pathogens, dysregulation of the degranulation process leads to allergy.

1.2 Adaptive Immunity

As evolution progressed, adaptive immunity evolved and consists of cells with antigen-specific receptors capable of somatic hypermutation (B lymphocytes) and rearrangement (B and T lymphocytes). The receptors can be secreted (B cells, humoral immunity) or surface bound, and they specifically recognize and "remember" an antigen (cellular immunity). The system is tightly controlled by both central and peripheral tolerance to prevent self-reactivity. B and T cells are produced from stem cells in the bone marrow and while immature T cells migrate to the thymus for further education/maturation, B cells mature in the bone marrow. B cells patrol our body and migrate to germinal centers located in secondary lymphatic tissue when they have encountered an antigen that stimulates their B cell receptor (BCR). After education/maturation in the thymus, naïve T cells also enter the bloodstream and lymph nodes where they encounter their target antigen.

1.2.1 B lymphocytes

B cells are initially named pro-B cells and during this stage rearrangement starts. When successful rearrangement has been performed and IgM is expressed the cell leaves the bone marrow and migrates to lymph nodes for further maturation. When a B cell develops a too high affinity receptor, death is induced by T helper cells. B cells proceed to somatic hypermutation and class switch to increase the antibody diversity as well as affinity to antigens (B cell development reviewed in [2]).

B cells express TLR-9, possess APC properties and CpG stimulation has a great maturation impact on B cells. Class switch and IgG secretion with a Th1 profile is greatly enhanced upon TLR-9 ligation [3]. CpG as immunotherapy will be discussed more thoroughly in Chapter 3. TLR-9 signaling in B cells can also cause dysregulated inflammation. This is thought to be mediated by unmethylated DNA that forms complexes with anti-DNA antibodies resulting in autoimmunity [4].

1.2.2 T lymphocytes

T lymphocytes can direct our adaptive immunity by receptor interaction and cytokine production. Phenotypically, T cells are described to express the T cell receptor (TCR), CD3 and CD4 or CD8 to amplify the TCR signal. T lymphocytes consist of subtypes and subclasses and the two major classes are CD4+ T helper cells (Th) and CD8+ cytotoxic T lymphocytes (CTLs).

Th cells are currently divided into four branches: Th1, Th2, Tregs and Th17. There is also a subgroup called the follicular helper T (Tfh) cells that regulate B cell development. However, there is still a debate whether this is a separate lineage or not [5]. Also, a new Th9 lineage has been proposed but the incorporation of this cell type into the branch tree is not yet completed [6, 7]. This could potentially be a subgroup of the Th2 lineage. In Figure 1, the Th cell plasticity and the cytokines responsible for the commitment process are shown.

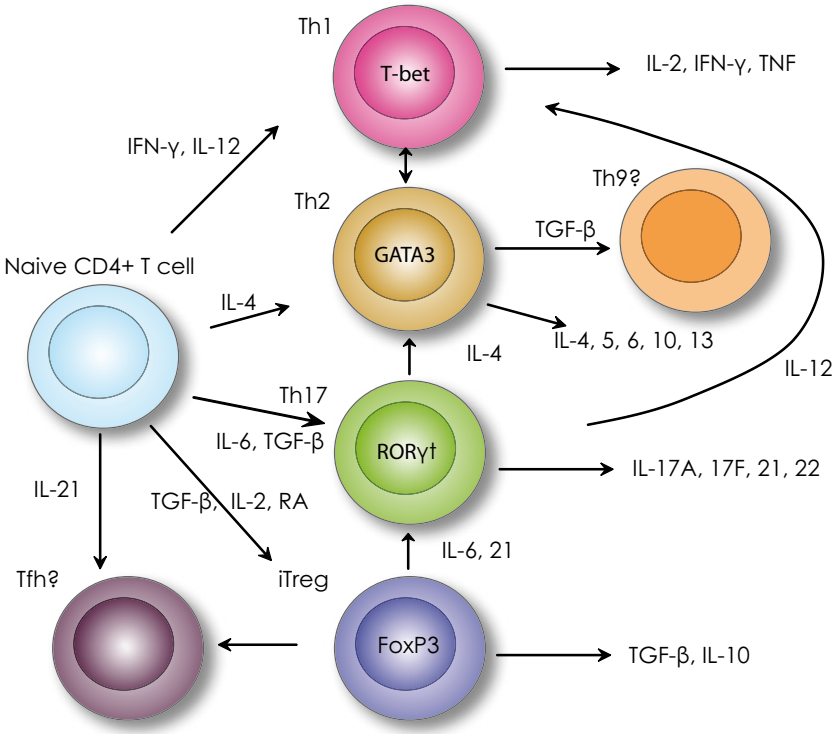

Figure 1. Naïve T helper cells can differentiate into various types of helper cells after cytokine stimulation. The four major groups of Th cells are presented above (Th1, Th2, Th17 and iTreg). The transcription factors that are the main definers of the subsets, as well as essential for the development into the specific cell types are stated inside the cells. The two subsets named Th9 and Tfh are not completely defined as definite lineages and do not have a known specific transcription factor coupled to their development. There is a great plasticity and depending on the cytokine milieu, cells can convert from one to another branch. Note that for Th17 development there is a discrepancy between mouse and human differentiation. While murine Th17 cells are induced as described above, human Th17 cells require IL-1, IL-6 and IL-23 but not TGF-β for their differentiation. Figure modified from [8-10].

T helper cells bind MHC class II plus the antigenic peptide through the TCR/CD4 complex and control immune responses by both receptor-ligand signals and cytokine production. The Th1 pathway controls cell-dependent responses and is the preferential type of Th response for virus clearance and tumor immunotherapy. The Th2 branch coordinates the humoral immune response and is the major player in bacterial infections, parasites and allergy.

Tregs include both naturally occurring Tregs (CD4+ CD25+ FoxP3+) and adaptive Tregs, also named inducible Tregs (iTregs). Tregs are also known to express CTLA-4 as well as GITR. These cells have a major impact on tumor growth since they infiltrate tumors, suppress T effector cells, NK cells, B cells and DCs and induce tolerance/anergy. There are also regulatory cells in the CD8+ lineage [11-13] which appear to play a role in both tolerance and control of autoimmune diseases [14-17]. The new Th17 lineage plays a role in chronic inflammation and can be found in patients with autoimmune diseases [18].

CTLs express CD8 and are major players in viral and tumor clearance. CTLs are promoted by Th cells to become cytotoxic and kill their target by releasing perforin and granzymes or by death receptors. CTLs recognize a peptide in the MHC class I context that is presented by a tumor cell, for example, and they are selective in their killing due to membrane polarization of secreted particles.

T cells have the capacity to become memory cells and there is a great heterogeneity in the memory pool. Both CD4+ and CD8+ cells are capable of becoming either central memory (T_{CM}) or effector memory T cells (T_{EM}). These cells mediate the long lived anti-tumor response which is warranted when developing tumor immunity. T_{CM} express CD62L and chemokine (C-C motif) receptor (CCR)-7 which mediate homing to lymph nodes where they reside and build up long term memory. They can convert into effector cells in response to their target antigen. T_{EM} do not express CCR-7 and have various levels of CD62L. They are more prone to circulate and can be activated immediately upon antigen encounter, thus mounting a rapid response in case of re-infection [19].

In addition, there are two other cell types that express TCRs, the γδT and NKT cells. Th, Tregs, CTLs and T memory cells all express the αβ TCR, but γδT cells express the γδ TCR chains instead. However, a population of γδT cells expressing FoxP3 and displaying suppressive capacities has recently been induced *in vitro* [20]. The importance of γδT cells and NKT cells in tumor surveillance has been acknowledged, and these subtypes can recognize unconventional antigens not identified by αβT cells. NKT cells can be divided into two branches where NKT type I cells possess anti-tumoral properties and NKT type II cells are immunosuppressive [21, 22].

1.3 Antigen presentation

Antigens can be proteins, carbohydrates or nucleic acids and they can derive from self, pathogens or non-pathogens. B cells bind antigens directly, but T cells require antigen presentation (usually peptides) via MHC for binding and recognition to occur. However, pathogens differ in their way of invading the host cell, and hence, there are different ways for cells to present pathogenic material to the immune system. Professional antigen-presenting cells (APCs), B-cells, DCs and macrophages, take up antigen by endocytosis into vesicles. The acidic environment in the endosome/lyzosome processes antigen proteins into smaller peptides which are assembled onto major histocompatibility complex (MHC) class II proteins and transported to the cell surface were they are recognized by Th cells.

Intracellular antigens produced by the cell (i.e., self or viral antigens) are presented by MHC class I to CD8+ T cells. In the cytosol of the cell, the proteasome degrades proteins. The transporters associated with antigen processing (TAP-1, 2) then transfer the antigens to the endoplasmatic reticulum (ER) were they are loaded onto MHC class I and further transported via Golgi to the cell surface. The stability of the MHC complex is increased by peptide incorporation. An important feature of tumor antigen presentation to cytotoxic T cells is the cross-presentation which refers to the possibility for endocytosed material to be transferred into the cytoplasm of the APC and loaded onto the MHC class I complex, even though it may not be a cytoplasmic antigen initially [23]. The exact mechanism of how exogenous material is loaded onto the MHC class I is still a matter of debate.

Of interest is that TAP and two of the subunits that make up the immunoproteasome (low molecular weight protein (LMP)-2, LMP-7) are clustered genes and are upregulated by IFN-γ [24, 25]. When cells are subjected to IFN-γ, the proteasome will change its proteolytic cleavage pattern and peptides with anchor residues preferably bound by both TAP and MHC class I will be formed. Note that all peptides are not efficiently presented by the IFN-γ-induced proteasome (immunoproteasome). Tumor evasion can be mediated by switching proteasome so that immunogenic peptides that are strongly presented by the proteasome become poorly presented by the IP and vice versa (reviewed in [26]).

1.3.1 Dendritic cells and subtypes

DCs belong to the innate part of the immune system but are an important link to adaptive immunity by peptide presentation via MHC molecules, as well as providing the ligands for costimulation. DCs are essential when discussing CpG, CTLA-4 and PD-1/PD-L1 (see Chapters 3 and 4). DCs, especially plasmacytoid DCs (pDCs) in humans, express TLR-9. In addition, DCs express the ligands for both CTLA-4 and PD-1 allowing for T cell fine-tuning.

DCs were first described by Steinman and Cohn in 1973 [27] and are not a homogenous cell population. They origin from hematopoietic stem cells (HSCs) and were thought to be part exclusively of the myeloid lineage, but this has been revised since lymphocytic precursor cells also generate DCs [28, 29]. Thus, although the conventional classification of murine DCs into myeloid DCs (mDCs) (CD11c+ CD11b+CD8α-), lymphoid DCs (CD11c+ CD11b-CD8α+) and plasmacytoid precursor DCs still exists, scientists also describe DCs as precursors that develop into immediate DC precursors and further into immature DCs. These immature DCs are then named plasmacytoid DCs (pDCs) or mDCs and further stimulation with TLR ligation, type I IFNs or CD40L, for example, induces the formation of mature DCs [30]. Zuniga et al introduced vertical plasticity in the horizontal lineage model by demonstrating that immature pDCs can become mDCs upon type I IFN and FMS-related tyrosine kinase 3 ligand (FLT3) stimulation [31]. FLT3L appears crucial for DC development since *in vivo* administration of FLT3L to humans results in a high quantity of DCs and mice lacking FLT3L have low DC levels [32-34].

One difficulty in experimental DC production is the use of monocytes, granulocyte monocyte colony stimulating factor (GM-CSF) and IL-4 in generating human antigen-presenting cells. These DCs are not comparable to mouse splenic DCs in cytokine production or phenotypical markers, thus have little relevance when extrapolating results from mouse to human and vice versa. In the case of TLR-9 stimulation, mice have a broader TLR-9 expression profile involving both conventional DCs and the inflammatory pDCs. Among human DCs, only pDCs have been demonstrated to express TLR-9, hence administration of TLR-9 ligands in mice results in a broader DC activation than in humans which could influence therapeutic efficacy. However, both our own and others observations indicate that along with pDCs, monocytes also express detectable levels of TLR-9 in humans, thus this dogma might be revised soon.

