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In vivo mechanism of antibody-based immunotherapy

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CHAPTER 1.

General Introduction

Introduction

Monoclonal antibodies (mAbs) are one of the most successful therapeutic agents for treating a wide range of hematological malignancies and solid tumors. Effective antitumor responses and long-lasting remissions can be achieved with antibody therapy, however antibody therapy has little value in many cancer types because a large percentage of the patients does not show clinical benefit. Understanding the mechanisms by which antibodies induce antitumor-responses and how antibody-mediated antitumor activity can be augmented by combining it with other therapeutics is critical in achieving more favourable treatment outcome. To design and improve antibody-based therapy for different cancer types, we heavily rely on preclinical research in mouse models. However, mouse models possess some limitations including the fact that they may not faithfully recapitulate the complexity of human malignancies and the immune contexture within the tumor microenvironment. Despite these limitations, different mouse models have been used successfully to evaluate the *in vivo* antitumor activities of many monoclonal antibodies. Depending on the specific research questions, choosing the right mouse models is essential to obtain preclinical results that are meaningful for clinical translation of improved antibody-based therapies. In this thesis, using multiple mouse models, we explore the following research questions with the goal to improve the effectiveness of mAb therapies: (i) Can we improve the therapeutic efficacy of an immunomodulatory antibody by increasing its engagement to the receptor for the Fc part of IgG (FcγR)? (ii) Can the tumor microenvironment be reprogrammed to a state optimal for mAb therapy? (iii) Does the tumor immunogenicity matter for effective tumor targeting mAb therapy? The following paragraphs describe how the immune system recognises cancer, how the tumor microenvironment affects tumor growth, and what are the mechanisms of action of different types of antitumor antibodies.

1. The Immune system

The immune system has evolved as a defence against infectious disease by discriminating self from nonself (1). Patients with markedly compromised immune responses are predisposed and easily succumbed to infections in early life. The immune system comprises both innate and adaptive immune responses which together enable the detection and elimination of foreign infectious agents, including viruses, bacteria, and fungi, from our body (2). While innate immunity is not specific against a particular pathogen, it detects pathogens via Pattern Recognition Receptors (PRR) such as toll-like receptors (TLRs) that recognize specific danger- or pathogen-associated molecular patterns (DAMPs or PAMPs) that are common to many pathogens but are absent in the host (3, 4). Recognition of the pathogen stimulates inflammatory responses and phagocytosis followed by intracellular killing; these responses are usually short-lived and cannot develop an immunological memory (5). Contrary to this, adaptive immunity involves the development of antigen-specific immune responses to help the clearance of antigen and form immunological memory to respond quickly against reinfection. Compared to innate immunity, adaptive immunity usually occurs over time in a slower manner due to the time required by adaptive T or B cells to undergo activation, differentiation and maturation into functional T cells or plasma cells (antibody-secreting B cells) respectively.

Adaptive responses comprise the cell-mediated immune response, which involves the activation of T cells, and the humoral immune response, which is mediated by activated B cells and antibodies (6).

1.1 T Cell mediated immune response

T cells are derived from the T cell progenitors in the bone marrow. These cells leave the bone marrow and migrate to the thymus to undergo T cell development. The developing progenitors within the thymus, also known as thymocytes, first undergo somatic recombination in their T Cell Receptor (TCR) genes to generate a large repertoire of individual T cell clones. Each T cell clone expresses an unique -TCR (7) which can recognise and bind to a specific antigen presented by other cells on MHC molecules (MHC class I and II) (8). All T cells in the thymus must go through both positive and negative selection (9, 10). In positive selection, T cells that bind moderately to MHC complexes receive a survival signal, giving rise to a population of T cells restricted to self MHC. Negative selection removes T cells whose TCRs can be activated by binding too strongly to self-antigens complexed with self-MHC molecules. These processes are critical to ensure that T cells can distinguish self from non-self-antigens without being self-reactive and causing autoimmunity. T cells which have completed their development in the thymus enter the bloodstream as naïve $CD4^+$ T cells and $CD8^+$ T cells (based on the expression of either the CD4 or the CD8 co-receptor as part of the TCR complex) and recirculate between blood and peripheral lymphoid tissues until they encounter their specific antigens presented by professional antigen-presenting cells (APCs; such as dendritic cells) on MHC molecules (11). An important role of APCs is to activate naïve $CD8^+$ T cell (CTL) by presenting exogenous antigens (taken up from the environment for example from apoptotic tumor cells) bound to their MHC class I molecules. This process is called cross-presentation/cross-priming (12). Subsequently the unique TCR on the activated $CD8^+$ T cells specifically recognize the target cells through the recognition of the antigen bound to MHC class I molecules on the surface of the target cells. This triggers the release of cytotoxic proteins such as perforin and granzymes to destroy the target cells. As for $CD4^+$ T cells, they are stimulated by binding of their TCR to antigen presented on MHC class II molecules of APCs. Depending on the cytokine milieu in the microenvironment, $CD4^+$ T cells can differentiate into the following subsets with a variety of effector functions: T helper 1 (Th1), T helper 2 (Th2), Th17 or immunosuppressive regulatory T cells (Tregs) (13).

