Antibody-based immunotherapy of cancer

From optimization to novel approaches

LUUK VAN HOOREN
Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbeck laboratory, Dag Hammarskjölds väg 20, Uppsala, Friday, 13 April 2018 at 13:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Professor Eugene C. Butcher (Stanford University, Department of Pathology).

Abstract

Antibody immunotherapy is a successful therapeutic approach to treat cancer. The overall aim of this thesis is to investigate the mechanisms of antibody-based immunotherapies and the role of the tumor microenvironment in mediating the anti-tumor immune response, in order to aid the development of improved immunotherapies for cancer patients.

Agonistic CD40 antibodies activate dendritic cells and improve anti-tumor T-cell responses. In Paper I we demonstrate that their efficacy can be enhanced by co-treatment with sunitinib, a multi-targeted tyrosine kinase inhibitor. The combination therapy restrains immunosuppression, synergistically increases endothelial activation and improves tumor T-cell recruitment, resulting in restrained tumor growth and prolonged survival.

CTLA-4 and PD-1 negatively regulate the anti-tumor T-cell response and blocking these immune checkpoints with antibodies enhances anti-tumor immunity. However, CTLA-4 checkpoint blockade is associated with severe adverse events. In Paper II, a local low-dose administration of CTLA-4 antibodies is demonstrated to be equally effective as systemic administration in treating experimental bladder cancer. Importantly, antibody spread is reduced, indicating that local administration may be an effective strategy to reduce side effects associated with CTLA-4 blockade.


Immunotherapy for glioma is constrained by the immunosuppressive microenvironment. In Paper IV we demonstrate that in vivo activation of B cells enhances tertiary lymphoid structure formation in the brain. Mice with induced tertiary lymphoid structures have an increase of B cells with a regulatory phenotype and CD8+ T-cell activation is suppressed. The response to PD-1 checkpoint blockade is also inhibited, suggesting tertiary lymphoid structures impair the response to immunotherapy.

This thesis demonstrates that immunotherapy can be improved by the addition of anti-angiogenic drugs and that local administration of antibodies is a feasible alternative to the systemic administration conventionally used in the clinic. In addition, therapeutic vaccination and induction of tertiary lymphoid structures by agonistic CD40 antibodies are novel approaches to employ antibodies to modulate the anti-tumor immune response.

Keywords: Cancer immunotherapy, CD40, CTLA-4, PD-1, Gal-1, tertiary lymphoid structures

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We are like sailors who on the open sea must reconstruct their ship but are never able to start afresh from the bottom

- Otto Neurath (1882-1945)
List of Papers

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


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### Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ALT</td>
<td>Alanine transaminase</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate transaminase</td>
</tr>
<tr>
<td>CCR7</td>
<td>C-C chemokine receptor type 7</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDC</td>
<td>Complement-dependent cytotoxicity</td>
</tr>
<tr>
<td>CXCL</td>
<td>C-X-C motif chemokine</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CTLA</td>
<td>Cytotoxic T lymphocyte antigen</td>
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<tr>
<td>DAMP</td>
<td>Damage associated molecular patterns</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>Teff</td>
<td>Effector T cell</td>
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<tr>
<td>EMA</td>
<td>European medicines agency</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor</td>
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<tr>
<td>Gal-1</td>
<td>Galectin-1</td>
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<tr>
<td>GBM</td>
<td>Glioblastoma</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GrzB</td>
<td>GranzymeB</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia inducible factor-1</td>
</tr>
<tr>
<td>irAEs</td>
<td>Immune-related adverse events</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibition motif</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>JAK3</td>
<td>Janus kinase 3</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>KO</td>
<td>Knock-out</td>
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<tr>
<td>LLC</td>
<td>Lewis lung carcinoma</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LAG-3</td>
<td>Lymphocyte-activation gene 3</td>
</tr>
<tr>
<td>LT</td>
<td>Lymphotoxin</td>
</tr>
<tr>
<td>MAC-1</td>
<td>Macrophage-1 antigen</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MB49</td>
<td>Murine bladder 49</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid derived suppressor cells</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappaB</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding oligomerization domain-like receptors</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer's patches</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death-1</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression-free survival</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>RCC</td>
<td>Renal cell carcinoma</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>SMAC</td>
<td>Supramolecular activation cluster</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TIM-3</td>
<td>T-cell immunoglobulin and mucin domain-3</td>
</tr>
<tr>
<td>TLO</td>
<td>Tertiary lymphoid structure</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor associated antigens</td>
</tr>
<tr>
<td>TME</td>
<td>Tumor microenvironment</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumor-infiltrating lymphocyte</td>
</tr>
<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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</tbody>
</table>
1. Introduction

The understanding of the importance of the immune response against cancer has not always been as clear as it is today. In 1909, the German physician and immunologist Paul Ehrlich suggested for the first time that malignant cells may arise continuously and if it were not for the immune system, cancer would develop at an “overwhelming frequency” (1). The concept did not gain much support among immunologists until 50 years later, when Thomas and Burnet refined the theory, coined the term immunosurveillance and provided experimental evidence that transplanted tumors can be repressed by the immune system (2). Although the repression of transplanted tumors was later found to be due to use of outbred animals and an allogenic immune response, the postulated existence of tumor associated antigens (TAAs) and the ability of the immune system to mount an immune response against these are important concepts first laid out by Thomas and Burnet.

Since the 1990s, experimental evidence in favor of immunosurveillance has accumulated through the use of immunodeficient mice. For example, Rag2 knockout (KO) mice lacking adaptive immunity (3), Perforin KO mice deficient in NK and T cell effector function (4, 5) and mice with impaired T helper 1 associated cytokine signaling such as IFNγ (6, 7) or IL-12 (8, 9) all have increased spontaneous as well as carcinogen-induced tumor development. Similarly, humans receiving long-term immunosuppressive medication after organ transplantation or due to HIV infection have an increased risk of developing tumors (10, 11). The fact that the immune system plays a crucial role in cancer development is now well understood. Current research is focused on understanding the interplay between the tumor, vasculature and the immune system and thereby support the development of interventions that can enhance the anti-tumor immune response.

1.1 Cancer and the tumor microenvironment

Cancer is a genetic disease, in which the normally highly controlled cell cycle of healthy cells has become dysregulated due to the accumulation of mutations. Decades of research have identified a plethora of oncogenes and tumor suppressor genes, which over time, as mutations accumulate, lead to the transformation of normally replicating cells into malignant tumor cells (12). These
genetic mutations provide the early developing tumor with cell-intrinsic traits such as unlimited proliferative potential and self-sufficiency in growth signals. Although cancer is commonly thought to emerge from a single cell that has acquired these characteristics, the extensive proliferation and the genomic instability which is characteristic of cancer results in highly heterogeneous tumors consisting of multiple different subclones (13). Moreover, cancer cells have a high degree of plasticity, as demonstrated by the ability to reversibly transition between different phenotypes (14). This clonal heterogeneity and plasticity has complicated the search for effective tumor-targeted therapies, since selection pressure causes tumors to select resistant clones or switch to alternative redundant driver pathways in order to evade therapy.

Another aspect of tumor heterogeneity is the tumor microenvironment (TME). As cancer cells grow into a solid tumor, a TME containing blood and lymphatic vessels, mesenchymal stem cells, pericytes, fibroblasts and immune cells develops (15). Tumor-associated fibroblasts and endothelial cells are responsible for the production of extracellular matrix proteins that provide structural and biochemical support to the tumor and consist of proteins such as proteoglycans, hyaluronic acid, collagens, fibronectin and laminin. Collectively, these extracellular matrix proteins and the nonmalignant cells of the TME are called the tumor stroma, which can make up 50-90% of solid tumors (16). The role of the tumor stroma in fostering tumor growth, metastasis development and resistance to therapy is increasingly recognized. Of particular interest is the immune compartment of tumors, which has opposing roles in maintaining tumor growth-promoting inflammation and eliminating tumor cells through immunosurveillance.

1.1.1 Melanoma

Cutaneous melanomas arise from pigment-forming melanocytes. Melanoma occurs mainly in white-skinned people and the incidence drops dramatically as skin pigmentation increases (17). An estimated 80% of melanomas is caused by exposure to ultraviolet light, resulting in the highest mutational load compared to other types of cancers (18). Melanoma is the deadliest form of skin cancer, responsible for 80% of all skin cancer related deaths (17). The incidence of malignant melanoma is 13/100 000 per year in the European Union and there are an estimated 133 000 new diagnoses worldwide every year. Advanced metastatic Stage IV melanoma patients have a poor prognosis, with a mean survival of 8–10 months and a 5-year survival rate of less than 10% (19). Treatment of primary melanoma is based on surgery and removal of the tumor. More advanced metastasized melanoma may be treated with targeted BRAF or C-KIT therapies in the patients who have mutations in these genes, or with antibody immunotherapy targeting PD-1 and CTLA-4, which will be discussed in more detail in later chapters.
1.1.2 Bladder cancer

Urothelial carcinoma of the bladder is a common cancer with an estimated 330,000 new cases and 130,000 deaths worldwide each year (17). Most bladder cancers are attributable to smoking or exposure to other types of carcinogens. The incidence is strongly male-biased, with three quarters of new cases diagnosed in males. At presentation, 70-80% of patients have superficial non-muscle invasive cancer, which is treated by transurethral resection followed by intravesicular chemotherapy (20). Higher-risk muscle-invasive bladder cancer and metastatic cancer are preferentially referred to adjuvant local Bacillus Calmette-Guerin (BCG) immunotherapy (21, 22). BCG instillation into the bladder has been used since the 1970s and results in remission for 70% of patients. With these treatments, patients and healthcare are faced with an extensive follow-up screening due to the high risk of relapses. More recently, in 2016, Atezolizumab was the first immune-checkpoint antibody targeting PD-L1 that gained clinical approval for patients with locally advanced or metastatic bladder cancer (23). Clinical trials employing a combination of antibodies targeting PD-1 and CTLA-4, which has proven to be more effective than PD-1 alone in melanoma, are currently underway [NCT02496208, NCT02553642].