Migrating DCs are transported in the blood stream, sampling antigens in the periphery. When an antigen is encountered together with activating signals (e.g., TLR stimulation), CCR-7 is upregulated and the ligation of this receptor leads to downstream signaling events resulting in cell skeletal rearrangement, actin polarization and finally motility. Subsequently, the cell homes to lymph nodes or spleen and presents the antigen. DC maturation also induces the upregulation of cluster of differentiation (CD) antigen-40, CD80, CD86 and CD83 [35] as well as increased production of cytokines and MHC class I expression.

Plasmacytoid DCs, as mentioned above, express high levels of TLR-9, secrete vast amounts of type I IFNs and can express indoleamine 2,3-dioxygenase (IDO), an enzyme catalyzing tryptophan to kynurenine as a step toward producing nicotinamide adenine dinucleotide. Type A (see Chapter

3) CpGs stimulate pDCs to secrete type I IFNs and upregulate costimulatory molecules on their cell surface, a feature essential for immunostimulatory therapies.

Plasmacytoid DCs are sensitive to autocrine IFN, CTLA-4 ligation, TLR-9 stimulation and are prone to induce expression of IDO. IDO+ DCs are suppressive cells and can be induced by Tregs [36]. T cells are especially susceptible to tryptophan deprivation which impedes their proliferation [37], thus IDO in the vicinity may cause cell cycle arrest in T cells. The circle of activation by certain stimuli that leads to IDO expression and T cell inhibition probably serves to dampen excessive tissue damage after viral clearance but can complicate tumor eradication.

An interesting approach in immunotherapy are the IDO inhibitors available on the market that can be applied for tumor therapy (see review discussing IDO and tumor formation/maintenance [38]). IDO negative pDCs have the opposite effect on the immune system, enhancing immune responses. Secretion of type I IFNs lead to production of IFN-γ, IL-6 and IL-15 [39]. Type I IFNs also increase IL-10 production and shift the anti-inflammatory properties of IL-10 to proinflammatory [40]. All these events counteract tumor growth, but could also mediate autoimmunity.

2. The scope of this thesis

This investigation aims toward finding new ways to manage and treat solid tumors in the clinic with the help of experimental animal models. The ultimate goal is to implement new immunotherapies into the clinic. The specific objectives of each paper are listed below:

2.1 Paper I

Since BCG is a toll agonist with potentially harmful effects for the patients, we decided to compare BCG to a murine TLR-9 agonist (CpG 1668) which is non-infectious. In addition, the study aimed at elucidating the effector mechanism behind CpG therapy in our experimental bladder cancer model.

2.2 Paper II

As paper I demonstrated that CpG was more effective than BCG in our murine model, we also sought to improve the therapy further by targeting immune checkpoint regulators to "release the breaks" in the immune system. Both CTLA-4 and PD-1 blockade were investigated alone or combined with CpG or BCG to assess anti-tumor efficacy. In addition, cytokine secretion, levels of regulatory cells and activated cells were investigated after therapy to explore how these factors were affected.

2.3 Paper III

As systemic aCTLA-4 therapy often results in adverse events, localized therapy may preserve the anti-tumor effect with less accompanying autoimmune events. Hence, the focus in this study was to investigate localized blockade of CTLA-4 as a means to control tumor growth, induce systemic anti-tumor responses as well as develop lasting tumor immunity.

2.4 Paper IV

TLR ligands have recently been discovered to interact with complement and the resulting immune response is affected by this interaction. Since CpG is a potential therapeutic agent for bladder cancer patients and also used in clinical trials to target various cancers, the objective of this study was to investigate how CpG interacts with human complement and the possible effects this may have on the subsequent immune response. The possible mechanisms of how CpG activates complement were also investigated.

3. Toll-like receptors

3.1 Pattern-recognition receptors

The existence of pattern-recognition receptors (PRRs) and their ligands were first hypothesized by Janeway in 1989 when he postulated that non-self is not only recognized by receptors that arise from rearranged genes. Instead, he proposed that the immune system had evolved to contain receptors specifically recognizing evolutionarily conserved patterns on infective agents. Currently 10 TLRs have been identified in humans and 13 in mice, however, the mouse TLR-10 is a pseudogene [41-43]. Toll-like receptors are type-1 integral membrane glycoproteins and have an intracellular Toll/interleukin (IL)-1R domain and an extracellular leucine-rich repeat (LRR) motif. Based on amino acid sequence, TLRs are divided into five subfamilies: the TLR-2, -3, -4, -5 and -9 subfamilies.

TLR-3, -7, -8 and -9 recognize nucleic acids and are intracellularly expressed while TLR-1, -2, -4, -5 and -6 are expressed on the cell surface (Fig. 2). Toll-like receptors have recently been discovered on endothelial as well as on tumor cells. Signaling by TLR-4 in tumor cells results in pro-inflammatory cytokine production, and upon TLR-4 blockade tumor growth is delayed [44]. The dual role of TLRs as both tumor suppressors and tumor growth enhancers is even more confounding because Tregs also express TLRs [45]. Further, FoxP3 (specific expression marker for Tregs) controls the expression of TLR-10 [46]. Proinflammatory responses induced by some TLR ligands can possess anti-tumoral properties if signaling promotes cytokine secretion in combination with adaptive immunity. If the same TLR signaling occurs in tumor cells as in Tregs, tumor progression may occur due to onset of chronic proinflammatory cytokine secretion (discussed in [47-49]).

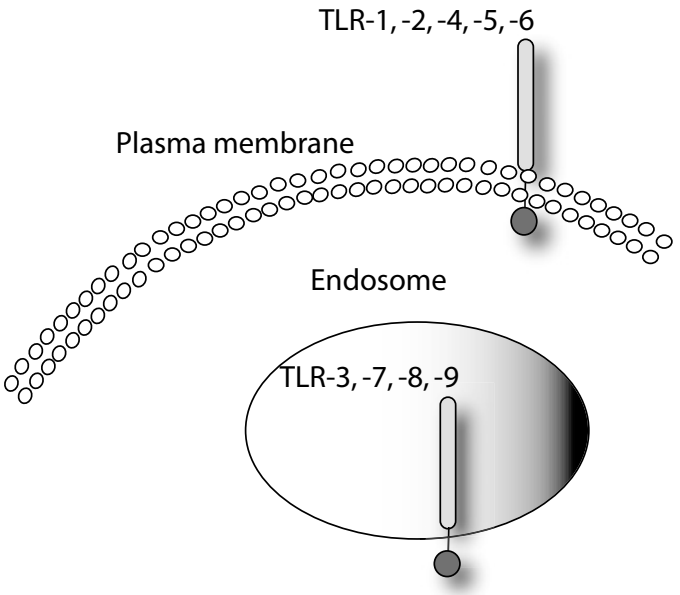

Figure 2. Schematic overview of the TLR expression in a cell.

3.2 TLR-9 and CpG

TLR-9 and its ligand CpG have shown encouraging pre-clinical results when anti-tumoral properties have been investigated. TLR-9 was discovered in 2000 [50] but the TLR-9 ligand, unmethylated CpG sequences (from e.g., bacterial genomes) had already been attributed to possess immunostimulatory properties.

There are areas in our own genome containing hypomethylated CpG islands that are thought to trigger the onset of for example, lupus erythematosus. However, a recent article states that DNA can trigger TLR-9 signaling by interaction of the 2' deoxyribose with TLR-9, mediating sequence-independent signaling [51]. The self and non-self theory is then challenged and the simple explanation for controlling TLR-9 activation could be compartment-restricted expression of TLR-9. TLR-9 expression is confined to the endosomes, thus activation can only occur when DNA is "faultily" located. In addition to the restricted localization of TLR-9 in cells, our own DNA can inhibit TLR-9 signaling and as a result prevent autoimmunity [52-55]. For example, it has been proposed that mammalian DNA contains repetitive elements that can inhibit TLR-9 signaling.

3.2.1 Cell restricted expression of TLR-9

Current literature describes TLR-9 expression in B cells, dendritic cells, monocytes and macrophages in the mouse. For humans, TLR-9 expression has been attributed to B cells as well as pDCs. This is the accepted view, however, reports indicate that human monocytes as well as monocyte-derived DCs and possibly T cells (see below) also express TLR-9 [56]. Of interest is the strong impact that many TLR-9 agonists have on NK cells; however, this effect appears to be mediated indirectly through DCs or macrophages since NK cells are not known to harbor the receptor themselves [57, 58]. Figure 3 illustrates some of the numerous effects that CpG has on the immune system.

Some groups have reported TLR and specifically TLR-9 expression in T lymphocytes (reviewed in [59]) and mouse CD4+ T cells have been demonstrated to be directly stimulated by CpG ODNs resulting in increased T cell proliferation and reduced anergy [60]. Further, CpGs can block immune suppression induced by Tregs in a T cell suppression assay with non-functional APCs (beads coated with antibodies against CD28 and CD3) [61]. In the direct T cell assay mentioned above, around 20 μg/mL CpG was added to cultures compared to 2-6 μg/mL in APC studies. This concentration difference could indicate that T cells have a higher activation threshold for CpGs or that the relatively low TLR-9 expression in T cells demands saturated levels of CpGs for activation to occur.

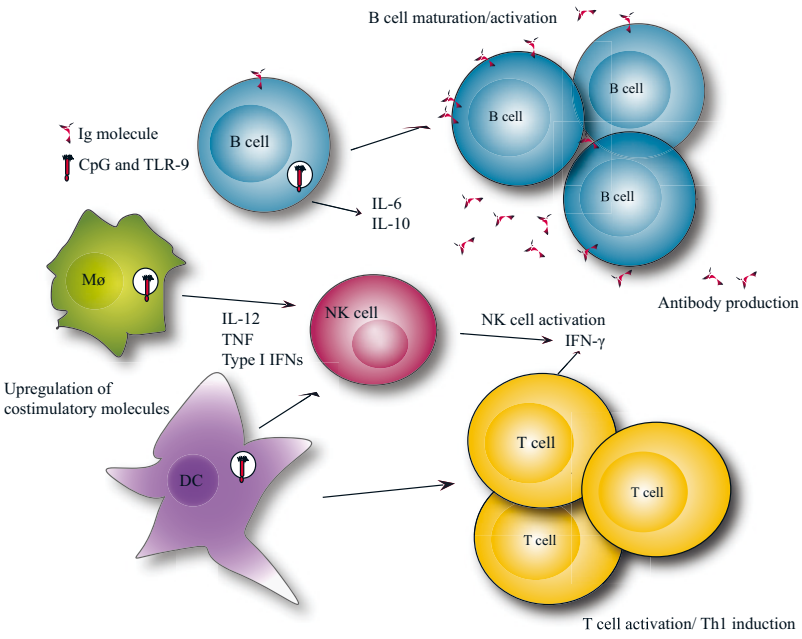

Figure 3. Possible immune effects of CpG. CpG is taken up by B cells, DCs and macrophages (Mo), leading to upregulation of costimulatory molecules such as CD80 and CD86 as well as CD40. IL-12, TNF and type I IFNs are secreted and the combination of a potential tumor antigen presented by the APC, cytokine secretion and enhanced costimulation leads to T cell activation and IFN-γ production. NK cells are also activated by the APCs to produce IFN-γ. This leads to a Th1 induction and subsequent tumor eradication. B cells are stimulated by CpG via TLR-9 and this leads to maturation and antibody production. IL-6 and IL-10 are also produced by the B cell in response to CpG.

3.2.2 CpG classification

Hartmann et al found that sequence, number and spacing between CG motifs determine the immune response [62]. Also, when modifying a phosphorodiester CpG oligo containing only one CG motif to a phosphorothioate oligo, the immune stimulatory effects were abolished. Phosphorothioate oligos require more then one CG motifs to be immunostimulatory. Tokunaga et al. were first to demonstrate and characterize anti-tumor effects from specific isolated *Mycobacterium bovis* fractions [63]. Krieg et al went further to characterize the specific effects induced by unmethylated CpG and did that by synthesizing short single-stranded unmethylated DNA sequences with CG nucleotides incorporated in the sequences [3, 64]. This work has also lead to a classification of CpG ODNs based on backbone and the ensuing immune response [65].