The activation and proliferation of effector T cells requires a series of independent signals. Recognition by the TCR of a specific antigen presented by MHC molecules on APCs provides “signal 1.” to the T cell. However, “signal 1” by itself is insufficient to initiate the proliferation of the activated T cell. “Signal 2” is provided by interactions between co-stimulatory receptors on T cells and their ligands on APCs. Based on their molecular structure, co-stimulatory receptors can be divided into two groups belonging either to the immunoglobulin (Ig) or to the tumor necrosis factor (TNF) receptor superfamily. The best characterized Ig superfamily members are CD28 and ICOS, binding to CD80/CD86 (B7.1/7.2) and ICOSL, respectively. The TNF-family molecules and their ligands include CD40-CD40L, CD27-CD70, 4-1BB-41BBL, OX40-OX40L, GITR-GITRL (14). In addition to signal 1 and 2, APCs also provide

signal 3 by secreting cytokines such as IL-12 or type 1 IFN to T cells in order to polarize them toward an effector phenotype (15). Not only the functional T cells are not only capable of inflicting devastating damage on invading pathogens, they can also do great harm to the host's tissues. To prevent this, the magnitude of the T cell response is regulated by a balance between co-stimulatory and co-inhibitory signals (14). These signals are collectively referred to as immune checkpoints. The co-inhibitory signals are conferred by co-inhibitory receptors on T cells which bind to ligands on APC or other cells in the surrounding microenvironment. This can result in the inhibition of T cell proliferation, differentiation, and/or cytokine secretion. The two of well characterised co-inhibitory molecules are cytotoxic-T-lymphocyte-associated protein 4 (CTLA-4) interacting with CD80/86 and programmed cell death 1 (PD-1) receptor interacting with PD-L1 ligand (16). CTLA-4 competes with CD28 for CD80/86 interaction on APC and down-regulates the early stages of T cell activation, proliferation and the secretion of IL-2 (17-19). On the other hand, the binding of PD-L1 to PD-1 on activated T cells confers inhibitory signals that impair the effector functions of T cells. While CD80/86 is expressed by APCs, PD-L1 can be found on many cell types, including immune cells, endothelial cells, and tumor cells (20). Under normal physiological conditions, immune checkpoint molecules are crucial to keep self-tolerance and protection of the host against tissue damage under control. Many tumor cell types have adopted inhibitory ligands of immune checkpoint receptors on T cells to evade eradication by the immune system. This can be reversed by using antibodies targeting immune checkpoints (21) as explained later.

1.2. Humoral immune response

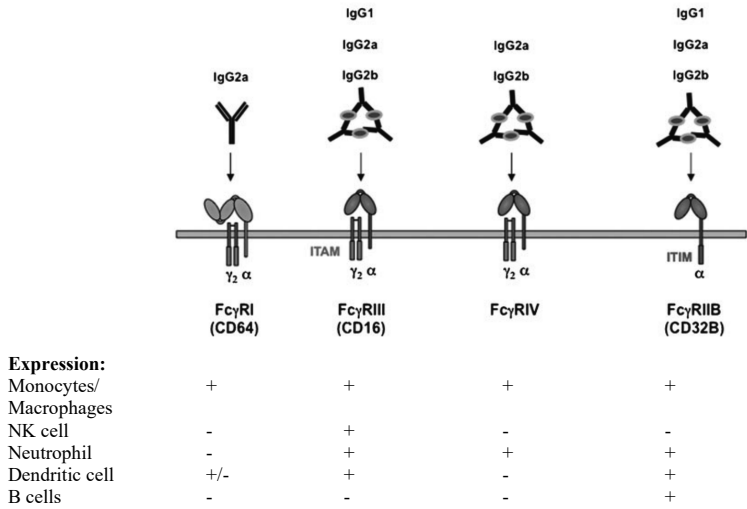
Unlike T cell development in thymus, the development and selection of naïve B cell repertoire occurs in the bone marrow and are subsequently recruited into the germinal centres within the spleen (22) and lymph nodes (23). Here, B cells can encounter soluble antigen or antigen presented on MHC II either by macrophages or follicular dendritic cells (FDCs) (24). Upon antigen recognition by the B cell receptor (BCR), antigen specific B cells migrate to the border of the follicle and the T cell area where they can receive signals from helper T cells that recognize antigen in MHC II on the B cells. This initiates B cells to undergo rounds of somatic hypermutation to select B cells with mutated high-affinity surface immunoglobulin by binding to antigen presented by FDCs. As well as undergoing somatic hypermutation, germinal centre B cells also undergo class switching of the antibodies. Some of the activated B cells then differentiate into high affinity antibody-secreting plasma cells in the periphery while others become memory B cells.

Antibody molecules (also named immunoglobulin) play a central role both in the specific recognition and elimination of foreign antigens by the stimulation of an immune response against the antigens (25). Antibodies are Y-shaped proteins with a molecular weight of ~150kDa and each molecule is composed of four polypeptide chains, two identical copies of heavy chains (50kDa) and two identical light chains (25kDa). Both the heavy and light chain consist of a variable (V_H and V_L respectively) and a constant region (C_H and C_L respectively) which are held together by disulphide bonds. Each antibody consists of two fragment antigen-binding regions (Fab), which are comprised of two variable domains and the CH_1 domains of

the heavy chains. The V_L and V_H domains contain the unique complementarity-determining regions (CDRs), which are responsible for the specific binding and the neutralization of the antigen. The CH_2 and CH_3 domains of the heavy chain make up the fragment crystallisable (Fc) region of the antibody, which elicits effector functions such as antibody dependent cell cytotoxicity (ADCC) and antibody dependent cellular phagocytosis (ADCP) by binding in the case of IgG to Fc γ receptors (Fc γ R) on immune effectors cells, as well as C1q of the complement system (26-28). The constant domains determine the antibody isotype (IgA, IgD, IgE, IgG, and IgM), which can be further divided in subclasses in the case of IgG (in humans IgG1, IgG2, IgG3 and IgG4 and in mice IgG1, IgG2a, IgG2b and IgG3) (29), and IgA (in humans IgA1 and IgA2) (30). Currently, although much efforts have been made to develop antitumor antibodies of the IgA or IgE isotype, most FDA-approved antibodies for use in cancer therapy are of either of the IgG1, IgG2 or IgG4 (31, 32) subclass. IgG2 and IgG4 with lower affinity for the Fc γ Rs and lower complement activation are preferred when Fc-mediated effector functions are not required or detrimental to the antitumor activity of the antibody (33, 34).