1.2.3 Glioblastoma

Brain tumors account for approximately 2% of all cancer cases with an incidence of 175,000 new cases worldwide each year (17). The most common brain tumors according to WHO classification are astrocytomas, oligodendro-gliomas, ependymomas, medulloblastomas and glioblastomas (GBM), which are named according to their cell type or tissue of origin. Unlike other cancers, brain tumors seldom metastasize to other parts of the body. Symptoms include headache, nausea, and visual or mental status impairment, although the severity of symptoms greatly depends on the location of the tumor in the brain.

Gliomas are the most common type of brain tumors, which originate from the glial cells in the central nervous system, and account for 80% of all malignant brain tumors (24). Tumors are classified according to their histopathological malignancy, ranging from WHO grade I to grade IV (25). Grade I gliomas are typically cured by surgical removal. Diffuse lower-grade gliomas (grade II-III) commonly occur in young patients and the median OS is more than 10 years (26). WHO grade IV gliomas are the most malignant tumors, and are called glioblastoma (GBM). Approximately 90% of GBM diagnoses are primary GBM, which occurs mostly in older patients. Lower-grade II-III gliomas may progress to high grade, in which case they are termed secondary GBM. Primary GBM is typically negative for mutations in isocitrate dehydrogenase-
1 (IDH-1) or IDH-2, while 70-80% of secondary GBM have a somatic mutation in these genes (27). IDH-1 and IDH-2 are catalysts of oxidative decarboxylation. IDH-1 expression is found in the cytoplasm, peroxisomes and endoplasmic reticulum, while IDH-2 is expressed in the mitochondria. Mutations in either IDH-1 or IDH-2 are associated with longer overall survival (OS) and progression-free survival (PFS) (27).

GBM is notoriously difficult to treat, partly due to its highly infiltrative nature, which makes complete surgical resection impossible. Instead, therapy is based on maximal surgical resection complemented with radiation therapy and chemotherapy. Newly diagnosed patients with GBM have a dismal prognosis, with a median OS of 14.6 months.

1.2 The importance of the immune system in cancer

The importance of the immune response against cancer is currently well recognized. The anti-tumor T-cell response in particular has proven to be effective at targeting and eliminating cancer cells. Adoptive cell transfer based on the isolation of tumor-infiltrating lymphocytes (TILs) followed by their expansion and re-infusion into the patient was proven to be an effective treatment of metastatic melanoma already in 1988 (28). The density of CD8$^+$ T cell in the tumor microenvironment is associated with positive prognosis for the majority of cancers (29) and a clonal expansion of T cells is associated with spontaneous regression of melanoma lesions (30, 31). The group of Galon provides another illustration of the importance of the immune response against cancer. Quantification of memory T cells (Tmem, CD3$^+$CD45RO$^+$) and effector T cells (Teff, CD3$^+$CD8$^+$) in the core and invasive margin of colorectal tumors is a better predictor of disease progression than the traditional TNM (tumor, lymph node, metastasis) staging system (32). A more recent development is the isolation of patients’ tumor infiltrating lymphocytes (TILs) followed by T cell receptor (TCR) gene capture and high throughput sequencing. This enables the characterization of which tumor associated antigens (TAAs) tumor infiltrating lymphocytes are reactive to, elucidating the heterogeneity and targets of the tumor-infiltrating T cell pool (33). Using this technique, the T cell clones and corresponding TAAs that are associated with an improved anti-tumor response and survival can be determined, which can enable the development of new effective therapies (34, 35).
1.2.1 The cancer-immunity cycle

In order to mount an effective endogenous anti-tumor T-cell immune response, a series of highly regulated events is required. This sequence of iterative events regulates the balance between recognition and elimination of cancerous cells vs. the prevention of autoimmunity, and is described by Chen D.S. and Mellman I. as the cancer-immunity cycle (*Figure 1*) (36).

During cancer development, genetic instability results in the expression of newly acquired antigens. These antigens are not present in other parts of the body and are called neoantigens. In addition, a global loss of DNA methylation in cancer cells results in the expression of embryonal antigens that are

*Figure 1. The cancer-immunity cycle.* The generation of an anti-tumor T cell response is a cyclic, iterative series of highly regulated events. The cycle is initiated by the release of TAAs (1). TAAs are captured and presented by dendritic cells, which then prime and activate T cells in draining lymphoid organs (3). T cells migrate through the vasculature to the tumor microenvironment (4). At the tumor, T cells migrate past the endothelial layer (5), recognize TAAs presented on MHC-1 (6) and exert their cytotoxic activity (7). TAA=tumor-associated antigens.
commonly referred to as cancer/testis antigens. These tumor-specific characteristics enable the immune system to distinguish cancer cells from healthy cells and to mount a specific immune response. In the first step of the cancer-immunity cycle, immunogenic cell death caused by the growing tumor results in the release of danger signals and the expression of cytokines. Phagocytosis of tumor debris along with signals inducing dendritic cell (DC) activation leads to a migration of DCs to the lymph node and a switch to processing and presentation of antigens rather than engulfing them. The dendritic cells migrate to secondary or tertiary-lymphoid structures, where antigen presentation takes place. During antigen presentation, naïve T cells carrying a TCR specific for the TAAs are primed and activated. This stage of the immune response is critical, since the nature of the immune response, resulting in a T effector or T regulatory response, is determined here. Activated effector T cells (Teff) exit the lymphoid organs during step 4, after which they migrate through the bloodstream. At the tumor, activated T cells transmigrate across the endothelial layer (step 5). Once in the tumor microenvironment, T cells recognize TAAs presented on MHC-I on tumor cells through their TCR and are able to exert their cytotoxic activity. The killing of tumor cells results in more release of TAAs and the start of a new cycle from step 1 of the cancer-immunity cycle.

Unfortunately, the cancer-immunity cycle is not functioning optimally in cancer patients, preventing an effective anti-tumor immune response (36). Tumors employ a wide range of mechanisms acting on different stages of the immunity-cycle, which will be described further in the chapter on tumor immune escape and immunosuppression. The aim of cancer immunotherapy is to enhance the cancer-immunity cycle and to initiate an effective and self-sustaining anti-tumor cycle.

1.2.2 Main cell types involved in cancer-immunotherapy

The number of tumor-infiltrating leukocytes varies between tumor type, stage as well as between individual patients. Depending on the type of immune cell and their activation state, leukocytes may have anti-tumoral effects or conversely, promote tumor growth. Tumors with high leukocyte infiltration are termed “hot” while tumors with low or no infiltration are commonly termed “cold” tumors. An alternative to these are the coined terms of inflamed, excluded and desert tumors, relating to leukocyte-infiltrated tumors, tumors with leukocytes in the border but not infiltrating into the tumor core, or tumors with no signs of immune cells within nor around them.

**T lymphocytes**

T cells arise from lymphoid progenitor stem cells in the bone marrow and are the main effector cells of the adaptive immune response. Precursor T cells migrate to the thymus, in which they initiate the expression of the α- and β-
chain that together form the T cell-receptor (TCR). A process of positive and negative selection called central tolerance takes place in the thymus, which is aimed at preventing autoimmune responses. Upon recognition of an antigen, the TCR associates with CD3 and the immunoreceptor tyrosine-based activating motifs (ITAMs) induce intracellular activation signals. The CD4 or CD8 co-receptors associate with MHC-II and MHC-I on antigen presenting cells and can be used to distinguish T helper cells (Th) and cytotoxic T cells (CTLs). CD8+ CTLs are effector cells that kill target cells by the release of perforin and granzymes after recognition of an epitope presented on MHC-1. Moreover, CTLs express FasL on their surface, which induces apoptosis upon binding to Fas on target cells (37). CD4+ Th cells can be subdivided in the main subclasses of Th1, Th2, Th17 and Tregs. Th1 cells are associated with IFNγ, IL-2 and TNFα production, and are the main effector cell in anti-tumor responses. Th2 cells are involved in supporting B cell responses and eliminating extracellular parasites, and are mainly characterized by production of IL-4, IL-5 and IL-10. Th17 cells produce IL-17 and form another proinflammatory arm of the T cell response, although they have been shown to have divergent roles in tumor progression, from pro-tumoral angiogenesis induction and promotion of cancer survival to anti-tumor effects (38, 39). Lastly, Tregs are characterized by expression of CD25 and the FoxP3 transcription factor and their main function is to suppress all other Th responses.

**B lymphocytes**

B cells are an essential component of humoral adaptive immunity through the production and secretion of antibodies. Like T cells, B cells also arise from common lymphoid progenitor stem cells in the bone marrow. The immunoglobulin (Ig) genes encoding for the heavy and light chain, which in a heterodimer form the B cell receptor (BCR), are rearranged during B cell development (40). This results in a wide variety of specificities of BCRs, and after central tolerance selection, the immature B cells are released into the bloodstream. The BCR directly binds antigens without the need of presentation by MHC, enabling B cells to respond to a wide variety of antigens. Upon recognition of an antigen through the BCR, B cells migrate to germinal centres in secondary or tertiary lymphoid structures (TLSs). In the germinal centres B cells undergo a process of clonal expansion, somatic hypermutation and class-switching, which results in highly-antigen specific antibody-producing plasma cells (41). In tumor progression, the role of B cells is diverse. Antigen presenting B cells have anti-tumor effects in some glioma models (42) while B cell infiltration in epithelial cancer models promotes tumor development by facilitating chronic inflammation (43).

**Monocytes and macrophages**

Monocytes are myeloid-derived cells that can give rise to multiple mature cell types. Monocytes migrate from the circulation into inflamed tissues where
they can differentiate into macrophages or DCs. Once in the tumor microenvironment, the cytokine milieu determines the polarization state of macrophages, which can be simplified into a pro-inflammatory M1 state or a more immunosuppressive M2 state. NFκB signaling in macrophages is an important regulator of this polarization, together with IFNγ signaling inducing a more M1-like state while IL-4 and IL-10 suppress NFκB signaling and promote an M2 phenotype (44). M1 polarized macrophages promote Th1 responses by the secretion of TNFα, IL-6 and IL-12. Most tumor-infiltrating macrophages are M2-polarized, and their infiltration in the tumor microenvironment is consistently associated with a negative prognosis (29). M2 macrophages produce CCL17, CCL2 and CCL24, which subsequently results in the recruitment of Tregs and Th2 cells to the tumor microenvironment and are part of the normal wound healing processes in normal homeostatic conditions (45).