The current classification divides CpG oligos into class A, B and C. Class A are IFN-α inducers, hence, the letter A. Class A CpGs stimulate pDCs to

produce IFN-α and also affects NK cells indirectly to produce IFN-γ but are poor stimulators of B cells. Class A induces a modest increase of costimulatory molecules on their target cells. Type B CpGs are strong B cell activators, (inducing B cell proliferation and maturation), hence, the letter B. Type B CpGs induce costimulatory molecules on pDCs, but effect only a modest increase in the IFN-α response. Class C stands for combined activities, and CpGs in this class induce a strong B and pDC activation with upregulation of costimulatory molecules as well as IFN-α secretion. Class B and C are based on the phosphorothioate backbone; the oligo is modified to contain a sulfur in one of the non-bridging oxygen atoms in between each nucleotide. Phosphorothioate oligos have a half life of about 2 days compared to few minutes for a phosphorodiester oligo (reviewed in [66]). Most data on mice and humans are based on type B CpGs. For mice there are two sequences that have been extensively studied and are referred to as CpG 1668 and CpG 1827. In human experiments, CpG 2006 (also named 7909 when applied as vaccine or PF-3512676 when administrated without adjuvant) is the most extensively studied ligand and has also entered several clinical trials.

Several CpGs have been assessed with success in experimental tumor models. A collective overview of these investigations is given in Table 1, and included are experiments where local CpG therapy has been used alone without the addition of peptides or other therapeutics. The B16 model demonstrates a NK dependent effector phase and no tumor immunity when using both a type A and B CpG, contrasting the other models which showed a T cell dependent effector phase and long-lived tumor immunity [67-71]. The T cell independent tumor eradication in the B16 model could be due to lack of MHC class I on B16 cells (seen by ourselves and others) as compared to, for example MB49 cells that do express MHC class I [72]. One of the papers demonstrates a macrophage (phagocyte) dependent effector phase of B16 eradication [58]. The effector phase has either been investigated in T cell deficient mice or by selective cell depletion. Depletion of cells during a tumor rechallenge have only been performed by us [71] and demonstrated a CD4+ T cell dependent immunity.

Table 1.

Tumor type	ODN	Effector phase	Tumor immunity	Reference
B16	1585, 1826	NK dependent (1585)	No (1585)	Ballas et al [67].
EL4	1585,1826	NK and T cell dependent (1826)	Yes (1826)	Ballas et al [67].
RMA	1826	CD8+ T cell dependent	Yes	Lonsdorf et al [70].
C26	1826	CD8+ T cell dependent	Yes	Heckelsmiller et al [68].
Renca	1826	n.i.	n.i.	Heckelsmiller et al [68].
B16	1826	Macrophage (phagocyte) dependent	n.i.	Buhtoiarov et al [58].[1]
B16	1668	NK dependent	No	Sfondrini et al [73].
AG104A	1668	NK and CD8+ T cell dependent	Yes	Kawarada et al [69].
IE7/IE7K^k	1668	n.i.	n.i.	Kawarada et al [69].
B16/ B16 OVA (MO4)	1668	n.i.	n.i.	Kawarada et al [69].
3LL.D12 (LLC-1)	1668	n.i.	n.i.	Kawarada et al [69].
MB49	1668	T cell dependent	Yes, CD4+ T cell dependent	Mangsbo et al [71].

CpG 1585 is a type A CpG (mixed PO and PS backbone), while CpG 1826 and 1668 are type B CpG (PS backbone). B16 is a murine melanoma cell line and B16 OVA (MO4) is a B16 transfectant expressing OVA. RMA is murine lymphoma, C26 is a colon carcinoma, Renca is a renal cell carcinoma and both AG104A as well as IE7 are fibrosarcomas. 3LL.D12 is a lung carcinoma, also named LLC-1 (Lewis Lung Carcinoma-1). n.i. (not investigated). Parentheses show the oligo that was used in the specific experiment. PS=phosphorothioate and PO=phosphorodiester.

3.2.3 PF-3512676

Various short oligo sequences have been used in animal models but based on *in vitro* data and later also human *in vivo* data one specific sequence has been taken into the clinic. PF-3512676 (also called CpG 7909 or 2006) was developed in 2000 [62, 74].

Melanoma trials (phase I or II) have documented clinical effects and combination therapies involve CpG coadministrated with Rituximab, Dacarbazine, Taxane/cisplatin, local radiation or other chemotherapies. Many trials are ongoing [66] and published trials are presented in Table 2. Taking into account the remarkable results achieved in animal models, the clinical trials have not lived up to the expected result. However, patients with metastatic melanoma might have poor survival independent of therapy. Thus, initiating CpG therapy in an early disease stage would be valuable. We know from animal data (our own and others) that local CpG injections work best

[1] Note that CpG was administrated i.p as a systemic therapy.

when used on small tumors and the bigger the tumor, the less effective therapy is. This is due perhaps to the accumulation of regulatory cells and that immune cells are outnumbered by the tumor cells.

Table 2. *Published results from clinical trials with PF-3512676 (Pubmed search September 9, 2009: PF-3512676 cancer)*

Study	Tumor type (stage)	Nr of patients (group)	Amount in mg	Dose schedule	Injection site	ORs?	Note
Pilot phase I [75]	MM (III-IV)	3 (CpG), 2 (peptide), 3 (CpG+ peptide)	2	Min. of six bi-weekly inj.	s.c	No	Tumor reactive immune cells expressed PD-1
Phase II [76]	NSCLC (IIIB-IV)	34 (Taxane-Platinum), 74 (Taxane-Platinum+ CpG)	~14	Adm. every 8 and 15 days of each cycle	s.c	Yes (PR and SD)	Fewer patients with stage IV disease in the control arm
Phase II [77, 78]	MM (I-III)	11 (CpG, 13 (saline)	8	1 inj. only	i.d adjacent to the scar of the primary melanoma excision	n.i	Patients that had previously undergone immunotherapy or that was on immunosuppressants were excluded
Phase I [79]	BCC or MM	5 (BCC), 5 (MM)	0.01-10	Increasing dosing schedule	Intralesional	Yes, 1 CR in each group and 4 PR in BCC and 1 SD i MM	
Phase I [80, 81]	CD20+ B cell NHL	50 (CpG+ rituximab)	See next column	Dosages varying between 0.04-0.48 (i.v) and 0.01-0.016 (s.c) mg/kg plus an extended cohort	s.c or i.v (comparison study)	24% OR (n=5)	”Although both i.v and s.c routes of administration were safe there was a higher frequency of symptomatic adverse events with s.c delivery, which, at high doses challenged patients tolerability over time”- author’s comment
Phase I [82]	MM	7 (CpG+ Melan A peptide)	0.5	4-13 inj. of the vaccine	s.c	SD that progressed into PD (n=3) and 1 NED	CpG and peptide vaccination induced circulating activated tumor specific cells, however, they did not appear to impact the clinical outcome
Phase I [83]	MM	8 (CpG+ Melan A peptide)	0.5	4 montly injections	s.c	n.i	

Phase II [84]	MM (IIIB/C-IV)	20 (CpG)	6	Every week for 24 weeks or until development of PD	s.c	2 PR and 3 SD	NK cytotoxicity was associated with a better clinical outcome
Phase II/III [85]	MM (III-IV)	46/46 (10/40 mg CpG), 45 (40 mg CpG+ DTIC), 39 (DTIC alone)	10 or 40	3 week cycle with inj. of CpG every week and DTIC on the first week of every cycle	s.c	16% OR in 40 mg CpG+ DTIC and 8% in DTIC only while 0-2% in CpG only	No difference in overall survival between the arms, hence the results did not support a continuation of the study into phase III

MM, malignant melanoma; NSCLC, non-small cell lung carcinoma; BCC, basal cell carcinoma; NHL, non-hodgkin lymphoma; s.c, subcutaneous; i.v, intravenous; i.d, intradermal; OR, objective response; PD, progressive disease; PR, partial response; SD, stable disease; CR, complete response; NED, no evidence of disease; DTIC, dacarbazine; Min., minimum; inj., injection.

4. Checkpoint blockade

4.1 Immunosurveillance

Ehrlich founded the theory of immunosurveillance in the early 20th century [86] when he proposed that transformed cells continuously arise in our body and are subsequently eradicated by our immune system before observed clinically. Later, Burnet and Thomas postulated the concept of immunosurveillance from experimental data demonstrating immune repression of transplanted tumors [87]. The validity of the theory has been questioned since much support came from virally transformed tumors and less evidence supporting eradication of spontaneous or chemically-induced tumors. Since the incidence of common tumor malignancies (except for Kaposi sarcoma) does not increase in the overall HIV population, there have been doubts regarding the theory. However, in the HIV population receiving combination antiretroviral therapy (cART), prolonged life span, and a higher incidence of non-AIDS-defining malignancies (nADM) then AIDS-defining malignancies (ADM) has been detected, which speaks in favor of the tumor immunosurveillance hypothesis [88]. Further, chemically-induced tumors have been studied and support the immunosurveillance theory with a central role of B, T, NK, NKT cells and type I/II IFNs as well as perforin as mediators of tumor repression [89, 90]. In 1980, Janeway proposed that the immune system is tolerized by default based on the Two Signal Model that Lafferty and Cunningham introduced [91]. Hence, a costimulatory signal is required to achieve proper lymphocyte activation. PPRs interact with pathogen-associated molecular patterns (PAMPs) and this interaction causes upregulation of costimulatory molecules on the antigen-presenting cells which serves as the second signal for lymphocyte activation [92, 93]. Matzinger proposed that tissue damage or stress is of importance for evoking immune responses. Tumors that do not threaten tissue integrity are well-protected from the immune system. When they grow, invading surrounding tissue, there is a release of heat shock proteins (HSPs), oxygen deprivation and an activation of surrounding endothelium that trigger danger signals, which ultimately activate the immune system. However, the tumor microenvironment suppresses immune cell activation and continued antigen exposure in the tumor area can lead to tolerized cells and failure of tumor eradication [94, 95].

4.1.1 Immunoediting

The theory of immunoediting describes three phases: Elimination occurs when immune cells eradicate newly transformed cells, a process that can lead to equilibrium since immune selection/pressure generates malignant cells that resist elimination. Resistant tumorigenic cells can later progress and spread when the tumor enters the escape phase with metastatic lesions and death as a consequence.

Mechanisms such as downregulation of MHC class I or tumor expression of MHC class I like molecules have been known tumor escape mechanisms for some time. Mutations in the TAP machinery or in apoptosis-inducing pathways are also ways of tumor escape. Cytokines secreted by the tumor such as TGF-β or IL-10 can inhibit immune responses and the lack of costimulatory molecules on APCs induces anergic T cells incapable of tumor destruction. Tregs, myeloid-derived suppressor cells (MDSC) and IDO+ DCs can be induced, or exist naturally, and may be attracted to the site of the tumor, leading to immune evasion.

4.2 Costimulation

As depicted above, tumor-reactive T cells exist but in order to avoid anergy and to sustain equilibrium or even achieve elimination of tumor cells there is a need for proper costimulation during the activation phase. Circulating tumor-reactive cells that have avoided negative selection in the thymus are likely to be anergic since costimulation is rare after MHC class I/peptide presentation by the tumor itself due to lack of B7-1 or 2 expression. T cells can also be ignorant due to low or no expression of the tumor associated antigen (TAA) or due to defective antigen presentation machinery. In tumor recognition, six categories of possible TAAs exist: tumor specific, germ-line encoded, differentiation, abnormally expressed, abnormally modified or oncoviral antigens [96]. The fact that many tumor antigens are expressed by normal cells makes immunotherapy as well as tumor vaccination problematic since central and peripheral tolerance is present. Also, in the case of a widely distributed antigen, the potential risk of inducing autoimmunity is increased.