In mice, there are 3 activating Fc γ R receptors (Fc γ RI, Fc γ RIII and Fc γ RIV) associated with a common signal transduction subunit, the FcR γ -chain, which contains an immunoreceptor tyrosine-based activation motif (ITAM). There is one inhibitory Fc γ R, Fc γ RIIb, which contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its intracellular domain (29). These activating and inhibiting receptors are orthologues to the human Fc γ RI, Fc γ RIIa/Fc γ RIIc, Fc γ RIIIa and Fc γ RIIb, respectively (35). The overall architecture of the Fc γ R system is similar between mouse and man but the expression patterns of the Fc γ R and their affinities for the different IgG subclasses vary substantially between these species (36, 37) (Figure 1). Notwithstanding these differences, Fc γ R deficient mouse models are useful in investigating the role of the Fc γ R-mediated effector functions of IgG antibodies.

(A) Mouse Fc receptors



(B) Human Fc receptors

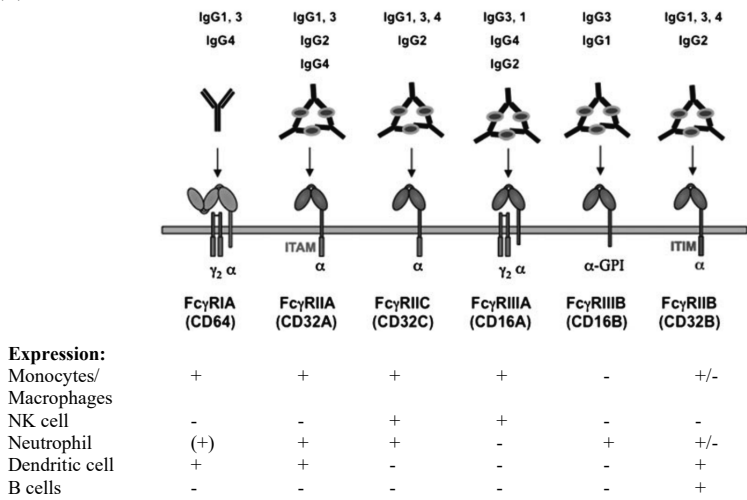


Figure 1. Mouse and human FcγR for IgG and their expression patterns on different immune cells. (A) There are four members of mouse FcγR that can be categorized by their ability to bind monomeric IgG2a (high affinity FcγRI) or IgG in the form of immune complexes (low affinity FcγRIII, FcγRIV, FcγRIIB). The activating FcγRI, III and IV signal via ITAM motifs located in the associated dimer of the common Fcγ chain, while the inhibiting FcγRIIB signals via an intracellular ITIM motif. (B) The mouse FcγRI, FcγRIII, FcγRIV, and FcγRIIB are orthologous to human FcγRIA, FcγRIIA/IIIC, FcγRIIIA, and FcγRIIB. Of note, the human FcγRIIIB, FcγRIIA and FcγRIIC do not exist in mice. mFcγRIII and hFcγRIIA are considered as functional orthologs as well as mFcγRIV and hFcγRIII. The IgG subclasses are labelled according to their binding affinity to their respective receptors. +: expression; +/-: expression on a subpopulation; (+): inducible expression. (Adapted from Lux and Nimmerjahn et al. 2013 (38); Bruhns et al. 2015 (36)).

2. The immune recognition of cancer cells

It has become clear that the immune system can function as an important defence against cancers (Immune surveillance) (39)(40). The generation of antitumor immune responses normally starts with the detection and killing of tumor cells by the innate immunity, followed by the uptake and presentation of tumor antigens by APC to T cells triggering the production of tumor specific CD4⁺ and CD8⁺ T cells and/or antibodies which help to destroy the remaining tumor. These stepwise events are referred as the Cancer-immunity cycle (Figure 2) (41). Immune recognition of tumor antigen is a prerequisite for the effective antitumor immune response (42). Tumor antigens can be classified into self and non-self-antigens.

Self-antigens can be expressed at increased levels in tumors compared with normal cells. One example of self-antigens are the cancer testis (CT) antigens which are normally found only in male germ cells in the testis (43). Because these cells do not express MHC molecules, peptides derived from these proteins are not presented to T cells. In malignancy, the gene regulation of CT antigen is disrupted, resulting in CT antigen expression in different tumor types (44). A second example of self-antigens are the differentiation antigens which are expressed in some types of tumors and the corresponding healthy tissues. Studies in melanoma have raised much interest in differentiation antigens as potential targets for immunotherapy (45), because researchers have documented spontaneous T cell responses against peptides derived from melanocytic protein gp100/Pmel17 (46, 47), MART-1/Melan-A (48, 49), and tyrosinase-related protein-1 and 2 (TRP-1 and 2) (50). CD19 is another differentiation antigen, in this case found on normal and malignant cells, that can be targeted in patients with B-lineage acute lymphoblastic leukemia and other B cell tumors (51). Another class of self-antigens are antigens overexpressed in tumor cells compared with the expression in their normal counterparts. Examples include HER2/neu (also known as ErbB2) and epidermal growth factor receptor (EGFR) (52, 53). Overexpression of these antigens is associated with the aggressivity of the disease and poor prognosis. Therapeutic vaccine targeting self-antigens have obtained poor results mainly due to central and peripheral tolerance mechanisms (54). Moreover, the strength of such responses is not fully clear as it is to be expected that high avidity T cells recognising self-antigens are subjected to negative selection to induce immune tolerance in the thymus, leaving predominantly low avidity T cells (55). In addition, targeting self-antigen may result in severe autoimmune toxicity (56).