**Dendritic cells**

Dendritic cells (DCs) are a heterogeneous population of myeloid or lymphoid origin that play a central role by connecting the innate and adaptive immune systems (46). T cells require 3 signals for optimal activation, which are antigen presentation on MHC molecules, co-stimulatory molecules and proinflammatory cytokine stimulation. DCs are efficient antigen presenting cells (APCs) capable of providing all these signals both *in vitro* and *in vivo* and are therefore termed professional APCs.

Immature DCs are scavenger cells that have a high endocytic capacity (47). In order to become immunogenic, immature DCs require maturation signals. These cues may be from danger signals such as damage associated molecular patterns (DAMPs) or pathogen associated molecular patterns (PAMPs) that stimulate toll-like receptors (TLRs) and NOD-like receptors (NLRs). Upon activation, DCs downregulate their endocytic activity and upregulate costimulatory molecules such as CD80, CD86, CD40 and secrete proinflammatory cytokines. Maturation also induces expression of C-C chemokine receptor type 7 (CCR7), which is required for the migration into secondary lymphoid tissues where they present antigens to T cells (48). Mature dendritic cells process endocytosed antigens by proteasomal degradation, after which the resulting antigens are loaded onto MHC-I and MHC-II molecules and presented on the surface (49).

Although most cancers have DC infiltration, the ability of tumor-infiltrating DCs to present antigen and initiate an anti-tumor T cell response is often impaired. Tumors employ various mechanisms, including signals that inhibit phagocytosis and antigen uptake and the secretion of IL-10 and TGFβ to inhibit DC function and maturation (50). Due to this, tumor-resident DCs typically have poor antigen presentation capacity.
Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a collection of heterogeneous, immature myeloid cells, which are defined by their immunosuppressive effects. MDSCs are comprised of monocytic and granulocytic lineages, which are both able to promote tumor progression via the suppression of anti-tumor immune responses. MDSCs exert their immunosuppressive effects through a wide variety of mechanisms, including the induction of reactive oxygen species (ROS) (51), consuming nutrients required for cytotoxic T cell activity through upregulation of arginase 1 (52), and by upregulating inducible nitric oxide synthase (iNOS) expression which results in production of the immunosuppressive NO in the tumor microenvironment (53). MDSCs have also been shown to support the activation of Tregs as well as to inhibit Teff trafficking (50). Although the granulocytic subset is typically more abundant in many tumor types, the monocytic subset has been demonstrated to have more potent immunosuppressive effects (54).

1.2.3 Immune escape and immunosuppression

Despite the ability of the immune system to recognize and kill cancer cells, tumors do arise in humans with a fully functioning immune system due to the tumor cells’ ability to decrease their inherent immunogenicity or by inducing peripheral tolerance through interactions with the immune system. This process, termed immunoediting, is caused by continuous selection pressure by the immune system on tumor cells which results in progressively immunoresistant cancer variants (55). Figure 2 illustrates the evolution of immunoediting in 3 phases of Elimination, Equilibrium and Escape.

In the first phase, Elimination, stromal rearrangement and tissue damage caused by the growing tumor cells results in the release of damage associated molecular patterns (DAMPs) and the recruitment of innate immune cells such as NK cells and γδ T cells. Perforin, FasL and TRAIL-mediated killing of tumor cells through their cytotoxic activity results in the release of TAAs and the secretion of cytokines such as IFNγ and IL-12, which activate the adaptive immune response (4, 56, 57). During Equilibrium, the tumor and an adaptive immune response co-exist, resulting in a Darwinian selection process on the genetically unstable cancer cells. Continuous pressure from the adaptive immune response prevents tumor outgrowth and kills tumor cells with a high intrinsic immunogenicity, resulting in the selection of non-immunogenic tumor cells (3). The equilibrium phase may continue for an extended period of time, and in fact, it is known that many individuals carry in situ tumors that may never progress to disease (58). In the third phase, Escape, the tumor has undergone genetic and epigenetic changes, which provide it with sufficient
mechanisms to evade and/or suppress immune destruction. Tumor cells express negative regulatory receptors for T cells, secrete immunosuppressive cytokines (e.g. TGFβ and IL-10) and have recruited immunosuppressive leukocytes (e.g. M2-polarized macrophages, Tregs and MDSCs) to the tumor microenvironment. These accumulated adaptations lead to tumor outgrowth and clinically apparent disease.

The immunoediting hypothesis

Immunoediting to avoid immune detection can be summarized into three main mechanisms, which are (I) avoiding immune detection, (II) desensitization to effector mechanisms and (III) creation of an immunosuppressive environment. Avoiding immune recognition (I) includes the downregulation of MHC I expression, thereby decreasing self-antigen presentation (59), the upregulation of immune checkpoint molecules such as PD-L1 to induce T cell exhaustion (60, 61), as well as the reversible inflammation-induced downregulation of TAAs such as gp100 for melanoma (62). Desensitization to immune effector mechanisms (II) includes the upregulation of FasL expression to induce T cell apoptosis (63, 64) or acquired mutations in apoptotic pathways to avoid T-cell induced killing (65). Lastly, the creation of an immunosuppressive tu-
*mor microenvironment (III)* involves the secretion of immunosuppressive cytokines and the recruitment or induction of immunosuppressive cells such as Tregs and MDSCs (66).

1.3 Tertiary lymphoid structures

Tertiary lymphoid structures (TLSs) are transient ectopic lymphoid structures that arise during chronic, non-resolving inflammation. Their organization resembles secondary lymphoid structures (SLS) such as lymph nodes or Peyer’s patches, and they can have immunosuppressive or inflammation promoting roles directly at the site of inflammation. TLSs arise at the site of inflammation during autoimmune diseases, chronic infection, allograft rejection, atherosclerosis and in cancer. TLS structures have been described in a wide variety of tissues, including the synovial membrane of the joints (rheumatoid arthritis), salivary glands (Sjögren’s syndrome), the thymus (Mysasthenia gravis and Grave disease), the meninges (multiple sclerosis), the liver (hepatitis C viral infection), the lung (Influenza A viral infection), in atherosclerotic vessels and in or at the border of tumors (67, 68). They facilitate the initiation and maintenance of both cellular and humoral responses against tumors and provide a specialized site of leukocyte recruitment into tumors through the formation of high-endothelial venules (HEVs). In more than 10 types of cancer, the presence of TLSs is positively correlated with survival (69). Conversely, in some cases of colorectal cancer and breast cancer their presence has been associated with higher tumor grade and tumor progression (70, 71).

1.3.1 Organization of tertiary lymphoid structures

TLSs encompass a spectrum of lymphoid tissue-like organization, with varying levels of maturity. They are described as primarily B-cell rich infiltrates, with varying degrees of T-cell infiltration. Mature TLS organization more closely resembles SLS, with a segregation between a densely packed follicle of B cells which is surrounded by T cell compartments. The vessels inside TLSs specialize into high endothelial venules, which are characterized by a plump morphology and expression of L-selected ligands such as peripheral node addressin (PNAd) (72). Unlike SLSs, TLSs are not encapsulated and are not supplied by afferent lymphatic vessels. Gene expression is characterized by the recruitment of leukocytes, with high expression levels of chemoattractants such as CCL19, CCL21 and CXCL13 (73).

Mature TLSs are further characterized by expression of the Bcl-6 transcriptional repressor, which is expressed in follicular B cells and T follicular helper (Tfh) cells (74). B cells in the core of the follicle are highly proliferative and are surrounded by a reticular network of CD21⁺ and CD35⁺ follicular dendritic
cells (75). In some TLSs, highly differentiated antibody-producing CD138+ plasma cells can also be found. A variety of TLSs have been found in cancer, ranging from immature TLSs consisting of only a few core components to mature TLSs which exhibit all of the key components (76).

1.3.2 Tertiary lymphoid structure neogenesis

SLSs are genetically preprogrammed to develop at specific sites in the body, with lymph node (LN) and Peyer’s patch (PP) development initiated prenatally while mucosal-associated lymphoid tissues (MALT) development is initiated postnatally (77). These SLSs form a network that facilitates continual immune surveillance in the body. TLSs develop in a similar manner to SLS, although they are transient, developing only at the site of chronic inflammation. In this way, TLSs are an extension of SLSs, facilitating immune function directly at the site of inflammation.

Formation of SLSs and TLSs is initiated by the engagement of the LTβR. The LTβR has two ligands, which are LTα1β2 and LIGHT (Figure 3). For the formation of LTα1β2, expression of both LTα and LTβ is necessary. LTα may be secreted as a soluble trimer which can activate TNFR signaling. It is also required for the formation of the LTα1β2 heterotrimer in intracellular vesicles and its translocation to the surface (78). Ectopic expression of LTα1β2 alone is sufficient to induce lymphoid neogenesis and for the generation of TLSs (79, 80). Germinal centers and follicular dendritic cell networks fail to form in LTα deficient mice, indicating the necessity of lymphotoxin signaling to induce lymphoid formation (81). Lymphotoxin expression directly induces expression of homeostatic chemokines such as CXCL13, CCL19 and CCL21, which are associated with TLS formation and are involved in the recruitment

![Figure 3. Selected members of the TNF receptor superfamily.](image)

**Figure 3.** Selected members of the TNF receptor superfamily. CD40 forms a trimer upon binding its ligand CD40L. TNF and soluble LTα3 are ligands of TNFR 1 and 2. LTα1β2 and LIGHT are ligands of the LTβR and LIGHT is also a ligand of the HVEM receptor.
of leukocytes (73). CD3−CD4+IL7Rα+ innate lymphoid cells called lymphoid tissue inducer cells (LTi) are the main cell type responsible for SLS development (80). For TLS development, also other cell types including Th17 cells, Tfh cells, NK cells and B cells have been shown to be able to replace LTis in TLS development (82).
2. The aims of this thesis

Since the clinical approval of CTLA-4 targeting antibodies in 2010 and the introduction of antibodies targeting PD-1 and PD-L1, immune-modulating antibodies have established themselves as a mainstay in cancer immunotherapy. The overall aim of this thesis is to investigate the mechanisms of antibody-based immunotherapies and the role of the tumor microenvironment in mediating the anti-tumor immune response, in order to aid the development of improved immunotherapies for cancer patients. This is pursued by investigating optimizations of existing antibody immunotherapies and by employing novel approaches for antibody-based immunotherapies. The specific objectives for each paper are listed below and a visual representation of the main focus of each paper represented on the cancer-immunity cycle is shown in Figure 4.