4.2.1 CD28

T cell activation is initiated when the TCR/CD3 complex interacts with the MHC/peptide. Upon TCR engagement, a second signal from CD28 interacting with B7-1/2 is necessary for T cell activation to occur as sole TCR engagement is not sufficient [97]. When CD28 binds to B7-1 or 2, the T cells go into a proliferative, activated state. Different therapies can upregulate B7-1 and 2, for example IFN-γ or agents inducing IFN-γ can be used. Another

approach is to utilize vectors carrying both the a TAA as well as B7 molecules into the tumor in order to initiate the expression [98], but sole antigen/B7-1 expression does not appear to cure patients. In addition, B7-1 and 2 expressing tumors in murine model systems do not lead to complete tumor regression for poorly immunogenic tumors [99], thus other factors play a role in the tumor escape mechanism scenario. In the case of TAA presentation, APCs possibly play a bigger role than the tumor itself and immunotherapy intervention should probably focus on these cells instead of the tumor.

4.3 Coinhibition

In order to assure that the immune system does not harm normal tissue upon activation there are control mechanisms that can prevent or minimize collateral damage. Two well studied molecules that regulate activation of T cells are the CTLA-4 and PD-1 receptors. They can be targeted in order to enhance immune activity. Their function and the result of their blockade in immunotherapy are described below.

4.3.1 Cytotoxic T lymphocyte antigen-4

The CD28 receptor belongs to the Ig superfamily and provides the second signal for T cell activation and is counteracted by its close relative Cytotoxic T lymphocyte antigen-4 (CTLA-4) [100]. Upon TCR engagement, CTLA-4 transcription and expression is enhanced. The clathrin adaptor complex AP-1 association in the Golgi apparatus directs CTLA-4 expression to the cell surface, and sustained expression is achieved by Lck and Zap-70 which phosphorylates tyrosines, that prevents clathrin proteins from binding to CTLA-4. Upon CTLA-4 expression, the stimulation threshold value required to induce optimal T cell proliferation is changed. CTLA-4 engagement also leads to decreased amounts of transcription factors followed by less cytokine production and specifically less IL-2 secretion. IL-2 secretion is essential for T cell expansion and the loss of IL-2R engagement leads to cell cycle arrest.

The importance of CTLA-4 as a co-inhibitory regulator was proposed after observing that mice lacking CTLA-4 die of massive lymphoproliferation of the CD4+ T cell population [101]. Numerous mechanisms have been demonstrated to account for the inhibitory outcome that is followed by increased CTLA-4 expression:

The preferential binding of CTLA-4 to B7-1 and CD28 to B7-2 results in a situation where, when only B7-1 is expressed, CD28 will be poorly localized to the immunological synapse and vice versa [102]. As a consequence, it follows that if B7-2 is expressed on the APC there is a higher probability that the signal will be activating since CD28 binds B7-2 with higher avidity

then B7-1 and is, therefore, less likely to be outcompeted by CTLA-4 [103] Thus, apart from the competition between CD28 and CTLA-4 for the same ligands [104] the different levels of B7-1 and 2 on the APC may also modulate the T cell response. CTLA-4 is also thought to physically block CD28 to enter the synapse, thereby preventing the second signal to occur. CTLA-4 interaction via Tregs with B7-1 or 2 on DCs can also induce lower expression of the ligands with poor costimulation as a result [105]. Further, CTLA-4 interaction with B7-1 or 2 can by bi-directional signaling induce the release of IDO which can cause tryptophan deprivation and a subsequent T cell inactivation. In an *in vivo* situation, CTLA-4 ligation on DCs causes reversed signaling through the B7 ligand into the DC which, together with type I and II IFNs, could induce IDO production and immune suppression [36, 106]. FoxP3, as well as TGF-β expression may also be upregulated by CTLA-4 ligation and this can lead to increased numbers of Tregs.

The importance of the cytoplasmic part of the CTLA-4 molecule for a complete inhibitory function was apparent when using a transgene expressing only the extracellular and transmembrane part of CTLA-4 [104]. CTLA-4 can also exert T cell inhibition even in the absence of CD28, strengthening the hypothesis that intracellular signaling can account for immune inhibition after CTLA-4 ligation [107]. It has also been established that CTLA-4 expression can lead to T cell inhibition via recruitment of the SHP-2 phosphatase that dephosphorylates the CD3/TCR chains [108, 109]. In addition, the serine/threonine phosphatase 2A (PP2A) interacts with CTLA-4 as well as with CD28 [110]. Baroja et al [111] propose that CTLA-4 activity is inhibited by this interaction via a regulatory subunit on PP2A called PP2AA, and that upon simultaneous ligation of both the TCR and CTLA-4, PP2AA will become phosphorylated and dissociate from CTLA-4 leading to decreased IL-2 transcription and T cell deactivation as a result.

CTLA-4 blockade as a therapy has been used with success in animal tumor models, both as monotherapy and in combination strategies [72, 112-127]. Table 2 gives a brief summary of the different experimental studies performed *in vivo* as well as the clone and therapeutic intervention scheme used. When examining previous experiments performed using antibody therapy and CTLA-4 blockade in experimental models, it is apparent that most studies have been performed using antibodies made in hamster. The 9D9 clone with a murine Fc region has not yet been compared to the hamster antibodies and may be more effective as a single therapy.

Much debate has taken place concerning the target cell of CTLA-4 blockade. Many reports support the notion that CTLA-4 expression in cis results in immune suppression (induced by the different mechanisms discussed above). But CTLA-4 is highly expressed on the Treg compartment and studies indicate that trans expression may also account for immune suppression. Recent advances have demonstrated CTLA-4 as a component of Treg mediated im-

mune suppression. In a conditional KO mouse, with a targeted CTLA-4 deficiency in the Treg compartment, the suppressive capacity of Tregs is severely diminished [105]. In a colitis model, where adoptive cell transfer of Treg (CD4+ CD25+) cells results in disease-free mice, CTLA-4 blockade breaks tolerance resulting in disease initiation [128, 129]. However, in this experiment there is no clear target population of the CTLA-4 blockade.

Quezada et al [130] recently provided data demonstrating that CTLA-4 blockade *in vitro* render Teff cells "resistant" to Treg-mediated suppression and that chronic CTLA-4 blockade does not diminish Treg levels but rather elevates functional Tregs in the lymph node compartment. Peggs et al [131] performed an elegant study where they demonstrate that CTLA-4 blockade *in vivo* has a synergistic effect when both Treg and Teff cells are targeted simultaneously. This was performed by constructing a transgenic (Tg) mouse expressing a gene consisting of the extracellular portion of human CTLA-4 and the mouse transmembrane and cytoplasmic domain of CTLA-4. The Tg mouse was then bred onto a CTLA-4-/- background. By making use of either an anti-human CTLA-4 antibody or an anti-mouse CTLA-4 antibody, and by sorting out Teff and Treg cells from both Tg mice and wild type mice, they could selectively target separate subpopulations. They reconstituted Rag-/- mice with different combinations of Treg or Teff cells from wt and Tg mice and subsequently challenged the mice with tumors. By selective CTLA-4 blockade of the Treg population, tumor growth was not affected. However, by selective CTLA-4 blockade on Teff cells, prolonged survival was seen. Targeting Tregs and Teff cells simultaneously resulted in the best overall survival. This work indicates that opposing immune responses may take place depending on if CTLA-4 is blocked or if there is a complete loss of the molecule.

There are two aCTLA-4 antibodies in clinical trials today, one is tremelimumab (Pfizer) which is a human IgG2 monoclonal antibody. The other antibody is ipilimumab (Medarex Inc.) which is a human IgG1 antibody and both antibodies antagonize the binding of CTLA-4 to the B7-1 and 2 molecules. Most studies enroll patients with advanced melanoma but there are also trials including patients with renal cell cancer, prostate cancer, bladder cancer, colorectal neoplasms, pancreatic cancer, lung cancer, breast cancer and lymphoma (clinicaltrials.gov). A large phase III study involving tremelimumab on advanced melanoma was stopped at the second interim analysis since tremelimumab was not evidently better than the standard regimen [132]. However, both a selective patient group as well as patients treated with combination regimens (including aCTLA-4) may benefit from the therapy and this must be further studied in detail. However, side effects such as severe enterocolitis are prominent with CTLA-4 blockade [133, 134] but are manageable with steroids that do not appear to interfere with clinical anti-tumor effects [135]. High-grade adverse events appear to correlate with a better clinical prognosis [136, 137]. However, this needs to be confirmed by more studies.

Table 3- Systemic anti-CTLA-4 therapy in murine models

Tumor	Clone	Amount (μg), injection site	Schedule	Delaying tumor growth	Tumor immunity	Note	
TRAMPC1	9H10	100, i.p	3x, 3 days apart	Yes	n.i.	MHC class I low tumors were more susceptible to therapy. 42% CR	Kwon et al [112].
CSA1M	UC10-4F10-11	1000, i.p	Once a week, 3x	Yes	Yes	Less effective when delaying therapy	Yang et al [116].
OV-HM	UC10-4F10-11	1000, i.p	Once a week, 3x	Yes	n.i.	Less effective when delaying therapy	Yang et al [116].
Meth A	UC10-4F10-11	500, i.v	1x	Only if administrated together with a GITR-depleting antibody	n.i.		Kuibeom et al [124].
B16	UC10-4F10-11	50-100, i.p	3x, 2 days apart	Yes if administrated with CpG/peptide vaccine	n.i	Requirement of both CD4+ and CD8+ cells for effective therapy	Davila et al [120].
51BLim10	Hamster	100, i.p	4x, 4 days apart	Yes	n.i.	B7-1 negative tumors were more affected by CTLA-4 blockage than B7-1 positive tumors	Leach et al [113].
SM1	9H10	100, i.p	3x, 3 days apart	Only if administrated with irradiated tumor cells expressing GM-CSF	Yes	During effector phase CD4+ and CD8+ T cells were required for tumor eradication	Hurwitz et al [117].
B16-BL6	9H10	100 and 50, i.p	3x, 3 days apart	,,	Yes	During effector phase NK cells and CD8+ T cells were required for tumor eradication	Van Elsas et al [118].
B16-BL6	9H10	100 and 50, i.p	3x, 3 days apart	Same as above and the effect was perforin and Fas-FasL dependent	,,	Both B and CD4+ T cells were dispensable for tumor rejection and in a prophylactic setting neither CD4+, CD8+ or NK cells were essential for tumor protection	Van Elsas et al [119].
HOPC and NSO	9H10	100, i.v	4x, 2 days apart or 2x 3 days apart	Yes	Yes	NK as well as CD8+ cells were required for tumor rejection	Murillo et al [125].

5TGM1	9H10	100, i.p	4x, 4 days apart.	Yes	n.i.	A metastatic model	Murillo et al [125].
B16-BL6	9H10	200, i.p	3x, 3 days apart	Only if administrated with irradiated tumor cells expressing GM-CSF	Yes	Therapy also included vaccination with irradiated GM-CSF-producing B16 cells and both CD4 as well as CD25 depletion enhanced therapeutic efficacy but not prophylactic efficacy	Sutmuller et al [127].
B16-OVA (MO5)	9H10	200, i.p	3x, 3 days apart	Only when combined with cryoablation	n.i		den Brok et al [121].
B16	9H10	100, i.p	Once	Yes of administrated with the vaccine	n.i	Xenogenic vaccination study. The vaccination was most effective when aCTLA-4 was administrated with the second vaccination.	Gregor et al [123].
Meth A	9H10	50-100, i.p	3x, 3 days apart	Yes and the anti-tumor effect was more effective when combined with a viral vaccine carrying p53	Yes		Espenschied et al [122].
CT26 or B16F10	9D9	10-15, or 100-150, i.p	2x, 3 days apart	Yes, in a high systemic dose with irradiated tumor cells	n.i	The paper also investigated aCTLA-4 expressing tumor cell lines and local aCTLA-4 therapy	Simmons et al [126].
MB49	9D9	200, i.p	3x, 3 days apart	Yes	Yes	The combination of local CpG-therapy and systemic aCTLA-4 resulted in enhanced therapeutic efficacy	Mangsbo et al [72].