Neoantigens, are nonself-antigen that arise from random tumor-specific-somatic mutations (caused by carcinogens or ultraviolet light which causes DNA mismatch repair defects) and are not present in normal cells (57). The probability of creating neoantigens is correlated with the mutational burden that varies strongly between tumor types. As a consequence, also the number of neoantigens varies strongly between tumor types (58, 59). For example, non-small cell lung cancer (NSCLC) and melanoma are immunogenic cancers with high mutation rates, whereas pancreatic ductal adenocarcinoma (PDAC) is a nonimmunogenic cancer with low mutation rate (57). Viral antigens found in virus-associated cancers (such as human papillomavirus (HPV)-positive cervical or head and neck cancer and Merkel cell polyomavirus (MCPyV)-positive Merkel cell carcinoma (MCC)) are the potential neoantigen and provide targets for T-cells (60, 61). Vaccines containing long HPV peptides have proven to be a promising therapeutic agents for HPV⁺ cancers for inducing HPV-16 specific CD4⁺ and CD8⁺ T cells

(62). Unlike the non-mutated tumor self-antigens, non-self-immunogenic neoantigens are more likely to be recognised by the host immune system leading to strong T cell responses. In fact, it has been shown that neoantigen-specific cytotoxic T cells represent the primary T cell populations against tumors (63, 64).

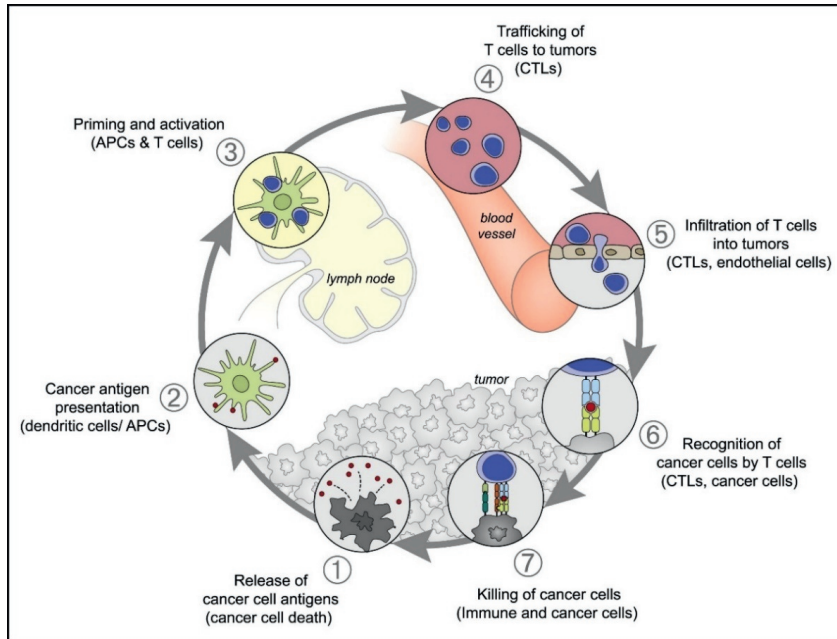


Figure 2. The cancer immunity cycle. The initiation of stepwise events of an antitumor responses. First, the released of tumor associated antigens are captured by antigen-presenting cells (APCs) (Step 1 and Step 2). Next, the captured antigens are loaded on MHCI and MHCII and presented by APCs to T cells in lymph nodes, leading to the priming and activation of T cells specific for tumor antigens (Step 3). The activated effector T cells then travel to the tumor site and infiltrate the tumor microenvironment (Step 4 and 5), TCR on T cells specifically recognise and interact with the tumor antigen-MHC complex on tumor cells (Step 6). T cells release the cytotoxic proteins which eliminate the tumor cells (Step 7). (Adapted from Chen et al. 2013) (41)

3. Tumor immune microenvironment

The tumor microenvironment (TME) is collectively formed by tumor cells, vasculature, fibroblasts and immune cells. Infiltration of tumor specific effector T cells into the TME is crucial for successful immune-mediated elimination of tumor cells. However, the levels of T cell infiltration vary across different tumor types. Highly immunogenic tumors with large mutational burden such as melanoma and certain lung cancers are commonly containing more T cell neoantigens/neoepitopes, resulting in immune tumor recognition and the priming of T cell responses. They contrast strongly with low T cells infiltrating tumors such as glioblastomas, ovarian, prostate, pancreatic, and most breast cancers (65). These weakly immunogenic tumors often exhibit low levels of neoantigens. Nevertheless, neoantigen targeting T cells can still be found in many different tumor types (66-68). However, their effector functions are regulated in the TME (69). Within the TME, tumors interact with many different immune cell types (e.g. tumor associated macrophages/neutrophils, regulatory T