2.1 Paper I

Agonistic CD40 antibodies have proven successful at generating an anti-tumor CD8$^+$ T-cell response in a wide variety of preclinical models and several clinical grade antibodies are currently in clinical trials. Anti-angiogenic therapies are approved in the clinic and have been suggested to have complementary and non-overlapping anti-tumor effects through the induction of vessel normalization and thereby enhancing CD8$^+$ T cell infiltration into the tumor microenvironment. The aim of paper I was to investigate if the efficacy of a local low-dose administration of agonistic CD40 antibodies can be improved by co-administration of anti-angiogenic therapy in the form of the tyrosine kinase inhibitor sunitinib.

2.2 Paper II

Checkpoint blockade of CTLA-4 introduced a new type of therapy for cancer patients into the clinic in 2010, and CTLA-4 checkpoint blockade combined with PD-1/PD-L1 antibodies has better efficacy than either monotherapy.
However, CTLA-4 antibodies are associated with severe adverse events, especially when combined with PD-1/PD-L1 checkpoint blockade. The aim of this paper was therefore to investigate the efficacy of local low-dose administration of CTLA-4 antibodies on anti-tumor efficacy and systemic immune effects.

2.3 Paper III

Monoclonal antibody immunotherapies are effective, but are expensive to produce and put a heavy burden on the healthcare economy. Therapeutic vaccination aimed at inducing endogenous anti-self antibodies is a potentially more economic approach. Galectin 1 is expressed by many cancers and promotes tumor progression through a wide variety of mechanisms, including the promotion of angiogenesis and the suppression of anti-tumor immunity. The aim
of paper III was to investigate the efficacy of a vaccination strategy aimed at inducing endogenous antibodies against Galectin-1.

2.4 Paper IV

Agonistic CD40 antibodies have a wide variety of therapeutic mechanisms, and their efficacy has not yet been investigated thoroughly for glioblastoma. The aim of this paper was therefore to investigate the efficacy of agonistic CD40 antibodies against glioblastoma, to investigate their effect on the formation of tertiary lymphoid structures (TLS) in the brain and the role of these structures in modulating the anti-tumor immune response.
3. Antibody immunotherapy

Monoclonal antibody immunotherapy has recently achieved clinical success with antibodies blocking immune checkpoint molecules CTLA-4 and PD-1. The CTLA-4 antibody Ipilimumab was approved for advanced malignant melanoma by the United States Food and Drug Administration (FDA) in 2011 (83) and by the European Medicines Agency (EMA) in 2012 (84). Two PD-1 antibodies called Nivolumab and Pembrolizumab gained clinical approval in 2014 for the indications of melanoma, non-small cell lung cancer and renal cell carcinoma (85). Since then, a number of other antibodies targeting CTLA-4 or the PD-1/PD-L1 axis have been approved for the clinic (Table 1) (86).

<table>
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<tr>
<th>Generic name</th>
<th>Trade name</th>
<th>Isotype</th>
<th>Target</th>
<th>Indications</th>
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<tr>
<td>Nivolumab</td>
<td>Opdivo</td>
<td>IgG4</td>
<td>PD-1</td>
<td>Melanoma, bladder cancer, squamous cell lung cancer, RCC</td>
</tr>
<tr>
<td>Pembrolizumab</td>
<td>Keytruda</td>
<td>IgG4</td>
<td>PD-1</td>
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<tr>
<td>Durvalumab</td>
<td>Imfinzi</td>
<td>IgG1</td>
<td>PD-L1</td>
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<tr>
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<td>IgG1</td>
<td>PD-L1</td>
<td>Merkel-cell carcinoma, bladder cancer</td>
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<tr>
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<td>CTLA-4</td>
<td>Melanoma</td>
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Historically, patients with grade IV melanoma had an average OS of 8 to 10 months and the 5-year survival from diagnosis was 10% for approved therapies. A recent long-term review which analyzed long-term survival data from Phase II and Phase III clinical trials with Ipilimumab indicated a median OS of 11.4 months, but with a plateau in the survival curve between 20% and 26% from 3 years up to 10 years after start of treatment (87). A recent phase III study of combined Ipilimumab and Nivolumab treatment or single agent alone resulted in a similar plateau around 20% for Ipilimumab but a plateau of 36%
for PD-1, although long-term survival remains to be confirmed for this trial (88). Thus, these new therapies do not just prolong life as is commonly seen for radio- and chemotherapy, but result in significant and durable clinical responses in 20-40% of the treated patients.

3.1 Antibody structure and isotypes

Antibodies belong to the family of globular proteins called immunoglobulins (Ig) that are characterized by their versatility in antigen binding, high affinity for their target antigen, and their biological activity. Based on their heavy chain structure, 5 subclasses of antibodies can be distinguished, namely IgG, IgA, IgE, IgM and IgD (89). The most abundant in the circulation are IgG antibodies, which are also the main form of antibodies during the secondary immune response and are responsible for humoral immunity (90). IgG are large (150 kDa) Y shaped molecules which consist of two heavy chains (H, grey) and two light chains (L, blue) which are linked together by disulfide bonds (Figure 5). The stem of the Y shape is called the Fc region, which is connected with a flexible hinge region to the Fab (fragment antigen binding) fragments.

Both the heavy chains and the light chains contain constant and variable domains. The variable regions of the heavy ($V_H$) and light chains ($V_L$) together form the antigen binding cleft which determines the specificity of the antibody. This binding specificity of the variable regions is determined by changes

![Figure 5. Structure of Immunoglobulin G.](image)

IgG consists of two heavy and two light chains which are connected by disulfide bridges. The antigen binding specificity is determined by the variable regions of both chains ($V$) The right panel is a schematic representation of an IgG molecule with constant (C) and variable (V) regions. The left panel is a schematic representation of human IgG subtypes illustrating the variation of disulfide bonds in the hinge, CH1 and CH2 regions.
in amino acid residues caused by a process called V(D)J or somatic recombination, which happens during the early stages of T- and B-cell development in the primary lymphoid organs.

In humans and mice 4 IgG isotypes exist, although in humans these are IgG1, IgG2, IgG3 and IgG4 while mice have IgG1, IgG2a, IgG2b and IgG3 (3). Class switching is the mechanism by which B cells change the constant regions of the antibody while keeping the variable region and thus preserving its antigen specificity (91). Class switching is influenced by factors such as the route of antigen exposure, the chemical composition of the antigen as well as stimulation of the antibody producing B cells through Toll-like receptors (TLRs), CD40-CD40L, cytokines and helper T cells (91). The IgG isotypes differ mostly in their hinge and C\textsubscript{H}2 domains, which is the region that determines their affinity of C1q as well as Fc receptors and thereby impart differential biological activities to the antibody subclasses.

3.2 Fc receptor functions and affinities

The antibody isotype influences its biological activity through differential affinity for Fc receptors as well as differential activation of the complement system. For example, the neonatal Fc receptor (FcRn) which is responsible for the transport of immunoglobulins across the placental barrier binds preferentially to IgG1, resulting in higher levels of maternal IgG1 in newborn babies (92). The long half-life of both endogenous antibodies and monoclonal therapeutic antibodies is also dependent on the FcRn interaction. Besides FcRn, there are 6 human Fc receptors from 3 different subclasses that bind IgG (Figure 6A) while in mice 4 Fc receptors from 4 different subclasses have been identified (Figure 6B) (93). Each receptor has a differential binding affinity for monomeric IgG isotypes and the expression level of Fc receptors on various leukocytes varies. Therefore, the biological activity is influenced by (I) the antibody isotype, (II) the affinity of the isotype for Fc receptors and (III) the bioavailability of Fc receptors. The affinity for an IgG will also change when it is present in a complexed format, such as when binding antigens (93).

In humans, Fc\textgammair, Fc\textgammairia, Fc\textgammairic and Fc\textgammairiaa are activatory Fc receptors that signal through the ITAM domains of either the common \(\gamma\) chain dimer (in the case of Fc\textgammair and Fc\textgammairiaa) or on their transmembrane \(\alpha\) chain (in the case of Fc\textgammairia and Fc\textgammairic). Conversely, Fc\textgammairib is an inhibitory receptor
which signals through its ITIM domain and does not have an intracellular signaling domain. In contrast, human FcγRIIIB and murine FcγRII have an inhibitory ITIM signaling domain.

3.2.1 Optimizing Fc receptor interactions for immunotherapy

Engagement of Fc receptors can mediate the biological functions of antibodies. For example, NK cells have high expression of FcγRIIA, and the binding of complexed IgGs results in antibody-dependent cell-mediated cytotoxicity (ADCC) (94). Activatory and inhibitory FcγRs activate opposing downstream
signaling pathways through their ITAM and ITIM domains. A balance between engagements of both types of receptors determines the outcome of the immune response on some cell types where both activatory and inhibitory FcRs are expressed, such as macrophages, neutrophils and dendritic cells. Human IgG1 or mouse IgG2a isotypes have a high binding affinity for activatory FcRs and a low affinity for inhibitory FcRs (Figure 6), and this high activatory compared to inhibitory ratio (A:I ratio) makes these isotypes the preferred choice for tumor-targeted antibodies. In contrast to antibodies reliant on ADCC, agonistic antibodies against members of the TNF receptor superfamily have enhanced downstream signaling with a higher affinity for the inhibitory FcγRIIB (83, 95) and alternative isotypes may be beneficial for these antibodies, as will be discussed in more detail later.