TRAMPC1, prostate cancer cell line; CSA1M, fibrosarcoma 9H10; OV-HM, ovarian carcinoma; 51BLim10, colon carcinoma; SM1, mammary carcinoma; B16-BL6, GM-CSF expressing melanoma cell line; HOPC, plasmacytoma; NSO, non-immunoglobulin plasmacytoma; 5TGM1, syngeneic disseminated myeloma; Meth A, fibrosarcoma; UC10-4F10-11 and 9H10 are hamster antibodies while 9D9 is a mouse antibody. Tumor immunity refers to lack of tumor growth after a tumor rechallenge, when the first tumor had complete regressed.

4.3.2 Programmed death receptor 1

PD-1 and its ligands PD-L1/L2 are related to the CD28 and B7 family, respectively. PD-1 is inducibly expressed on T cells, B cells, NKT cells and monocytes while PD-L1 is expressed on B cells, DCs, macrophages, bone marrow-derived mast cells, T cells and non-hematopoietic cells. The expression of PD-L1 on tumor cells and ligation of PD-L1 to its receptor PD-1 on T cells cause signals that generate anergic T cells [138]. Islets of Langerhans express PD-L1 and loss of expression in an experimental animal model which results in type I diabetes [139]. PD-L2 is inducibly expressed on mast cells, DCs and macrophages and the limited expression pattern has rendered this ligand less studied.

The molecular mechanism behind the suppressive effect of PD-1 is likely different from CTLA-4. While both pathways converge to affect cell metabolism, they do it differently. As discussed above, CTLA-4 has numerous ways of inhibiting cell responses, but one of them being the PPA2 pathway that ultimately affects IL-2 production as well as glucose metabolism via the seronine/threonin kinase Akt. PD-1 signaling affects PIK3, resulting in a net effect on Akt as well. The two pathways might synergize when applied simultaneously [140].

The binding of PD-1 to its ligands most likely serves to regulate T cell activation as well as APC function since ligation signals bi-directionally [141, 142]. Both chronic viral infections and tumors can evade the immune system by utilizing PD-1/PD-L1 expression. Exhausted CD8+ T cells have a higher expression of PD-1 on their cell surface causing defective viral clearance, and blocking this pathway improves T cell function in HIV infected cells [143]. Linkage analysis demonstrates that tumors with poor prognosis have a higher expression of PD-L1 on their surface [144-147] and targeting tumors with aPD-L1 antibodies or T cells with aPD-1 is an interesting approach to prevent tumor growth. Data demonstrate that in PD-1 deficient mice tumor engraftment is severely hampered and PD-L1 blockade dampens tumor growth [148]. However, as with aCTLA-4 therapy, the potential risk of developing autoimmunity is high since PD-L1 is important for maintenance of peripheral tolerance. In mice, the lack of PD-1 results in spontaneous induction of a lupus-like syndrome [149]. There appears to be no spontaneous aberrant phenotype in mice lacking both PD-L1 and L2 [150]. However, if PD-L1-/- mice are infected with lymphocytic choriomeningitis virus (LCMV) clone 13 they die due to uncontrolled immune activation, thus PD-L1 preserves/protects surrounding tissue integrity [151].

PD-1 or PD-L1 blockade has not been as extensively studied as CTLA-4 blockade. Different strategies have been utilized to prevent the interaction between the receptor and ligand. Both PD-1 and PD-L1 have been targeted with antibodies. The antibodies used have until recently been of hamster or rat origin. Recently, a PD-1 blocking antibody (clone 4H2) was generated by

first immunizing rats with mouse PD-1-immunoglobulin fusion protein. By determining the variable regions of this antibody and grafting these regions onto a murine IgG1 Fc and Cκ region, a chimeric antibody was developed that should be less prone to bind to murine Fc receptors. This antibody alone could not delay tumor growth in the B16 model, while there was a slight delay of tumor growth in the CT26 model. However, as with aCTLA-4, tumor growth was vastly inhibited when combining the blockade with a vaccination using irradiated GM-CSF expressing tumors [152]. Using a hamster anti-mouse PD-1 antibody, Iwai et al [153] demonstrated that metastases were diminished after systemic administration of the antibody. Another strategy of PD-1/PD-L1 blockade has been to express the extracellular portion of PD-1 so that this molecule occupies the binding site and prevents ligation of endogenous PD-1 with PD-L1 and L2. Local gene transfer of a plasmid containing this soluble PD-1 molecule slightly regressed growth of the murine hepatoma cell line H22 [154]. Another group utilized a hamster anti-PD-L1 antibody and demonstrated that tumors expressing PD-L1 were susceptible to PD-1/PD-L1 blockade; however, the effect on tumor growth was only present when PD-L1 blockade was combined with a 41BB agonist [155]. In adoptive transfer experiments of tumor-specific CTLs, both aPD-L1 and aPD-1 antibodies abrogated the growth of a mastocytoma (P815) expressing PD-L1.

There is one phase I study published with a humanized aPD-1 antibody (CT-011) tested in patients with different hematological malignancies. They report that the dose escalation was well-tolerated. The study also included a follow-up period where they followed the patients for survival beyond the initial 21 days. One of the patients was reported as a complete responder. Interestingly, this patient did not receive any prior treatment before enrolling in the study. Computer tomography (CT) confirmed CR 10 months after treatment and during this 10 month period she did not receive any other therapy [156]. When surveying the Clinical Trials database at the National Cancer Institute two other studies are listed with the goal to block PD-1. These studies list the aPD-1 antibody MDX-1106 (ONO-4538) as the drug, and represent phase I studies on patients with metastatic castration-refractory prostate cancer, renal cell carcinoma, melanoma or non-small cell lung carcinoma A study on Japanese patients with advanced malignant solid tumors is also ongoing with the same drug. A few abstracts related to these studies have been listed at conferences and they report that the doses are well-tolerated and that there are possible anti-tumor effects related to therapy [157].

5. Complement system

5.1 Complement pathways

An important feature of innate immunity are the around 30 proteins and glycoproteins [158] that build-up the complement system. Complement activation can occur via three different mechanism which all lead to C3 convertase build-up and eventually the formation of the membrane attack complex (MAC) that has the potential to lyse target cells/foreign organisms. The three pathways are a) the classical pathway, b) the lectin pathway and c) the alternative pathway (Fig. 4).

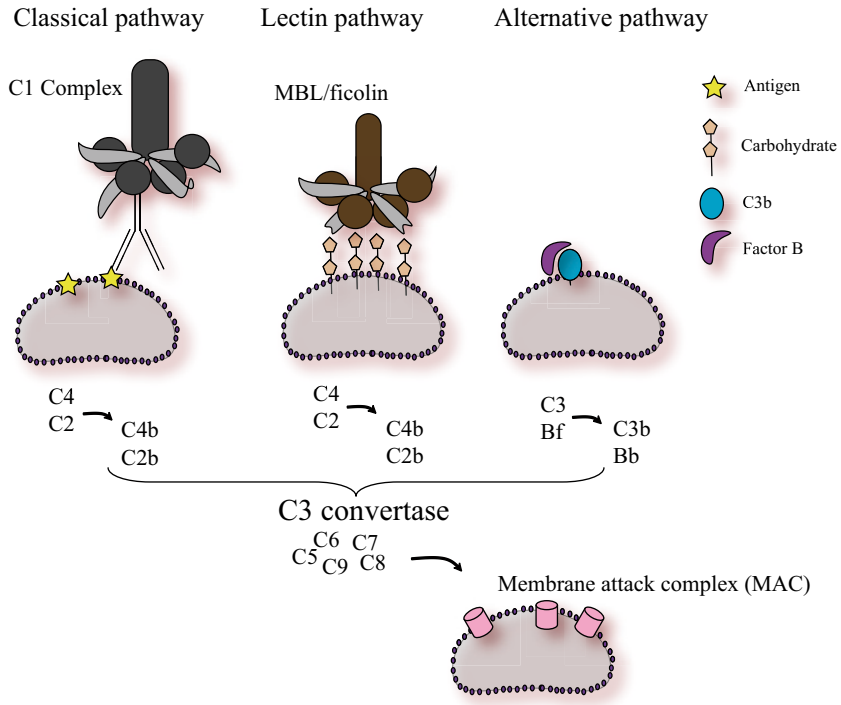

Figure 4. Simplified schematic drawing of complement cascade activation by the three different pathways known to mediate complement activation.

During classical complement activation, C1q builds up a complex together with serine proteases (C1r and C1s). The binding of C1q to a target surface most likely induces mechanical stress leading to activation of C1r that sequentially cleaves off C1s. C1s can then cleave C4 and 2 which leads to the build-up of the C3 convertase. C1q can bind directly to a non-self surface or via an antibody that has recognized its target. In addition, C-reactive protein (CRP) can function as a link between the target and C1q [159].

The lectin pathway is activated by recognition of carbohydrates on the surface of an organism [160]. The mannose binding lectin (MBL) or ficolins interact with carbohydrates and together with MBL-associated serine proteases (MASPs), homologues to C1r and C1s, they form a recognition complex that can initiate complement activation. The lectin pathway may be initiated by sole activation of MASP-2 [161].

Spontaneous hydrolysis of C3 to C3b and the covalent attachment of C3b to various surfaces can also lead to C3 convertase build-up. Once C3b is bound to the surface, Factor B is cleaved by Factor D and the remaining part of Factor B, Bb, is bound to C3b generating the AP convertase. The activation can be amplified since the AP convertase continues to cleave C3 to C3b. To prevent tissue damage, this pathway is controlled by inhibitory proteins such as Factor F, decay-accelerating factor (DAF), membrane cofactor protein (MCP) as well as the murine inhibitor Crry. The two other pathways, the classical and the lectin pathway, are mainly regulated by the C1 inhibitor that acts as a substrate for the proteases and arrests the catalytic cycle upon interacting with the enzymes. Until recently, another protein in the alternative pathway called properdin was thought only to stabilize the AP convertase. However, it has been shown recently that properdin interacts with non-self surfaces initiating a platform where C3b can bind, with a resulting complement activation [162-164]. Figure 5 outlines the proposed mechanism of how CpG ODN 2006, a synthetic human TLR-9 agonist may activate complement (paper IV).

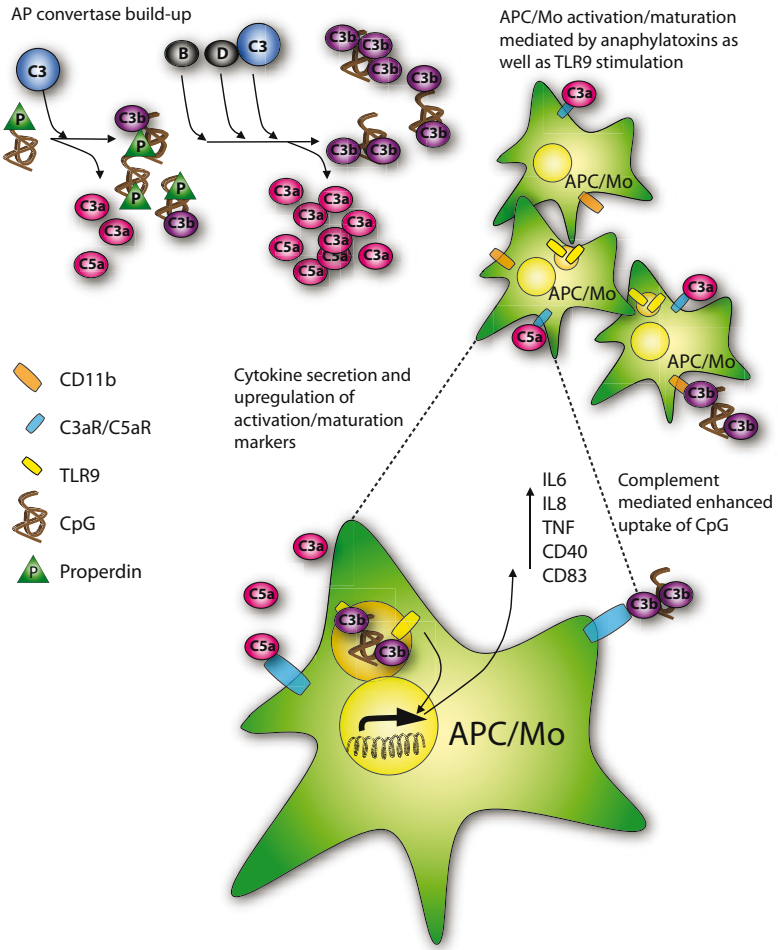

Figure 5. Depicted pathway of complement activation by the TLR-9 agonist CpG 2006. Complement and CpG interact and this affects both uptake and immune responses. The figure is extracted from Mangsbo et al [165] (paper IV, Fig. 7).