cells), stroma cells (e.g. tumor-associated fibroblasts) and cytokines (e.g. interleukin-10, transforming growth factor- β (TGF- β)) to continuously promote a chronic inflammatory and immunosuppressive TME (70). There are different immunosuppressive mechanisms (71, 72) in the TME which hamper the antitumor immunity and promote the differentiation of intratumoral effector T cells into “exhausted T cells” (73), which are highly positive for the expression of multiple inhibitory receptors including CTLA-4, PD-1, TIM-3 (T-cell immunoglobulin mucin 3), and LAG-3 (lymphocyte activation gene-3) (74-76). Binding of these inhibitory receptors to their responsive ligands inhibit the T cells ability to proliferate and produce cytokines (i.e. IFN γ , IL-2 and TNF α) (77, 78), resulting in cancer immune evasion. T cell dysfunction can be resolved by the use of blocking antibodies specific for immune checkpoint axes. However, the respond rates of anti-CTLA-4 or PD-1/PD-L1 Ab is only ~15-40% of cancer patients. Thus, the significance of tumor immune phenotype on prognosis has been examined in many clinical research studies to identify potential biomarkers to predict the response to immune checkpoint inhibitors.

Indeed, high IFN γ and cytotoxic proteins (perforin, granzyme and granulysin) expressing human tumors were more likely to respond favourably to anti-CTLA-4 mAb (ipilimumab) (79-81). Recently, the quantification of T cells both at the tumor centre and invasion margin of tumor have been proven to be a more reliable prognostic tool for colorectal carcinoma (CRC) patients (82). This qualification method is currently tested to predict the response to anti-PD-1 therapy in patients (NCT02274753). It was reported that the neoantigen landscape has a strong association with the treatment response to immune checkpoint blockades (83). Some studies indicate that PD-L1 expression can be used to identify tumors that are likely to respond to anti-PD1 mAb therapy (84). However, this is not conclusive as some PD-L1⁺ tumors do not respond to anti-PD1 therapy (85), suggesting other factors should be taken into consideration while identifying predictive markers. Tumors with an immune-excluded phenotype, such as ovarian cancer and pancreatic ductal adenocarcinoma display a low response rate to checkpoint inhibition (86, 87). These tumors are characterized by the presence of T cells in the tumor stromal regions but not the tumor beds. Interestingly, Tauriello et al. (88) and Mariathasan et al. (89) indicated that TGF- β signalling promotes T cell exclusion from the TME and reduces the response rate to anti-PD-L1 mAb therapy.

In the TME, TGF- β is a well-studied pleiotropic cytokine which is produced in latent isoforms (TGF- β 1, - β 2, TGF- β 3) by cancer cells, immune cells and stromal cells. The active form of these TGF- β isoforms is generated by cleavage by integrins or matrix metalloproteinases. Upon TGF- β binding, the TGF- β type II receptor phosphorylates the TGF- β type I receptor and initiates either SMAD- or non-SMAD-mediated transcription of TGF- β target genes (90). TGF- β signalling promotes antitumor effect during the early stages of tumorigenesis, but this function is mitigated during later phases of tumor progression. Instead, it promotes cancer cell migration and invasion, extracellular matrix (ECM) remodelling, epithelial-to-mesenchymal transition (EMT), promoting immunosuppressive TME that ultimately suppress T-cell immunosurveillance (91). For example, TGF- β signalling silences the expression of transcription factor T-bet which are responsible for the differentiation of CD4⁺ T cells into effector cells, while promoting FoxP3 expression and inducing the transition of CD4⁺ T cells into Tregs (92). Moreover, Tregs can augment immunosuppression by

communicating with other immunosuppressive cells within the tumor microenvironment (93). For instance, Tregs produce high levels of TGF- β and triggers the conversion of stromal fibroblast into CAFs that are capable of inducing T cell apoptosis, thereby inhibiting cytotoxic T cells from killing tumor cells (94). Moreover, TGF- β expressing CAF has been showed to promote the formation of physical barriers and limit the T cell infiltration into the tumor (88, 89). Indeed, blocking of the TGF- β signalling in the TME by neutralizing antibodies to TGF- β or small molecule kinase inhibitors can reverse the antitumor activity in both highly and weakly immunogenic tumor tissues by improving the infiltration of T cells into the tumor (95, 96), indicating that some weakly immunogenic tumors may retain some tumor antigens but manage to evade antitumor immunity by promoting an immunosuppressive TME and physical barrier. The complex and dynamic interaction between immune system and tumors are called “cancer immunoediting”, which explains that the immune system cannot only recognise and eliminate transformed malignant cells but also plays a critical role in selecting tumors with decreased immunogenicity and therefore promote tumor progression (97). Furthermore, the ability of tumors to avoid destruction by immune system has been proposed as one of the hallmarks of cancer (98).

4. Antibody-based cancer immunotherapies and their mode of action

Cancer immunotherapy comprises various strategies to enhance the host immune system’s ability to specifically recognise tumor cells and attempt to eliminate them. This approach has been studied for over a century (99, 100). In the year 2013, due to the success of a series of proof-of-concept clinical trials, especially with antibodies targeting immune checkpoints, cancer immunotherapy was named by the Science’s editors as “Breakthrough of the Year” (101). The successful clinical results have brought cancer immunotherapy to the forefront in cancer treatment. Examples of cancer immunotherapy are cancer vaccines, adoptive T cell transfer, treatment with cytokines and monoclonal antibodies (mAbs). MAbs have become essential therapeutic agents for the treatment of haematological malignancies and solid tumors. They can be used to target tumor antigens and trigger immune effector functions; block/neutralize proteins necessary for the cell growth or development of new blood vessels; target the inhibitory/activating immune molecules to activate antitumor immunity; deliver chemotherapeutic drug/radioactive particle to the cancer cells (102). In this thesis, we focus on antibody-based cancer immunotherapies, in particular the tumor targeting and immunomodulatory mAbs (103).