3.3 T cell exhaustion and immune checkpoint molecules

In chronic inflammatory conditions such as chronic viral infections and cancer, persistent antigen exposure and inflammatory stimuli result in a loss of T cell effector function, which is termed exhaustion. Exhausted T cells exhibit altered transcriptional programming, resulting in sustained expression of negative regulatory molecules (e.g. PD-1, CTLA-4, TIM-3, LAG-3, BTLA, TIGIT), decreased effector cytokine production (e.g. IL-2, TNFα, IFNγ) and decreased cytolytic activity (e.g. GranzymeB) (96).

T cell exhaustion was first identified in mice with chronic viral infections (97, 98). Transcriptional analysis of CD8+ T cells during acute vs chronic infections has revealed that exhaustion negatively regulates the effector function, involving the upregulation of molecules such as PD-1, LAG-3 and TIM-3, the soluble mediators IL-10 and TGFβ, and the transcription factors Prdm1 (Blimp-1) and Batf (99). In contrast, there is a transcriptional upregulation of activation and effector function transcriptional programs in CD8+ effector cells during acute infections. During chronic viral infections, exhausted CD8+ T cells downregulate effector-like transcriptional programs (involved in cell cycle, DNA replication and RNA processing) while maintaining increased expression of activation transcriptional programs (e.g. transcription factors T-bet (Tbx21), Batf, Stat3, and Blimp-1 (Prdm1)) (100). These data suggest that the inability to resolve a chronic inflammation results in an active regulation into an exhausted phenotype of T cells, which is partially overlapping with effector cells, but distinct from memory T cells that arise after the resolution of a normal infection.

Although exhausted T cells are not considered completely inert, blocking immune checkpoint molecules such as CTLA-4 and PD-1 has shown that reversing the T cell exhaustion status to a more effector function is a successful
strategy for immunotherapy. An understanding of the phenotypic changes T cells undergo due to the chronic inflammatory stimuli provided by tumors will (I) assist in improving staging and diagnosis through immunoscopying (as discussed previously), (II) provide tools to aid in the evaluation of the efficacy of immunotherapy and (III) may lead to the development of new targets for immunotherapy.

3.3.1 Cytotoxic T lymphocyte antigen-4

Positive and negative co-stimulatory molecules regulate the activation of T cells by APCs (101). During antigen presentation, antigens are presented on MHC molecules by APCs. The recognition of MHC with its bound antigen by a T cell receptor (TCR) represents the first signal required for T cell activation. Co-stimulatory signaling between CD28 on the T cell and B7 molecules CD80 or CD86 on APCs represents the second required signal. Members of the TNF receptor superfamily such as CD40 and CD137 provide co-stimulatory signals while CTLA-4, PD-1, TIM-3 and LAG-3 provide negative co-stimulatory signals. These negative co-stimulatory signals are termed immune checkpoints. A selection of the main molecules involved in the interaction between an APC and a T cell during antigen presentation are shown in Figure 7.

Upon TCR engagement, the Src family kinases phosphorylate the Y-165 and Y-182 sites on the YVKM motif of the cytoplasmic tail of CTLA-4, which abolishes its association with the clathrin adaptor protein AP. This results in the translocation of CTLA-4 from intracellular vesicles to the surface membrane (102, 103). CTLA-4 is a member of the same family as co-stimulatory molecule CD28, although it has a higher affinity for their ligands, the B7 co-stimulatory molecules CD80 and CD86. CTLA-4 thereby competes with CD28 for binding to B7 molecules and acts as a negative feedback loop on T cell activation (104, 105). Moreover, ligation of CTLA-4 abrogates the ZAP70 microcluster formation responsible for stabilization of the cSMAC, reducing Ca^{2+} influx and the contact time between APCs and T cells (106). Downstream signaling of CTLA-4 attenuates T cell activity through the inhibition of cell cycle proliferation, IL-2 production as well as IL-1 receptor expression (107). Treatment with anti-CTLA antibodies blocks the interaction of CTLA-4 with B7. This results in an increase of CD4^+ICOS^+ Tregs and increased IFNγ expression by these cells in both the circulation and the tumor microenvironment (108). The ICOS/LICOS co-stimulatory pathways are demonstrated to be partially required for the anti-tumor effect of CTLA-4 checkpoint blockade in experimental animals models (109).
Another mechanism of anti-CTLA-4 therapy is the depletion of T regulatory cells, which express high levels of CTLA-4, in the tumor microenvironment. ADCC of intratumoral Tregs and overall therapeutic efficacy is superior with IgG2a antibodies compared to IgG2b and IgG1, which is in line with the higher affinity of IgG2a for activatory Fc receptors (110). Interestingly, this depletion is dependent on intratumoral macrophages expressing FcyRIV, but not FcγRI (111). Humans do not have an equivalent Fc receptor for the FcγRIV in mice and whether CTLA-4 antibodies affect Tregs in humans remains to be elucidated.
Clinical success and side effects of CTLA-4 antibodies

In 2010, a landmark phase III clinical trial enrolling more than 600 melanoma patients demonstrated improved survival for patients treated with Ipilimumab (112), which lead to the clinical approval for first-line treatment of advanced malignant melanoma in the U.S.A. in 2011 (83) and in the European union in 2012 (84). Although anti-CTLA-4 blocking therapy can induce anti-tumor responses, systemic anti-CTLA-4 therapy does not selectively enhance only the tumor-specific immune response, resulting in autoimmune related adverse events (113). The most prevalent side effects are observed in the skin, intestine, liver and endocrine glands. Skin rash is the most common symptom with the earliest onset at 2 weeks after treatment initiation. Elevation of hepatic AST and ALT are also commonly found, although hepatotoxicity remains asymptomatic in most patients (114). Endocrinopathies of the pituitary, adrenal and thyroid glands include symptoms such as fatigue, nausea and headaches. Although not the most common, colitis / diarrhea is perhaps the most clinically relevant side effect which has resulted in some treatment related deaths in early clinical trials (112).

Immune related adverse events can be managed clinically by immunosuppressive medication, such as with corticosteroids, TNF antagonists or mycophenolate mofetil. Prolonged immunosuppressive medication which is often required for Ipilimumab treatment may however result in opportunistic infections (115). A correlation between the occurrence of immune related adverse events and the clinical efficacy has been reported in clinical trials (116, 117). Due to this fact, clinicians face a delicate balance between maintaining anti-tumor responses and managing adverse events associated with anti-CTLA-4 treatment.

3.3.2 Programmed death 1

Similarly to CTLA-4, programmed death 1 (PD-1) is a member of the family of B7/CD28 costimulatory receptors. PD-1 has two ligands, PD-L1 and PD-L2, and it negatively regulates T-cell proliferation, survival, and the production of proinflammatory cytokines such as IFNγ, TNFα and IL-2 (118). Coincident engagement of the TCR and PD-1 prevents phosphorylation of signaling molecules downstream of TCR activation and thereby prevents T-cell activation (119). Unlike CTLA-4, which functions during the T-cell priming phase, PD-1 therefore inhibits T-cell function during the effector phase.

PD-1 expression is upregulated in activated T cells that have received insufficient CD4+ T-cell help and is a characteristic of an exhausted T-cell phenotype (99). The ligands PD-L1 and PD-L2 are expressed in a wide variety of cells.
PD-L1 expression is found on leukocytes, nonhematopoietic cells and in non-lymphoid tissues and is induced by inflammatory IFNγ signaling (120). PD-L2 expression is more restricted, being predominantly expressed in DCs and monocytes. Besides IFNγ signaling, PD-L1 expression is also induced by PI3K-Akt kinases, which is a commonly affected pathway in cancer development. PD-L1 expression is therefore found in many different types of cancer, and its expression by tumors is associated with increased T cell infiltration and a poor prognosis (60).

The first PD-1 targeting antibody approved for the clinic was Nivolumab, which gained FDA approval in 2014 and in the EU in 2015. Since then, antibodies targeting both PD-1 and PD-L1 have been approved for a wide range of cancers, including melanoma, non-small cell lung cancer (NSCLC), bladder cancer, head and neck squamous cell carcinoma (HNSCC), Hodgkin's lymphoma and Merkel-cell carcinoma (Table 1) (86).

3.3.3 Galectin-1
Galectin-1 (Gal-1) is a member of the family of β-galactoside-binding proteins. It contains a carbohydrate recognition domain which enables binding to N- and O-linked glycans (121). Gal-1 can be found intracellularly or extracellularly and mediates a wide range of functions, from neural stem cell growth, to hematopoietic differentiation, muscle differentiation, endothelial cell functions and angiogenesis (121, 122). In cancer, Gal-1 is found on the surface of tumor-endothelial cells as well as in the serum, and increased systemic levels of Gal-1 are associated with decreased survival (123, 124). Gal-1 is upregulated in tumors refractory to antiangiogenic therapy, and it can directly bind complex N-glycans on VEGFR2, activating the signaling downstream of VEGFR2 in the absence of its VEGF (125). Besides its proangiogenic role, Gal-1 also promotes tumor growth through immune evasion. Expression of Gal-1 by both tumor cells and tumor endothelial cells induces apoptosis of T cells (126, 127). Furthermore, Gal-1 regulates effector T cell polarization and induces a tolerogenic phenotype in DCs (128, 129). Expression of Gal-1 is also required for regulatory B-cell function in response to agonistic CD40 antibodies (130).