5.2 Anaphylatoxins

Anaphylatoxins (C3a, C4a and C5a) are also formed during complement activation and the split products enhance cell migration and maturation. As a result, innate immune components may influence the adaptive immune system via, for example dendritic cells that respond to factors released at the site of complement activation. Tumors may activate complement but avoid this by making use of complement inhibitors to control and maintain a quiescent environment [166-168]. The role of complement activation in the tumor milieu is controversial. There are articles arguing that the anaphylatoxins, C3a and C5a, may influence and create a more Th1-like immune response [169-172] but a recent publication indicates that C5a promotes tumor growth [173].

6. Bladder cancer

6.1 Epidemiology and etiology

In 2007 in Sweden, 2402 new cases of bladder cancer and cancer in the urinary tract were documented, making this cancer type the 6th most common in our country. Western Europe and North America demonstrate the highest prevalence while bladder cancer is relatively uncommon in Japan [174]. Males have elevated incidence rates (70% of affected individuals are males). The overall five year survival rate is 60-70% and diet as well as smoking are major risk factors for developing bladder cancer [175]. Studies have also shown that nulliparous women have an higher risk of developing bladder cancer then parous women, something that has been attributed to hormonal changes [176, 177] while others could not establish this correlation [178]. While smoking is a major risk factor in developed countries, the ongoing Schistosoma infections are the leading cause for squamous cell bladder carcinomas in Africa [179] and this cancer type accounts for around 30% of all cancers in this population [180]. In the case of Schistosomiasis, one important factor regarding transformation into malignant cells is the immune cells migrating to the area of infection. Infiltrating cells release cytokines, as well as oxygen and hydroxyl radicals, causing increased cell growth and cell transformation [179, 181].

6.2 Pathology

The bladder surface consists of a cell layer named transitional epithelial cells (or urothelium) which is expandable, reflecting a need for volume changes due to urine production. The urothelium can be 5-6 cell layers thick in an unextended bladder but reaches 3-4 cell layers at maximal distension. Beneath the urothelium there is a thin layer of connective tissue called lamina propria which is surrounded by smooth muscle. Most tumors arise from the transitional cells giving rise to transitional cell carcinoma (TCC) while adenocarcinoma and squamous cell carcinoma accounts for a minority of incidence in the developed world.

Tumors are divided into stages reflecting invasiveness and the patient's chance of survival. The Tumor-Node-Metastasis (TNM) system is used to evaluate the stage of disease where intraepithelial tumors (Ta or Tis) are

locally situated and thus have better prognosis. Tumors invading lamina propria (T1) or muscle (T2-4) have poorer prognosis and can become metastatic (Fig. 6). WHO's grading system is also used to evaluate malignant cells under a microscope. This system grades cellular differentiation and abnormality from G1 to G4. G1 often correlates to localized disease, while G3 usually corresponds to muscle-invasive malignancies.

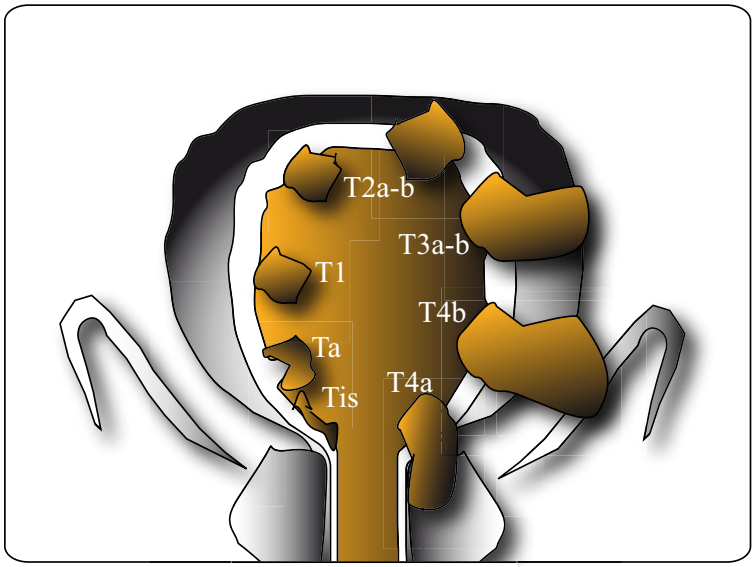

Figure 6. Illustrative figure showing the steps of tumor progression in bladder cancer. The black/grey zone represents the muscle layer and the white zone lamina propria.

6.3 Symptoms, diagnosis and treatment

Initial symptoms of bladder cancer are mainly blood in the urine as well as a burning sensation much like a urinary tract infection. Pain can be associated with muscle invasive disease. Diagnosis methods include cytoscopy, urography and ultrasound, but CT as well as magnetic resonance is also used nowadays. Around 10-20% of the tumors have infiltrated the surrounding tissue upon diagnosis [175]. Depending on the invasiveness of the cancer, different therapies are applied. For invasive tumors, surgery and palliative radiation therapy are applied, while systemic immunotherapies are still being evaluated in clinical trials. Localized tumors are treated with surgery, chemotherapy, radiotherapy as well as immunotherapy and occasionally with a combination of these therapies.

6.3.1 BCG therapy

Current immunotherapy for non-invasive bladder cancer is based on live attenuated BCG, which is instilled into the bladder. BCG is resolved in appropriate media, instilled and the patient is rolled around to increase bacterial contact with the urothelium. BCG has been administrated to patients with superficial bladder cancer for over 30 years and enhances survival rate compared to patients not receiving BCG instillations [182, 183]. The effector mechanisms that resolve bladder tumors after BCG therapy are several but overall the therapeutic success is still confounding researchers. Until today, published studies indicate that instillation of BCG into the bladder cavity causes urothelial cells to secrete IL-1, IL-6, TNF-α as well as IL-8. Cytokine release promotes cell migration and neutrophils, monocytes as well as macrophages infiltrate the area. Later, CD4+ T cells migrate into the tissue and this cell type dominates the granulomata [184]. If these cells represent effector cells or are actually suppressor cells remain to be elucidated, but Loskog et al [185] demonstrated Treg infiltration in human bladder cancer tumors. Both CD4+, CD8+ as well as NK cells appear responsible for tumor eradication [184] but also neutrophils play a role [186]. Immunological responses have also been attributed to TLR stimulation [187-194]. Since TLR ligands are powerful stimulators of APCs and they are available in clinical grade purity, there is an opportunity to refine bladder cancer treatment with targeted TLR stimulation, thereby avoiding toxic live bacteria.

7. Methods

7.1 Experimental bladder cancer

For new immunotherapies to be implemented in the clinic, a bladder cancer model is needed for experimental purposes. For this reason, Mouse Bladder-49 (MB49) cells were utilized in syngeneic C57BL/6 female mice. MB49 cells possess many characteristics of human bladder cancer such as expression of TGF-β, activation of IL-10 producing cells [195] and expression of PD-L1 (paper II). There are controversies regarding the use of this model since the cell line was isolated from male mice and consequently expresses the male transplantation antigen, HY [196]. Expression of the male HY antigen in MB49 cells may have implications since the tumor cells may be intrinsically immunogenic when implanted into female mice. However, the HY expression in this cell line can be used as a surrogate tumor antigen and studied with HY peptide-specific tetramers or pentamers. For MB49 tumors to grow in male mice, only 1/10 of the cells are required for tumor growth (compared to female mice), demonstrating its immunogenicity. Nevertheless, administrating CpG to male mice results in the same tumor regression pattern as in female mice. Thus HY alone is not responsible for the powerful anti-tumor responses seen in female mice (Fig. 7).

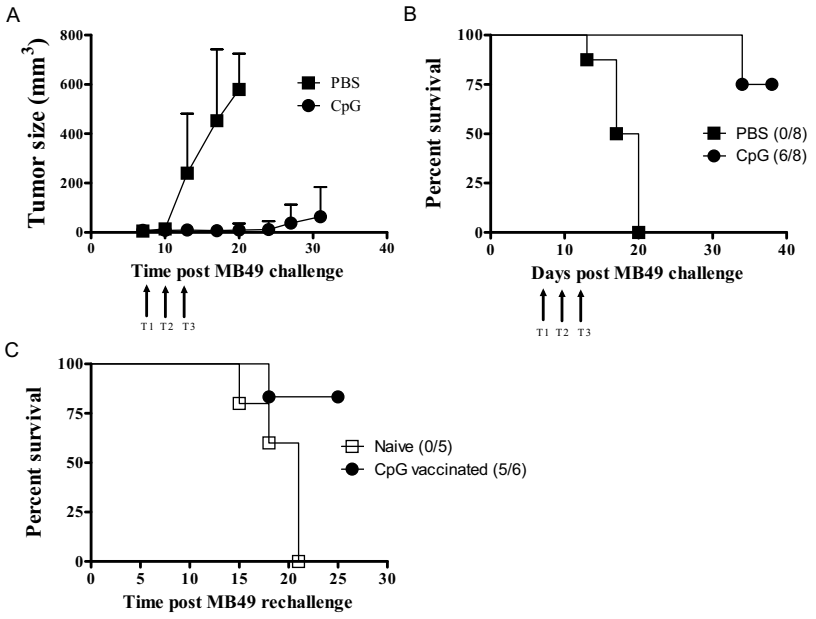

Figure 7. Subcutaneous MB49 tumors growing in male mice are susceptible to CpG therapy, thus the effect of MB49 regression by CpG is not solely dependent on the HY antigen. $1x10^4$ MB49 cells were injected into the right flank of male C57BL/6 mice. Treatment with CpG was performed as described in paper I and tumor growth (A) as well as survival (B) was monitored. Surviving mice were rechallenged with MB49 cells and compared to naïve control mice for survival (C) and 5 out of 6 mice demonstrated tumor immunity, with no palpable tumors, despite the lack of an HY antigen mismatch.

MB49 cells can be implanted in the bladder, but can also be injected to grow subcutaneously or intravenously leading to lung metastasis [197]. Figure 8 outlines the different experimental models used when studying bladder cancer in an experimental setting. The anatomically correct orthotopic model, with local instillation of therapeutics, is superior since the pharmacological situation is similar to therapy of human bladder cancers.

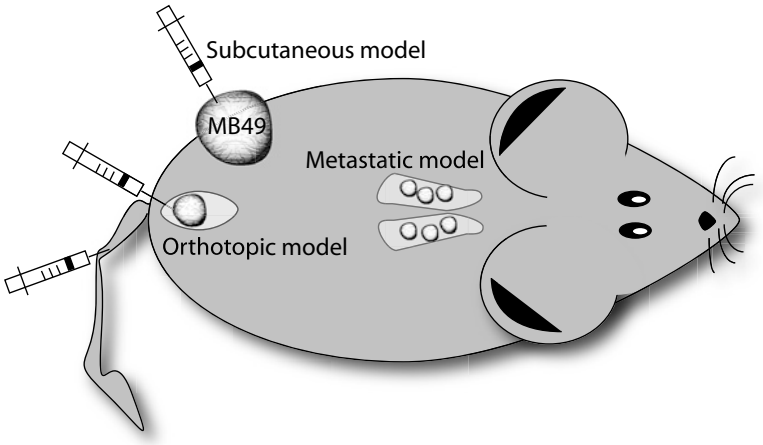

Figure 8. In the subcutaneous MB49 model, tumors grow under the skin and therapeutics are injected either locally or systemically (i.p or i.v). For the orthotopic model, tumors are implanted into the bladder lumen via a catheter and therapy is also instilled in the same manner. To mimic metastatic diseases, tumor cells can be injected i.v., whereby cells travel to the lungs and form small tumor nodules. The therapeutic agent is then injected either i.v or i.p. The metastatic model has not been used in this thesis work.