4.1. Tumor targeting monoclonal antibodies (mAbs)

Tumor-targeting mAbs target the tumor cells directly by binding to cell surface antigens that are overexpressed or ectopically expressed or specifically expressed by the tumor cells. The killing of antibody-coated tumor cells can be induced via the blockade of the pathophysiological function of their target proteins. Examples are trastuzumab and cetuximab which bind and block HER2 and epidermal growth factor receptor (EGFR) (104) respectively. Blockade of HER2 or EGFR signalling by antibodies causes diminished cellular activity and

inhibit cell proliferation resulting in reduced survival of the tumor cells (105, 106). Mouse models are indispensable tools for the development of anticancer antibodies. For example, human xenograft tumor models, in which human tumor cell lines are implanted in mice, have been used successfully to demonstrate the direct growth inhibitory activity of tumor targeting antibodies such as anti-CD20 mAb (107) and anti-Her2 mAb (108, 109). These studies have helped to provide impetus for clinical evaluation of tumor targeting antibodies as monotherapy or in combination with chemotherapy (110-114) which has led to the approval of Rituximab (anti-CD20 mAb) and Trastuzumab (anti-Her2 mAb) by the US Food and Drug Administration (FDA) at the end of 1990s for follicular lymphoma (FL) (115) and Her2-overexpressing breast cancer (116), respectively. While the direct cytotoxic effect was thought to be the central mechanism of action for tumor targeting antibodies, a number of *in vitro* studies suggest that tumor-bound mAb can elicit several immune effector mechanisms (109, 117-119) (Figure 3), which are induced by the binding of the Fc region of the antibody to the FcγRs, expressed on a variety of immune cells, or to C1q molecules of the complement system. These mechanisms include Antibody Dependent Cell mediated Cytotoxicity (ADCC) by NK cells and phagocytes directly lysing the opsonized tumor cell, Antibody Dependent Cellular Phagocytosis (ADCP) or Complement Dependent Cell Mediated Phagocytosis (CDCP) by phagocytic cells such as macrophages devouring the opsonised target cells (117, 120) and Complement Dependent Cytotoxicity (CDC). The latter is induced when the complement factor C1q binds to the Fc part of the tumor cell bound antibody, this leads to the formation of a membrane attack complex that punches holes into the surface of a tumor cells. These preclinical findings are supported by human studies showing that an activating FcγRIIIA allelic variant with higher affinity for IgG is associated with enhanced responses to tumor directing mAbs in patients (121-123). These findings highlighted the Fc mediated effector functions by NK cells, which predominantly express only FcγRIIIA. However, it is important to note that macrophages and monocytes also express FcγRIIIA, and for this reason, larger studies have failed to confirm this finding (124, 125). Perhaps the most convincing data for elucidating the role of Fc-mediated effector functions for tumor targeting antibodies are generated from Fc receptor knock-out mouse models. The antitumor effects of anti-Her2 and anti-CD20 mAb were lost in common γ-chain knock-out mice (FcRγ^{-/-}, mice lacking expression of activating IgG Fc receptors) (126). This strongly suggested that that activating FcγR substantially contribute to the therapeutic effect of tumor targeting antibodies at least in mice (126). It is believed that the induction of ADCC by antibodies indirectly contributes to tumor destruction by the initiation of cross priming by APCs triggering antitumor T cell responses. Indeed, both CD4⁺ and CD8⁺ T cells were required for the anti-CD20 mAb-induced antitumor protection in mice (127). In addition, utilising immunocompetent breast cancer models (either transplantable tumor cell line or oncogene driven tumor models), studies have revealed that effective anti-Her2 mAb therapy requires various immune effector pathways such as activation of type I and II IFN responses; stimulation of CD4⁺ T cells and CD8⁺ T cells (128, 129). In the clinic, increased levels of TILs were associated with higher response rate to anti-Her2 mAb but not tyrosine kinase inhibitor (lapatinib) therapy (130), highlighting the importance of immune system to anti-Her2 mAb therapy.