3.4 Agonistic CD40 targeted immunotherapy
CD40 is a member of the TNF receptor superfamily which is constitutively expressed on B cells, dendritic cells, macrophages, T cells as well as non-hematopoetic cells such as the endothelial cells, fibroblasts and epithelial cells (131). Its ligand, CD40L, is expressed mainly on activated T cells or by platelets, the latter of which are the main source of soluble CD40L (132). Soluble
CD40L is secreted as a trimer and its activation requires clustering on the cell membrane, which involves association of CD40L with p53 resulting in the formation of ceramide-enriched sphingolipid membrane micro domains which function as signaling platforms (133). Trimerized CD40L in either its soluble or membrane-bound form can effectively cluster CD40 on the target cell, which subsequently enables downstream activation of CD40 signaling (Figure 8). The intracellular domain of CD40 contains binding domains for Janus kinase 3 (JAK3), TNF receptor associated factor (TRAF) 2, TRAF 5 and TRAF 6, through which CD40 activates signaling pathways including NF-κB (Nuclear Factor-KappaB), MAPK (Mitogen-Activated Protein Kinase) and STAT3 (Signal Transducers and Activators of Transcription-3) (134, 135). Downstream activation is dependent on cell type. CD40 signaling on B cells is required for survival and regulates humoral immunity through regulation of affinity maturation and heavy chain class switching. Dendritic cell stimulation of CD40 is an important regulator of cellular immunity, as it licenses dendritic cells to initiate a T-cell response through the induction of co-stimulatory molecules and the secretion of cytokines such as IL-12 (136, 137).

3.4.1 Antibody design for agonistic CD40 antibodies

Antibodies targeting CD40 have increased efficacy when their Fc domain has a higher affinity for FcγRIIB, which is paradoxical given the activation of inhibitory pathways downstream of this receptor (83, 95). Experiments with co-administration with TLR3 agonists that induce activatory Fc receptor expression indicate that these receptors also provide the Fc receptor cross-linking and enhance downstream CD40 signaling, albeit less efficiently than FcγRIIB (138). In addition, cross-linking antibodies with anti-Fc F(ab)2 fragments or designed multimeric anti-CD40 antibodies also impart a higher agonistic activity (138, 139). These findings therefore indicate that the improved efficacy

**Figure 8. CD40 signaling by CD40L and agonistic CD40 antibodies.** Activation of CD40 signaling is mediated by clustering of CD40 on the membrane, which can occur either by trimerized CD40L or by agonistic CD40 antibodies.
for antibodies with a high affinity for FcγRIIB does not rely on the signaling provided by FcγRIIB nor on the cell expressing FcγRIIB. More likely, FcγRIIB cross-linking provides the clustering of CD40 required for optimal downstream signaling in a similar fashion as soluble CD40 ligand trimers or clustering of CD40L in sphingolipid membrane micro domains (Figure 8).

The group of White et al. has demonstrated a second mechanism of increased agonistic potency of human IgG2 agonistic CD40 antibodies (140). Human IgG2 is unique in its ability to rearrange disulfide bonds between its CH1 and hinge region after synthesis, resulting in isoforms with distinct conformations, called IgG2a and IgG2b. The IgG2b form has a disulfide bond conformation that does not exist in murine antibodies. This conformation reduces the flexibility of the hinge region and is thereby imparts Fc-receptor independent agonistic properties to the antibody (140).

Another important determinant of agonistic properties of CD40 antibodies is the epitope specificity. CP-870,893 is a fully humanized IgG2 antibody with superagonistic properties that are independent of Fc receptor cross-linking or antibody isotype (139). A Phase I clinical trial with CP-870,893 had promising results with a partial response observed in several melanoma patients, and the dose limiting toxicity of CP-870,893 was determined to be 0.3 mg / kg body weight (141). Other examples of agonistic CD40 antibodies tested in clinical trials to date are dacetuzumab and Chi Lob 7/4, which can be given at higher doses, with dose limiting toxicities of 12 mg / kg (142). Thus, the optimal design of agonistic CD40 antibodies needs to take into consideration the isotype, Fc receptor cross-linking dependency as well as epitope specificity.

**Local administration: boosting the T cell response while reducing side effects**

In line with the expression of CD40 on different cell types, a wide range of effects of agonistic CD40 treatment have been reported in experimental animal models. These reported mechanisms include apoptosis induction by direct binding to CD40+ tumor cells, activation of CD40 expressing macrophages in the tumor microenvironment as well as increased NK cell activity following CD40-induced activation on dendritic cells (143). The most important therapeutic mechanism however is the CD40-induced activation of dendritic cells, which increases their cross-presentation of antigen and licenses a cytotoxic anti-tumor T-cell response (142, 144, 145).

An important consideration for the clinic is the dose limiting toxicity reported in early clinical trials. High CD40 stimulation results in systemic cytokine release. Early administration of IL-6 neutralizing antibody Tocilizumab is an effective strategy if administered early at the onset of symptoms. Regardless, experimental models indicate that local administration decreases circulating
antibody levels and side effects while maintaining the anti-tumor effect (146-148). In line with this, a recently executed phase I clinical trial with ADC1013 (now JNJ-64457107) employed intratumoral injections of an agonistic CD40 targeting antibody (149, 150). Intratumoral injections into non-hepatic lesions at doses up to 400 μg/kg were well tolerated, whereas hepatic injections were associated with a higher adverse event profile (151).
4. Targeting the tumor vasculature

The vascular system is a tree-like structure, which pervades the entire body and transports oxygen, nutrients, signaling molecules, circulating cells and waste products to and from the organs. The inner surface of vessels is lined with endothelial cells which are supported by extracellular matrix proteins called the basal lamina. Larger vessels are surrounded by smooth muscle cells for further support and for regulating blood pressure and vascular tone, while the smaller vessels or capillaries are covered by pericytes (152). Angiogenesis, the growth of new blood vessels, is required for tumors to grow larger than approximately 1-2 millimeters in size due to a requirement of nutrient and oxygen for further growth (153).

4.1 Tumor angiogenesis and anti-angiogenic therapy

The induction of angiogenesis is regulated by hypoxia, which induces the expression of vascular endothelial growth factor (VEGF) and other pro-angiogenic growth factors (154, 155). Hypoxia inducible factor-1 (HIF-1) is a heterodimeric transcription factor of which the subunits HIF-1α and HIF-1β are constitutively expressed in most cells. Under normoxic conditions, HIF-1α is rapidly degraded by the proteasome preventing HIF-1 transcriptional activation. However, under low oxygen pressure HIF-1α is stabilized and accumulates, resulting in heterodimer formation with HIF-1β and transcriptional induction of pro-angiogenic factors (156). The main pro-angiogenic factor is VEGF-A (commonly called just VEGF), and its binding to VEGFR2 leads to dimerization and phosphorylation of this tyrosine kinase (157). VEGFR2 signaling in endothelial cells regulates cell division, survival, sprouting and migration. Other important angiogenic growth factors include the family of angiopoietins and their cognate receptor TIE-2 (158) as well as the fibroblast growth factor (FGF) family (159).

4.1.1 The angiogenic switch and anti-angiogenic therapy

The angiogenic switch is a change in the angiogenic signature of early developing lesions, by which increased VEGF signaling enables the tumor to grow. Several mechanisms that contribute to the angiogenic switch have been reported, such as an oncogenic mutation in the gene coding for the von Hippel-
Lindau protein which is responsible for the ubiquitin ligase complex-dependent degradation of HIF-1α and thereby results in sustained angiogenic signaling (154). In addition, myeloid cells including macrophages, neutrophils, mast cells and MDSCs which are recruited to the peritumoral margins stimulate angiogenic signaling and thereby sustain angiogenesis and tumor growth (160-162).

In 1971, Folkman proposed that given the dependence of tumors on angiogenesis for their supply of nutrients and oxygen, anti-angiogenic treatment could be employed to arrest tumor growth and treat cancer (163). This has led to the clinical approval of several anti-angiogenic agents. The first was Bevacizumab (Avastin), an antibody targeting VEGF that prevents binding of VEGF to its receptor and thereby inhibits angiogenesis (164). Another class of anti-angiogenic treatments is small molecule tyrosine kinase (RTK) inhibitors, which block the ATP binding pocket of VEGFR and thereby prevent downstream signaling, regardless of VEGF stimulation. A disadvantage of RTK inhibitors is their limited specificity and therefore wide range of side effects. For example, the clinically approved sunitinib does not just block VEGFR2 but also other members of the VEGF family, PDGFR-α/β, FLT-3, c-KIT and c-RET (165).

A total of 10 anti-angiogenic drugs have been approved to date (166). The first clinically approved drug, Bevacizumab, slows tumor growth and prolongs survival in patients with non-small cell lung and colorectal cancer, though with only a marginal improvement of long-term survival (167, 168). In other types of tumors including breast, melanoma, pancreatic and prostate cancer no improvement of OS has been observed (169).

4.1.2 Resistance to anti-angiogenic therapy

Treatment of colorectal cancer patients with Bevacizumab results in an initial response with decrease tumor growth or even regression. However, relapse is common and tumor regrowth is often more aggressive than before anti-angiogenic treatment (170). In glioblastoma patients, Bevacizumab results in an improvement of PFS in glioblastoma patients, but not in an improvement of OS (171). Several mechanisms have been proposed for the resistance to anti-angiogenic treatment, which include co-option of normal vessels from local tissue, recruitment of pro-angiogenic myeloid cells and an upregulation of alternative pro-angiogenic signals such as angiopoietins, FGFs, EGFs and HGFs (153). In addition to resistance, anti-angiogenic treatment by sunitinib has been suggested to increase invasiveness and metastasis formation in experimental models (172), although this has not been observed in patients with renal cell carcinoma which have received sunitinib treatment (173).
4.2 Endothelial activation and leukocyte recruitment

Endothelial cells form a barrier between the circulation and the underlying tissue. They are directly involved in the regulation of inflammation through the facilitation of leukocyte transmigration as well as the expression of immune modulatory factors including cytokines and chemokines. Endothelial activation is a crucial and well-characterized change in endothelial phenotype which facilitates the transmigration of leukocytes into underlying tissues such as the tumor microenvironment (174). The most rapid type of endothelial activation which is independent of gene expression is the G protein coupled receptor (GPCR)-mediated translocation of P-selectin to the surface membrane and the activation of nitric oxide (NO) synthesis (174). NO induces vasodilation and increases blood flow, increasing the amount of leukocytes passing the activated endothelium. A more sustained form of endothelial activation can be induced mainly through TNFa and IL-1, which activate the downstream canonical NF-κB pathway and lead to upregulation of selectins and adhesion molecules (175). Although the genes upregulated by these factors differ due to endothelial heterogeneity (176), commonly upregulated adhesion molecules include Intercellular adhesion molecule-1 (ICAM-1), Vascular cell adhesion molecule-1 (VCAM-1) and E-selectin while upregulated chemokines include CXCL10 and CXCL11 (177, 178).