7.2 Human whole blood loop system

The whole blood loop system is based on a heparinization process developed by Corline Systems AB. Immobilized heparin on the inside of PVC tubing allow us to study the effects of substances on whole blood, with a functional cascade systems. The tubings as well as metallic connectors are coated with heparin to avoid both coagulation and complement activation from the plastic and metal. Whole blood is collected by an open system where the blood is directly transferred into a surface-heparinized Falcon tube. The blood is then quickly pipetted into the tubings and they are connected via the specially made connectors, forming a loop. The loops are then attached to a wheel with the capacity to rotate. The wheel is placed inside a 37°C incubator and set to rotate (Fig. 9). At chosen time points, blood is collected by temporarily opening the loop. Clotting is monitored by measuring platelet levels at every sampling procedure for every loop. To avoid complement activation and coagulation after sampling, all samples are directly mixed with EDTA.

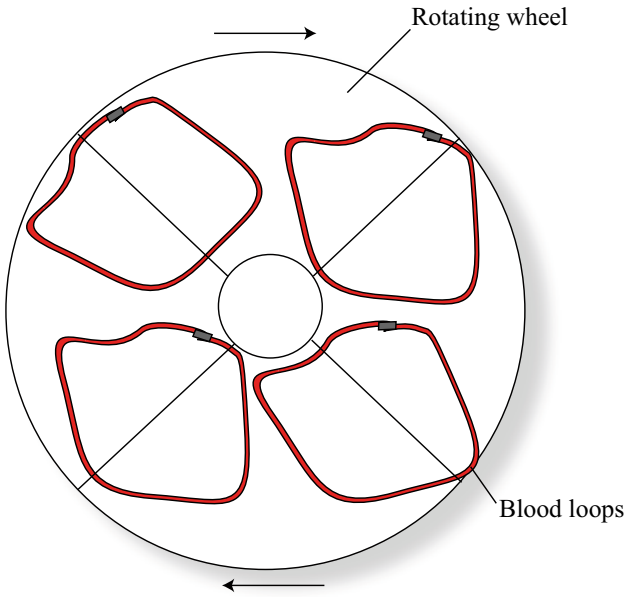

Figure 9. Illustration of how the loops are attached on the spinning wheel.

7.3 Quartz Crystal Microbalance with Dissipation monitoring

The interaction of complement with the CpG oligo was studied using Quartz Crystal Microbalance with Dissipation monitoring (QCM-D). This method can be used to study small mass changes and are readily applied when studying protein interactions. The crystal sensor can be set to oscillate using electrodes that are incorporated into the sensor. Upon binding of a protein onto the sensor, the resonance frequency will be dampened and the mass change corresponds to the frequency shift (Fig. 10). If water is incorporated together with the molecule, this will also affect frequency. The second component of the QCM-D technique is dissipation. Dissipation measures how "soft" the structure is that adheres to the sensor. Thus, if the molecules that bind to the sensor are large and non-rigid, there will be greater energy losses in every oscillating cycle compared to a rigid and small molecule that dampens the oscillation less. These two components can be studied together to assure proper readout of the experiment.

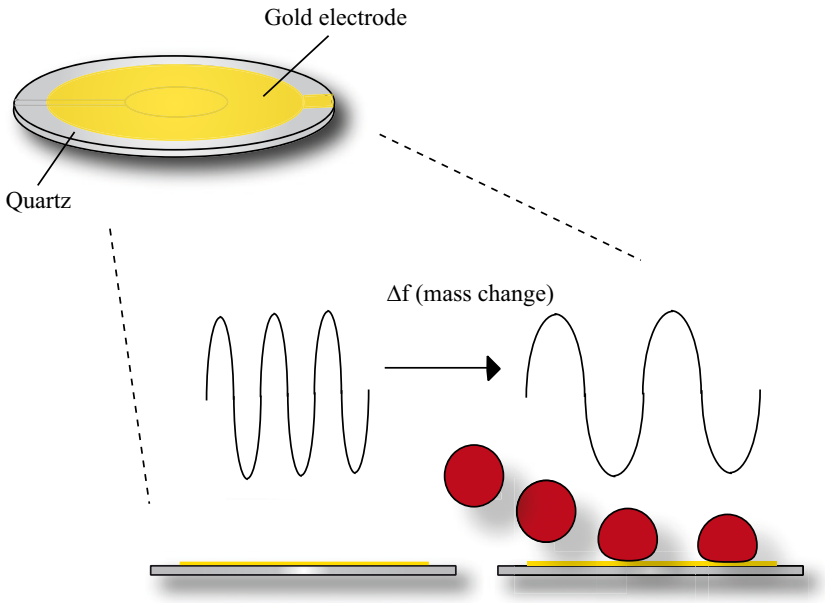

Figure 10. Schematic drawing of the sensor illustrating what happens when the sensor binds, for example a protein. The sensor is a crystal that oscillates when a current is applied through electrodes. Upon mass deposition there is a decrease in frequency which is proportional to the mass change.

In paper IV, this method was applied to study AP convertase build-up onto the CpG oligo. In order to investigate this, a pre-coating of the sensor was performed to avoid unspecific binding of the protein-of-interest (C3b). Figure 11 illustrates the layers that were built-up onto the sensor in the QCM-D apparatus. First, a layer of fibrinogen was used since this is known to bind C3b the least (out of fibrinogen, albumin and immunoglobulin) and then biotinylation of the fibrinogen was performed in the chamber. Sequentially, neutrAvidin was added and after that biotinylated CpG. As a control, the whole process was performed with the exclusion of biotinylated CpG to control for unspecific binding. Lastly, excess amounts of C3b were added to ensure a proper initiation surface for AP convertase build-up. Factor B, D and finally C3 were then added to form the AP convertase.

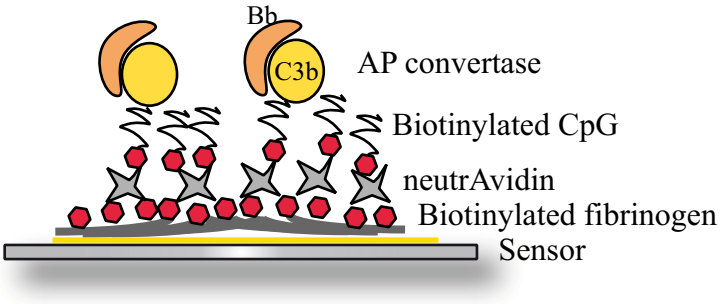

Figure 11. Schematic drawing of the components that were used in the QCM experiment in paper IV [165].

8. Results & Conclusions

8.1 Paper I

The fact that CpG is superior to BCG in our experimental animal model may be a result of species barriers, but could also reflect a true efficacy difference whereby a specific TLR-9 agonist induces a more refined and potent Th1 immunity. In this paper, we demonstrate that CpG is superior to BCG in an orthotopic bladder cancer model and that T cells are required for efficient tumor eradication during the effector phase. In the memory phase, we show that CD4+ T cells are required for tumor protection after rechallenge. We conclude from this work that CpG has an excellent anti-tumor effect in our murine bladder cancer model and represents a potential drug candidate for bladder cancer patients in the clinic. Safety and efficacy are the main arguments for the use of CpG instead of BCG in human bladder cancer therapy.

8.2 Paper II

For improved immunotherapy, one must not only achieve APC activation and maturation but also control checkpoint regulators that balance the immune response. In order to investigate if we could block the two major immune checkpoint regulators, CTLA-4 and PD-1, we used antibodies that selectively target these molecules. CTLA-4 blockade alone prolonged survival of mice with experimental bladder cancer. Further, we demonstrate that, by a simultaneous APC activation/maturation and blockade of checkpoint regulators (CTLA-4 or PD-1 [Fig. 12]), tumors were eradicated in a more demanding experimental model. Intriguingly, the use of an antibody targeting the ligand of PD-1 (PD-L1) does not add to therapeutic efficacy when combined with CpG. The combination of CpG plus aCTLA-4 or aPD-1 result in elevated levels of circulating tumor-reactive CD8+ T cells as well as activated CD4+ splenocytes. In addition, levels of Tregs in the tumor area are decreased after therapy.

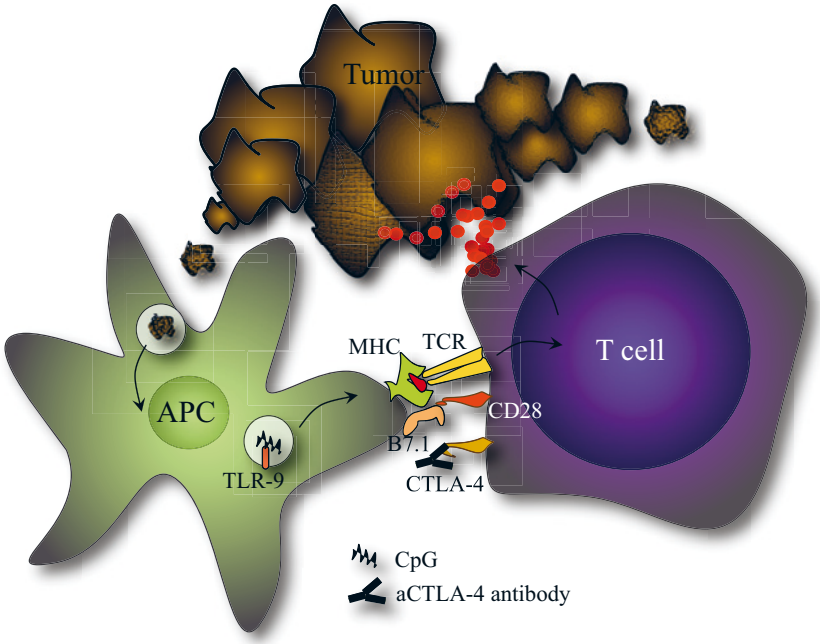

Figure 12. A two target model where APCs are activated by CpG, for example, and blockade of checkpoint regulators such as CTLA-4 allows for costimulation to occur. As a result, tumor eradication takes place via T cell-mediated mechanisms such as perforin/granzyme release.

8.3 Paper III

Systemic blockade of the checkpoint regulator CTLA-4 has been linked with severe side effects in humans. Hence, local administration of the antibody could resolve this. We demonstrate that by administrating aCTLA-4 in the tumor vicinity, a systemic anti-tumor response is mounted which eradicates both local and distant tumors. In addition to the anti-tumor effects, there is also a decrease in autoimmune events when injecting aCTLA-4 locally. This is of major importance for the clinical application of aCTLA-4 therapy and may resolve many of the current problems with this therapy. The impressive anti-tumor effects induced by local injections of aCTLA-4 could in the future also be combined with locally injected immunostimulators.

8.4 Paper IV

As TLR biology has recently been linked with complement, we sought to investigate if and how the human TLR-9 agonist CpG 2006 interacts with

complement. CpG 2006 is the candidate of choice for treatment of bladder cancer when initiating a clinical trial. Using a human whole blood loop system, we demonstrate that CpG-induced cytokine production is complement-dependent. Further, we demonstrate AP convertase build-up onto the CpG. In order to establish how CpG initiates complement activation we studied binding of different proteins onto the oligo. We demonstrate that CpG binds both IgM and properdin, both of which could initiate complement activation. The findings highlight the importance of investigating the role of complement activation in CpG therapy.

9. Future Investigations

9.1 Paper I

In paper I, specific cells responsible for tumor immunity in the memory phase were investigated and CD4+ T cells were found to be of importance. In addition, we demonstrated that T cells were important for CpG to exert effect during therapy. However, if it was CD4+ or CD8+ cells was not evident. In order to investigate this, a pilot experiment was performed using the subcutaneous tumor model where $2.5x10^5$ MB49 cells were injected day 0 and therapy started day 7 and was repeated 3 times every third day. Depleting antibodies (500 μg, i.p) were administrated day 6, 9, 12 and 16 after tumor injection. Depleting efficacy was investigated by tail-vein bleeding. For this analysis, staining antibodies from the same clones (GK1.5 and 53-6.7) as the depleting antibodies as well as staining antibodies from different clones (YTS191.1 and 53-5.8) were used in order to investigate if cells were completely depleted or if they were covered with antibodies.