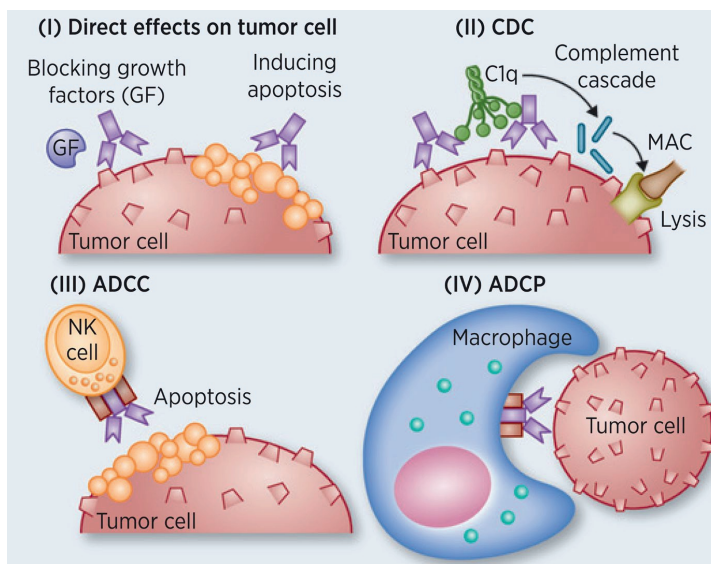


Figure 3. Mechanisms of action of tumor targeting monoclonal antibodies. (I) Tumor targeting mAbs can have a direct antitumor effect by inhibiting/triggering possible downstream signalling events that lead to target cell death. (II) mAbs can trigger complement-dependent cytotoxicity (CDC) to kill the target cells through the development of membrane attack complex (MAC). MABs bind to the FcγR expressing effector cells to mediate antibody-dependent cellular cytotoxicity (ADCC) (III) or antibody-dependent cellular phagocytosis (ADCP) (IV). (Adapted from Gul et al 2015) (117)

4.2. Immunostimulatory monoclonal antibodies (mAbs)

Among all the antitumor responses, tumor killing by cytotoxic T cells is the key for tumor control. As mentioned earlier, besides first signal through the MHC-peptide-TCR axis, the activation of T cells is tightly regulated by both co-inhibitory and co-stimulatory pathways. Therefore, blocking/antagonistic antibodies targeting co-inhibitory/immune checkpoints and agonistic antibodies targeting co-stimulatory pathways are attractive candidates for cancer immunotherapy. Unprecedented clinical success has been achieved with antibodies targeting immune checkpoints (Figure 4) (131, 132). In 2011, anti-CTLA-4 mAb (ipilimumab) was the first checkpoint inhibitor approved by the FDA for the treatment of patients with advanced melanoma (133). Recently, anti-PD-1 and PD-L1 (31% objective response rate) have induced better antitumor efficacy compared with ipilimumab (6% objective response rate) (134, 135) (136-138). Interestingly, the combination of anti-CTLA-4 and PD-1 has further increased the antitumor efficacy (53% objective response rate) (139). Other blocking antibodies targeting co-inhibitory molecules such as T-cell immunoglobulin mucin-3 (TIM-3) and lymphocyte-activation gene 3 (LAG-3) are being studied extensively (140). Apart from blocking co-inhibitory molecules, activation of antitumor T cell responses can also be achieved with agonistic antibodies targeting co-stimulatory members (e.g. CD40, CD137, OX40) of the TNF receptor family. Whereas the therapeutic effect of anti-CTLA4 and anti-PD-L1 is based on blocking their interaction with their ligands, the therapeutic effect of agonistic antibodies such as anti-CD40 mAb is based on cross linking the target molecule initiating an activation signal.

The transmembrane protein receptor CD40 is a member of tumor necrosis factor receptor superfamily and is primarily expressed on dendritic cells (DCs), B cells, macrophages and monocytes. Agonistic antibody targeting CD40 stimulates antitumor T cell responses and promotes cytotoxic myeloid cells in preclinical tumor models and a variety of cancer patients, including those with melanoma and pancreatic adenocarcinoma (141, 142). OX40 and 4-1BB/CD137 represent another promising target for agonistic antibodies. These molecules are found on activated T cells and are key mediators of costimulatory signals. Both agonistic anti-CD137 and OX40 mAb support T cell survival and proliferation, which in turn induces potent antitumor immune responses (143, 144). Agonistic anti-CD137 and OX40 mAb have been advanced into clinical development (145, 146).

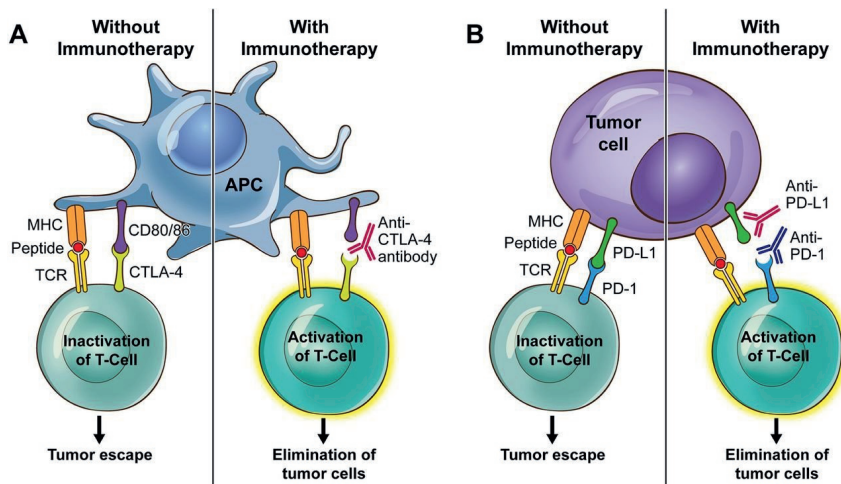


Figure 4. Targeting CTLA-4 and PD-1. (A) Binding of CTLA-4 on APC to CD80/86 on T cells downregulates T-cell activation, this can be abolished with anti-CTLA-4 mAb. (B) Antitumor immune reaction can be provoked with anti-PD-1 blocking mAb which blocks the binding of PD-1 on T cells to PD-L1 on tumor or tumor infiltrating immune cells. (Adapted from Soularue et al 2018) (132)