The process of leukocyte rolling, arrest, adhesion and transmigration following endothelial activation has been well characterized and reviewed by the group of E.C. Butcher (Figure 9) (179). Leukocyte recruitment to tissues is mediated by interaction of specific combinations of chemoattractants with their receptors. The subsequent capture and rolling is mediated by endothelial E-selectin and P-selectin with P-selectin glycoprotein ligand 1 (PSGL1), E-selectin ligand 1 (ESL1) and CD44 on leukocytes. The interaction between selectins and their ligands is relatively weak, allowing for a loose interaction and rolling along the endothelial cell membrane. Next, slow rolling and arrest are mediated by integrins such as lymphocyte function associated antigen-1 (LFA-1), very late antigen-4 (VLA-4 or α4β1-integrin) and α4β7-integrin which interact with, respectively, ICAM-1, VCAM-1 and mucosal vascular addressin cell adhesion molecule (MAdCAM-1) expressed on endothelial cells. Following firm adhesion, leukocytes transmigrate through the endothelial cell layer via either the paracellular route or the transcellular route. The paracellular route is thought to be the main route of transmigration, for which interaction between ICAM-1 and macrophage antigen-1 (MAC-1) is required to induce endothelial cell contraction and opening of endothelial cell junctions, allowing the leukocytes to pass through.
Figure 9. Leukocyte-endothelial cell interactions during recruitment. Schematic representation of the leukocyte adhesion cascade and the major molecular regulators involved in each step.

The expression of adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 on the tumor vasculature is correlated with T-cell recruitment to the tumor microenvironment, underscoring the importance of endothelial activation for immunotherapy (181-183).

The tumor microenvironment plays an important role in modulating endothelial activation and the recruitment of leukocytes to the tumor. For example, angiogenic growth factors such as FGF and VEGF inhibit TNFα-induced expression of ICAM-1 and VCAM-1 on endothelial cells (184) as well as the expression of chemokines that attract T cells such as CXCL10 and CXCL11 (177). Tumors can induce endothelial cells to suppress cytotoxic cell activity, as tumor conditioned medium from Lewis lung carcinoma (LLC) induces endothelial cells to inhibit NK and T cell functions (185). Moreover, endothelial cells directly mediate tumor immune evasion through expression of T-cell inhibitory molecules such as PD-L1, PD-L2 and TIM-3 (186, 187). The type of leukocytes recruited to the tumor by the endothelium is also important to consider, as an increased infiltration of macrophages and immature myeloid cells has been associated with decreased survival (162).

4.3 Vascular abnormalization
In normal conditions such as wound healing, the formation of new vessels will ameliorate tissue hypoxia resulting in a decrease of pro-angiogenic signaling and a maturation of the blood vessels. Due to persistent proliferation of tumor cells, hypoxia and the pro-angiogenic signals provided by the inflammatory tumor stroma remain high. This continuous pro-angiogenic milieu results in
vascular malformations and a hierarchically disorganized, tortuous vascular phenotype (188). Tumor vessels can further be characterized by their inefficient perfusion, rough endothelial lining including endothelial projections into the vessel lumen, lack of pericyte coverage and increased permeability (189, 190).

4.3.1 Vessel normalization

Despite the limited success of anti-angiogenic treatments as single agent therapies, inhibition of angiogenic signaling may result in a normalization of the vasculature and can thereby be used to improve the efficacy of other treatments, as was proposed by Jain in 2005 (191). Anti-angiogenic therapy provides a window in which the relief of continues angiogenic signaling results in vessel pruning, maturation, and improved perfusion. Increased tumor perfusion can enhance delivery of chemotherapy, as suggested by Bevacizumab and another anti-angiogenic drug called Aflibercept, which combined with chemotherapy improve PFS and OS in metastatic colorectal cancer (192, 193). The dose of anti-angiogenic treatment has been suggested to be important for realizing optimal vessel normalization that improves macrophage polarization and T cell recruitment (194). Anti-angiogenic therapy has also been reported to improve endothelial activation, as tumor endothelial cells have suppressed ICAM-1 expression and decreased leukocyte rolling, which can both be ameliorated by in vivo treatment with VEGF antibodies (184, 195). Importantly, the combination of antibodies targeting either VEGF or VEGFR2 with immunotherapies such as adoptive T cell transfer (196) as well as PD-1 blockade (197) enhances anti-tumor efficacy in experimental models.
5. Results and discussion

5.1 Paper I

Effective cancer immunotherapy approaches in the clinic are based on enhancing the anti-tumor T-cell response, which is initiated by antigen-presenting dendritic cells in tumor-draining lymph nodes (36). Agonistic CD40 therapy activates CD40+ DCs and thereby enhances T-cell priming and the initiation of the anti-tumor T-cell response in the tumor-draining lymph nodes (146). Tumor endothelial cells form a barrier to T-cell infiltration and our group has previously demonstrated that short-term anti-angiogenic therapy can relieve this endothelial anergy and improve T cell infiltration into the tumor microenvironment (177). Sunitinib is a tyrosine kinase inhibitor (TKI) which inhibits VEGFR2 signaling and in addition exerts anti-tumor effects through reduction of immunosuppressive regulatory T cells and myeloid-derived suppressor cells (MDSCs). We therefore hypothesized that sunitinib has complementary mechanistic effects and can improve agonistic CD40 antibody immunotherapy. In order to evaluate this hypothesis we studied the anti-tumor effects of combined agonistic CD40 immunotherapy with sunitinib in the B16 and T241 experimental cancer models.

The combination of agonistic CD40 antibodies with sunitinib administration decreased tumor growth and prolonged survival in both B16 melanoma and T241 fibrosarcoma. Agonistic CD40 antibody therapy resulted in upregulation of the co-stimulatory molecule CD86 on dendritic cells in tumor-draining lymph nodes while sunitinib decreased vessel surface area, improved perfusion and decreased hypoxic areas in the tumor microenvironment. Agonistic CD40 antibodies induced an accumulation of MDSCs in tumor draining lymph nodes, which was reversed by co-administration of sunitinib. Sunitinib administration alone or in combination with agonistic CD40 antibodies also reduced the monocytic MDSC to granulocytic MDSC ratio in T241 tumors. CD8+ T-cell infiltration and endothelial activation by ICAM1 and VCAM1 endothelial expression was increased in tumors of mice treated with the combination of agonistic CD40 antibodies and sunitinib, but not for either monotherapy. Continued treatment of agonistic CD40 antibodies with sunitinib for 5 weeks resulted in a significant improvement in survival and a reduction of tumor size. These results demonstrate that the combination of agonistic CD40 antibodies with sunitinib is superior to either therapy alone.
5.2 Paper II

CTLA-4 is translocated to the T-cell surface upon T-cell receptor (TCR) signaling and functions as a negative feedback loop on T-cell activation by preventing engagement of the co-stimulatory molecule CD28 by CD80/86 (198). CTLA-4 checkpoint blockade has proven successful at enhancing the anti-tumor T-cell response in the clinic and results in long-term survival in some patients (87). PD-1 checkpoint blockade is enhanced by co-administration with CTLA-4 antibodies, although this approach is limited by severe or disabling immune-related adverse events which require (prolonged) hospitalization in approximately 24% of patients receiving the combinatorial therapy (114). The aim of this project was to investigate if local low-dose administration of CTLA-4 antibodies in experimental bladder cancer induces an effective anti-tumor immune response while decreasing systemic antibody release and immune-related side effects compared to systemic treatment.

Local low-dose administration of CTLA-4 antibodies reduced tumor growth in both subcutaneous and orthotopically growing MB49 tumors. Subcutaneous administration of CTLA-4 antibodies reduced circulating antibody levels compared to systemic administration. Intratumoral injection into orthotopically growing MB49 tumors further decreased circulating antibody levels more than 10-fold while retaining anti-tumor efficacy. These results indicate that local administration of CTLA-4 antibodies can be used instead of systemic administration as a means to reduce immune-related adverse events in the clinic.

5.3 Paper III

Galectin-1 (Gal-1) is a glycan-binding protein, which promotes tumor growth by enhancing angiogenic signaling through binding to VEGFR2 and by immunosuppressive effects on cytotoxic and regulatory T cells. Many tumor types have elevated Gal-1 expression, which is associated with a decreased prognosis in cancer patients. The aim of paper III was to examine if vaccination for Gal-1 is an effective strategy for decreasing tumor growth in experimental melanoma.

Vaccination using the TRX-Gal-1 construct with M720/CpG adjuvant induced antibodies after 1 immunization and was followed by 2 booster immunizations to increase antibody titers. The growth of the Gal-1-secreting B16 melanoma model was significantly decreased in mice vaccinated against Gal-1 compared to control immunization. Gal-1 serum levels were increased in B16 tumor-bearing mice compared to healthy mice. Importantly, the vaccinated B16 tumor-bearing mice had significantly reduced levels of anti-Gal-
l antibodies, indicating that the fusion protein technique is able to induce anti-Gal-1 antibodies that can bind to Gal-1 \textit{in vivo}. Gal-1 vaccination resulted in increased vascular perfusion and increased infiltration of both CD68$^+$ macrophages and CD8$^+$ T cells. An increase in GrzB$^+$ area was found in vaccinated mice. GrzB$^+$ areas negatively correlated with tumor growth and co-localized with CD8$^+$ T cells, indicating that Gal-1 vaccination enhanced the cytotoxic T cell response and thereby decreased tumor growth. High Gal-1 serum levels have been associated with poor prognosis in cancer patients (123, 124). The results herein establish that therapeutic vaccination for Gal-1 is a feasible strategy and may help relieve Gal-1 induced angiogenesis and immunosuppression in cancer patients.