Results of depletion efficacy demonstrated that CD4+ cells were completely depleted day 9 (not shown). CD8+ cells where still present when staining with a different clone. When staining with the same clone as the depletion was performed with, no cells were visible (day 10). We concluded that all cells were covered with depletion antibodies, but they were still in the circulation. However, since clone 53-6.7 is known to inhibit IL-2 induced T cells responses [198], T cell inhibition is likely to occur when the antibody has bound the CD8 molecule.

Figure 13 indicates that both CD4+ and CD8+ T cells are responsible for tumor eradication during the effector phase. Nevertheless, there is still an apparent anti-tumor effect in both groups despite depletion of effector cells and a combined block of both CD4+ and CD8+ cells should be performed in order to conclude that these two subsets together are important for tumor eradication in our experimental animal model.

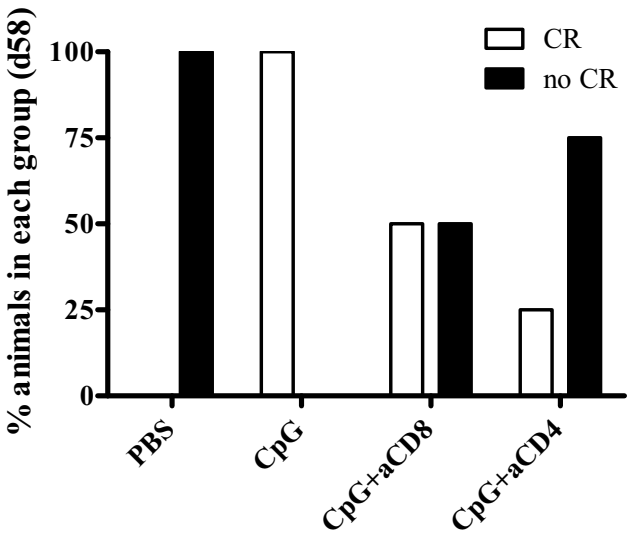

Figure 13. The role of CD4+ and CD8+ T cells for the therapeutic effect of CpG therapy was investigated in the subcutaneous tumor model using depleting antibodies. CR= complete remission (i.e., no visible or palpable tumor) and no CR (i.e., still a palpable tumor or a sacrificed animal due to tumor growth). The group that received CpG therapy also received i.p injections of rat IgG. aCD4= depletion of CD4+ cells using anti-CD4 depletion antibodies (clone GK1.5) and aCD8= depletion of CD8+ cells using anti-CD8 depletion antibodies (clone 53-6.7). Data represent d.58 post initial tumor challenge (n=4/group).

9.2 Paper II

In paper II, the combination of CpG with aCTLA-4 or aPD-1 shows promising anti-tumor effects and the vitiligo induced by this therapy indicates a break of tolerance. The vitiligo symptoms warrant an investigation of the combined therapies in the B16 model. It would be of interest to perform this study in a model where tumor antigens potentially match skin antigens. In addition, investigating the combination of all three therapies in the B16 model may provide important complimentary information to the current data. B16 tumors are extremely aggressive and also known to be weakly immunogenic, hence, there is room for improvement and one can more easily distinguish the effect of different therapies in the B16 model than in the MB49 model where the HY antigen is a foreign immunogen in female mice.

In this paper, aCTLA-4 as well as aPD-1 therapy was used systemically. Paper III, as discussed previously, demonstrated that local aCTLA-4 injections gave beneficial therapeutic effects both in the MB49 and the Panc02 tumor model. Thus, it would also be of interest to see if a combination of

local CpG and local aCTLA-4 or aPD-1 injections could result in the same effect as systemic CTLA-4 or PD-1 blockade combined with local CpG therapy.

In addition, when focusing specifically on bladder cancer, one should perform the same experiment in the orthotopic tumor model. In this experiment, local CpG instillations into the bladder, and simultaneous treatment of the mice with systemic aCTLA-4 or aPD-1 could be performed to evaluate the effects of these therapies in a more anatomically correct model.

9.3 Paper III

Systemic CTLA-4 blockade had a remarkable effect on tumor growth when used alone in paper I. Paper III aimed at investigating local CTLA-4 blockade. The effects of local CTLA-4 blockade were notable on both MB49 and Panc02 tumors. Interestingly, a dose escalation of the antibody did not increase anti-tumor efficacy but rather appeared to be less potent with respect to anti-tumor efficacy, which we ascribed to Treg induction. We also noted a distant tumor effect by local aCTLA-4 therapy. In the future, it would be of interest to study the biodistribution of the specific aCTLA-4 antibody we used in order to see if the cells that bind the antibody travel to both the tumor-draining LN and to distant LN compartments and tumors. Potentially, cells that are activated by the local therapy activate other cells that are responsible for the systemic effect. It would also be interesting to combine local aCTLA-4 therapy with an agonistic antibody such as aCD40 or a41BB injected peritumorally. This combination therapy, injected in proximity to the tumor, could possibly enhance therapeutic efficacy with less adverse events.

9.4 Paper IV

Recent advances in the TLR complement field indicate that the two systems may interact under certain circumstances and that this interaction modulates the immune response. Our findings in a human model system confirm the murine experiments. The data are of importance since complement may have dual effects on anti-tumor responses. It would be of great interest to evaluate local CpG injections with and without complement inhibition in humans to see if complement is essential for TLR activation, or if for example C5a induction is detrimental for a Th1 response as described by others. Another investigation that would strengthen the importance of complement induction for efficient TLR stimulation would be to evaluate the effect of CpG therapy in C3-/- mice on a C57BL/6 background. In addition, *in vitro* cultures of

monocytic DCs indicate that LPS, the TLR-4 agonist, stimulates cells to produce different complement factors [199, 200], thus allowing for complement activation to occur even in cultures lacking exogenous complement proteins. Hence, it would be interesting to evaluate if CpG also has this effect on mDCs, and if so, this could affect our interpretation of the data generated *in vitro*.

In addition, bone marrow DCs could be generated from mouse strains with complement deficiencies. Using CpG combined with complement products, as a maturation stimulus, cells could be analyzed for the expression of surface maturation markers. This analysis would then hopefully indicate if complement is important during TLR-9 induced DC maturation.

Summary of the Thesis in Swedish

Populärvetenskaplig sammanfattning på svenska

Målet med denna avhandling har varit att undersöka nya metoder för att angripa cancerceller med hjälp av vårt eget immunförsvar. Siffror tyder på att var tredje person kommer att drabbas av cancer i någon form och varje år avlider över 20 000 personer i Sverige i en cancer sjukdom. Dock har 5-års överlevnaden i Sverige höjts från 35 % för män och 48 % för kvinnor under 1970 talet till ungefär 70 % i dag. Detta inger hopp, men fortfarande finns ett stort tomrum att fylla för de cancerpatienter vars behandlingar inte fungerar.

Vi immunologer anser att det finns ett ypperligt anti-tumör försvar inom oss. Genom att utnyttja immunförsvaret tror vi att nya cancerbehandlingar kan utvecklas som ger en förbättrad långtidsöverlevnad, eftersom det minne som utvecklas i immunsystemet kan utnyttjas. De flesta är bekanta med konceptet vaccinering och vet att vacciner kan ge oss ett skydd mot infektioner under en kortare eller längre tid. Att cancer också kan behandlas med infektiösa bakterier är kanske inte lika känt, men ända sedan 1970 talet så har urinblåsecancer behandlats med en så kallad immunterapi. Denna terapi har bestått i att urinblåsan har fyllts med en bakterielösning (med levande attenuerad tuberkulosbakterie). Patienten har sedan rullats runt en timme för att öka bakterie-yt-kontakten. Bakterien initierar sedan en immunreaktion i urinblåsan och detta attraherar immunceller. I slutändan kommer dessa immunceller även att komma i kontakt med den tumör som växer i blåsan. Tumörer har ofta proteiner på sina yta som inte helt liknar de proteiner som finns på friska celler och genom dessa proteiner kan immunförsvaret specifikt angripa de sjuka cancercellerna men utan att angripa frisk vävnad.

Att angripa den sjuka vävnaden, men undvika den friska, är ett av många viktiga mål med en specifik tumörbehandling. Men problemen som infinner sig är att de proteiner som finns på ytan av tumörcellerna ibland är så lika de som finns på de friska cellerna att en korsreaktion sker och autoimmunitet uppkommer. Vi känner autoimmunitet som olika sjukdomar exempelvis reumatoid artrit eller MS, och dessa sjukdomar är ofta allvarliga. Därför är det av största vikt att väga nyttan mot de eventuella negativa effekter som kan uppstå om autoimmunitet uppkommer, men också att skapa behandlingar där specificiteten mot tumörcellerna är stor. Vårt immunsystem har också en unik kapacitet att skilja på små strukturer och också att känna igen struk-

turer efter lång tid. Vårt immunsystem har alltså god strukturkännedom men också gott strukturminne.

För att förbättra dagens behandling av urinblåsecancer, och generellt också behandlingen av andra solida cancerformer, så har vi undersökt möjligheten att använda en liten del av den tuberkulosbakterie som används i kliniken idag. Det är nämligen känt att den mest immunstimulerande delen i tuberkulosbakterien är dess DNA. DNA från just bakterier kan stimulera receptorer i vårt immunsystem, uråldriga receptorer som känner igen olika strukturer hos bakterier eller virus. Dessa receptorer signalerar in i den cell de sitter på och detta kan skapa en aktivering av vårt immunförsvar när de binder den struktur de känner igen. Vi kan syntetiskt framställa DNA sekvenser som är immunstimulerande och injicera dem lokalt vid tumören i hopp om att det ska dra till sig immunceller som beskrivet ovan. För att ytterligare förbättra denna behandling prövade vi även att sätta till specifika antikroppar som kan binda andra receptorer på så kallade T-celler. T-cellerna i vår kropp är viktiga för att skapa det minne som krävs för att tumören inte skall kunna börja växa igen. T-cellerna har många receptorer på sin yta och när de binder sin ligand så kan det antingen aktivera T-cellen eller blockera T-cellen. Om vi då blockerar den receptor som är hämmande så kan vi styra T-cellen mot aktivering.

Vad vi såg i våra studier var att i vår simulerade urinblåsecancermodell är de syntetiska DNA sekvenserna mycket bättre än tuberkulosbakterien för att förlänga överlevnaden hos möss. Mätningarna visade att det skapades ett immunologiskt minne mot tumörcellen och att detta minne gjorde att tumören inte kunde börja växa igen. Vidare studerade vi effekten av att blockera de inhiberande signaleringsvägarna hos T cellerna. Också denna blockering gav ett lyckat resultat med förlängd överlevnad hos mössen. Denna blockering kunde också göras lokalt vid tumören med ett gott resultat och detta innebar att autoimmunitetsrisken minskade. Vi försökte också kombinera DNA sekvenserna med blockeringen av de inhiberande receptorerna och såg att kombinationen gav ett bättre resultat än var terapi för sig.

Slutligen undersökte vi hur DNA sekvenserna påverkar olika kaskadsystem i kroppen. Vår lever producerar över 30-talet olika proteiner som hamnar i vårt blod. Dessa proteiner kan snabbt klyvas om de möter en bakterie eller ett virus som känns igen som främmande och detta startar en immunaktivering som syftar till att eliminera det främmande ämnet. Syntetiska ämnen kan också aktivera detta kaskadsystem och därför undersökte vi också hur syntetiskt DNA påverkade kaskadsystemet. Testerna visade att det skedde en aktivering vid höga doser av DNA och att denna aktivering påverkade immunresponsen. Vi drog slutsatsen att det krävs ytterligare forskning inom detta område för att ta reda på om den förändring av immunresponsen som sker vid kaskadaktiveringen kan påverka cancerbehandlingen.

Denna avhandling lägger en grund för fortsatt arbete mot att börja använda kombinationsbehandlingar i klinisk verksamhet. Både DNA sekvenserna, och de blockerande antikropparna, testas idag i kliniska prövningar för olika cancerformer, främst mot melanom (hudcancer). Dock har ingen startat en klinisk prövning för just urinblåsecancer och ingen har heller kombinerat de olika terapierna. Jag hoppas att delar av detta arbeta kommer framtida patienter till del och kan hjälpa dem i deras kamp mot sin cancersjukdom.

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