5.4 Paper IV

Glioblastoma is the most common and the most aggressive brain tumor with a dismal prognosis. Newly diagnosed patients have a median survival of 14.6 months based on the current standard of care of maximal surgical resection, radiotherapy and chemotherapy (199). Immunotherapeutic strategies against glioma are limited due to immunosuppressive brain microenvironment, the blood-brain barrier and abnormal tumor vascularization. A recent clinical trial with PD-1 checkpoint blockade did not improve survival of patients recurring from radio- and chemotherapy [NCT02017717]. There is an urgent need for novel therapeutic strategies designed specifically to circumvent the immunospecialized brain microenvironment and thereby mount an effective anti-tumor immune response. The aim of the project was to evaluate the efficacy of agonistic CD40 therapy in glioma, and more specifically the role of TLSs induced by CD40 stimulation on modulating the anti-tumor immune response.

Agonistic CD40 antibody therapy induced the formation of dense CD45$^+$B220$^+$ tertiary lymphoid structures (TLSs) in the brain of glioma-bearing mice. TLSs were found peritumorally, adjacent to the meninges and were increased by agonistic CD40 antibodies in both the GL261 and CT-2A glioma models. Further characterization of the TLSs in the GL261 model showed a characteristic cell composition of T cells, CD11b$^+$ macrophages and CD11c$^+$ DCs and that the clusters formed preferentially around CD31$^+$ vessels. Staining for the therapeutic CD40 antibodies showed that antibody was predominantly present on B220$^+$ B cells. CD40 stimulation of B cells induced LT$\alpha$ expression \textit{in vitro} and \textit{in vivo}, while LT$\beta$ was increased \textit{in vitro} and constitutively expressed \textit{in vivo}. These results indicate that direct CD40 stimulation of B cells results in the expression of LT$\alpha_1\beta_2$ and the induction of TLSs in glioma-bearing mice.
In line with published data on agonistic CD40 antibody therapy enhancement of APC induction of a CD8+ T cell response, we observed an increase of CD8+ T cells and a decrease of FoxP3+CD25+ Tregs in tumor-bearing mice. Despite this, the activation status of CD8+ T cells was decreased and CD40 therapy did not affect survival in either the GL261 or CT-2A models. Further examination revealed that despite a decrease in the total number of Tregs in the brain, CD3+ T cells in the TLSs were predominantly CD4+FoxP3+. In addition, CD40 antibody therapy induced an increase of CD1d fluorescence intensity as well as percentage positive CD19+B220+ B cells. These results suggest that even though CD40 activation induces a CD8+ T cell response, the CD8+ T cells in the tumor are anergic due to immunosuppressive environment of Treg-infiltrated TLSs and regulatory B cells.

To investigate the potential immunosuppressive effect on the anti-tumor immune response, agonistic CD40 antibodies were co-administered with PD-1 antibodies. TLS structures were induced in both the CD40 monotherapy and the CD40 and PD-1 combinatorial regimen. Survival of mice receiving the combinatorial treatments was significantly decreased compared to PD-1 antibody administration alone, indicating that CD40-induced TLSs impair the cytotoxic T cell response.

Lastly, we examined if the TLSs we found in experimental murine models of glioma could be found in en bloc resected tumors from low-grade glioma patients. Interestingly, dense clusters of nuclei could be found in the cerebral sulcus or meningeal space in 2/4 of examined patient samples. Similarly to the TLSs found in mice, the clusters were in proximity to IDH1+ tumor cells and in close association with the meninges. Further examination of the clusters in sequential sections of a grade II oligodendroglioma patient revealed that the clusters were CD45+, consisting mostly of CD20+ B cells, CD8+ T cells and CD4+ T cells. The presence of CD35+ dendritic cells and CD138+ plasma cells could also be detected, and vessel structures stained positive for the HEV marker PNAd.

In literature, the role of TLSs and B cells in promoting vs. suppressing inflammation are varied. In most types of cancer, the presence of TLS markers is positively correlated with survival (200). The presence of B cells in TLSs is also positively correlated with survival in some models of cancer (201). However, in experimental models of multiple sclerosis, B cells can adopt a regulatory phenotype and suppress the initiation of disease (202). Moreover, artery TLSs have been found to have high Treg infiltration and suppress the progression of atherosclerosis (203). Importantly, the immunomodulatory role of TLSs in cancer is susceptible to manipulation, as evidenced by a potent CD8+ T cell mediated tumor destruction after Treg depletion in an experimental model of lung adenocarcinoma with abundant Treg-infiltrated TLSs (204).
Although further work remains to be done to investigate the mechanisms of the immunosuppressive microenvironment in murine glioma models and to what extent this immunosuppression is present in glioma patients, this work has significant implications for future glioma immunotherapy. The results on the inhibition of the response to PD-1 checkpoint blockade by CD40-induced TLSs presents a possible explanation for the lack of efficacy of clinical trials employing checkpoint inhibitors for glioma patients. Moreover, the discovery of the presence of TLSs in glioma presents novel therapeutic options for manipulating the anti-glioma immune response.
6. Future perspectives

6.1 Paper I

In Paper I we demonstrate that immuno-vascular targeting using the TKI sunitinib in combination with agonistic CD40-antibody immunotherapy can result in complementary and synergistic anti-tumor immune responses. Although the results indicating a synergistic anti-tumor T-cell response are promising, further investigations employing the combinatorial therapy in a metastatic cancer setting are warranted. Anti-angiogenic therapies including sunitinib have previously been implicated in inducing cancer dissemination, primarily due to tissue hypoxia-induced increase in cancer cell invasiveness (172). In addition, the observed systemic expansion of myeloid-derived suppressor cells with agonistic CD40 antibody therapy may also have negative consequences on cancer dissemination given the role of these cells in cancer metastasis (205). Our research focus is therefore now on cancer models that better resemble human disease and are more suited for studying metastasis formation in vivo (206).

In addition to studying metastasis formation, the synergistic induction of endothelial activation found in the combination therapy but not either monotherapy is of interest. We have previously demonstrated that sunitinib can relieve VEGF-induced suppression of genes involved in T cell recruitment in vitro (177). We now demonstrate that sunitinib monotherapy does not result in endothelial activation in vivo but that it requires the addition of agonistic CD40-antibodies. Based on these results we are continuing in a project in which we are investigating tumor endothelial gene expression using TRAP mice, which enable the analyses of active gene expression by isolation of fluorescently tagged endothelial ribosomes (207). Using this technique, we have identified endothelial gene expression changes in response to agonistic CD40 antibodies and have identified novel therapeutic targets for combinatorial treatment regimens, which are currently under investigation.
6.2 Paper II

At the time of publication of our paper on local low-dose CTLA-4 antibodies for bladder cancer, the FDA had approved the anti-PD-L1 antibody aezolizumab as second-line therapy [NCT02108652]. Since then, Atezolizumab has been approved as a first-line treatment. In addition, other drugs targeting the PD-1/PD-L1 axis have gained clinical approval for bladder cancer, including pembrolizumab, avelumab and durvalumab. For melanoma patients, the efficacy of PD-1 therapy can be improved by addition of CTLA-4 blockade, most likely due to the different mechanisms of PD-1 and CTLA-4 on T cell activation (88, 208). This therapeutic approach of combined PD-1 and CTLA-4 targeting is currently underway for bladder cancer patients in several clinical trials [NCT02496208, NCT02553642]. Although the efficacy against bladder cancer remains to be determined, combined PD-1 and CTLA-4 blockade results in a significant increase in grade 3-4 immune-related adverse events (irAEs), which are severe adverse events that require hospitalization. These adverse events may prevent optimal assessment of therapeutic efficacy. Considering the results of Paper II and that CTLA-4 antibodies are associated with more severe adverse events than PD-1 antibodies, a clinical trial investigating systemic PD-1 therapy combined with local administration of CTLA-4 is warranted in order to reduce systemic side effects.

6.3 Paper III

Paper III establishes that immunization against Gal-1 using fusion proteins consisting of Gal-1 and a part of a foreign bacterial-derived protein can induce an effective endogenous antibody immune response against Gal-1. This approach of therapeutic vaccination is an attractive strategy to cost-effectively target tumor-associated self-proteins and thereby relieve immunosuppression or induce vascular normalization. Interestingly, a recent publication demonstrates that Gal-1 expression by B cells is required for IL-10 and Tim-1 expression induced by agonistic CD40 antibodies and suggests that Galectin-1 is required for regulatory B cell function, bringing up interesting implications considering our results in paper IV (130). A follow-up to this study, in which the efficacy of vaccination against other cancer-associated self-proteins is tested, is currently underway in the lab.

6.4 Paper IV

In paper IV we demonstrate for the first time the presence of TLSs in murine models of glioblastoma via the induction with agonistic CD40 antibodies and
in low-grade glioma patients. The observed increase of CD8$^+$ T-cell infiltration in response to agonistic CD40 therapy indicates that CD40-induced DC activation results in an enhanced initiation of the CD8$^+$ T-cell response. However, further work remains to be done to characterize the immunosuppressive microenvironment, including its effect on antigen presentation and the contribution of Tregs and Bregs to immunosuppression. Of particular interest is whether CD1d$^+$ B cells in the brain are inherently immunosuppressive and to what degree CD40-based activation induces a more suppressive phenotype. In line with this, it is of interest to investigate whether TLSs can be induced by alternative mechanisms other than agonistic CD40 antibodies and whether alternative induction strategies can result in a more anti-tumor TLS phenotype.

Although availability of samples from patients is a limiting factor, a further characterization of TLSs glioma patients is of relevance. In particular, it would be interesting to quantify the presence of TLSs in gliomas of different malignancy grades and molecular subtypes and to examine if the presence of TLSs is associated with tumor progression and survival.
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