The studies on immunomodulatory antibodies have been mostly conducted in syngeneic mouse tumor models on different backgrounds (e.g. C57BL/6, BALB/c and FVB mice), these tumor models have been successfully used to identify the antitumor activity of currently FDA approved anti-CTLA-4, anti-PD-1 and anti-PD-L1 mAb (147). In addition, preclinical studies have revealed an unexpected role of both activating and inhibiting FcγR (148) in the efficacy of immune checkpoint blockade. For example, activating FcγRs appeared to be essential for the antitumor activity anti-CTLA-4 mAb in mice (149, 150). Together, anti-CTLA-4 mAb elicits antitumor activities by blocking CTLA-4 on T cells and/or Fc-mediated deletion of intratumoral immunosuppressive regulatory T cells (Tregs), thus (re)activating cytotoxic CD8⁺ T cells in the tumor microenvironment. Ipilimumab, a human IgG1 mAb targeting CTLA-4, improves the therapeutic outcome in patients with advanced melanoma. Interestingly, Fc polymorphisms in melanoma patients with higher affinity to activating FcγR have now been associated with better clinical outcome to ipilimumab (151). Not only the therapeutic effect of anti-CTLA-4, but also from anti-4-1BB; anti-GITR and anti-OX40 mAbs involves depletion

of Tregs dependent on the co-engagement of activatory FcγRs (152-154). While the presence of inhibitory FcγRIIb was detrimental to the therapeutic effect of rituximab (anti-CD20) and trastuzumab (anti-Her2) (126), the optimal activity of agonistic anti-CD40 mAb has been proposed to dependent on FcγR-mediated antibody cross-linking that promotes signalling in CD40⁺ immune cells (142, 155) (156). However, not all immunostimulatory mAbs can benefit from FcγR interaction. Several studies have clearly reported that engagement of FcγR could potentially hamper the antitumor activity of anti-PD-1 mAb by depleting intratumoral CD8⁺ T cells by ADCC (157) or ADCP (158). Also, anti-PD-1 mAb can be removed from the surface of T cells by the interaction with FcγR on macrophages, resulting in poorer antitumor activity in mice. This was resolved by blocking FcγR with antibodies (159). These observations have significant implications for identifying the relevant FcγRs required for the optimal outcome of mAb therapy. To date, most of the approved anti-PD-1 mAbs for clinical use are of human IgG4 isotype with S228P mutation (which substitutes a serine residue with the proline in the hinge region) to abolish FcγR binding. This results in reduced Fc-mediated killing of PD-1⁺ immune effector cells while retaining its blocking function of the PD-1 (160) (Figure 5).

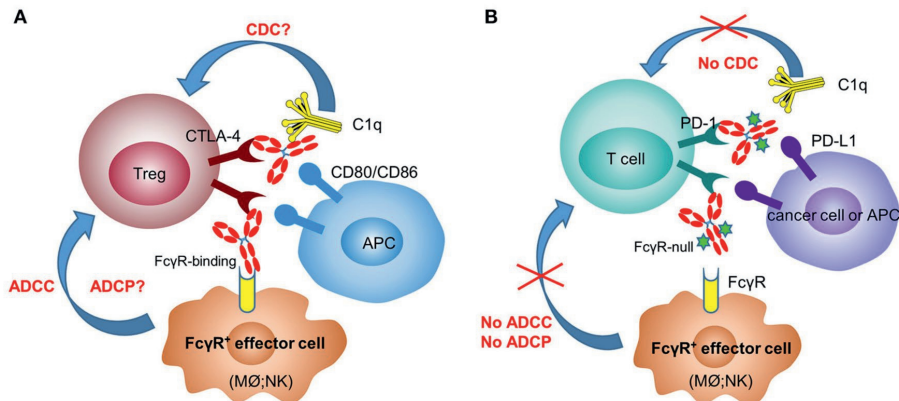


Figure 5. Anti-CTLA-4 and anti-PD-1 mAbs have differential FcγR-binding requirement for optimal therapeutic activity. (A) The additional therapeutic efficacy of anti-CTLA-4 mAb is achieved by depleting intratumoral regulatory T cells (Treg) through the interaction of activating FcγR on immune effector cells. (B) Interaction to activating FcγR is detrimental to the antitumor efficacy of anti-PD-1 mAb due to the elimination of PD-1⁺ T cells. (Adapted from Chen et al 2019) (160)

Outline of the thesis

Using a variety of relevant preclinical mouse models, this thesis not only showed several ways to improve antibody-based cancer immunotherapy but also demonstrates the importance of the choice of the model for the translational relevance of the preclinical observations. In **chapter 2**, the question whether engaging FcγR can enhance the therapeutic efficacy of anti-PD-L1 mAb therapy was studied. For this, the Fc region of rat IgG anti-PD-L1 mAb was replaced with the Fc regions of mouse IgG1, IgG2a, IgG3 and the D265A mutant of mouse IgG2a, which differ from each other in their binding affinity to mouse FcγR. The therapeutic efficacy of these four anti-PD-L1 mAb variants were analysed in two different colon adenocarcinoma tumor models. In **chapter 3**, two mouse tumor models were used to explore if the antitumor effect of anti-PD-L1 mAb can be improved by the blockade of TGF-β, a potent immunosuppressive cytokine. The impact of the combination therapy on the frequency of immune cells in the tumor microenvironment was analysed. In **chapter 4**, the synergy between the tumor targeting anti-Trp1 mAb (TA99) with the innate stimulating compound imiquimod and IL-2 was assessed in a therapeutic setting of the B16F10 melanoma model. The involvement of CD8⁺ and CD4⁺ T cells, NK cells, macrophages, neutrophils and FcγR in the therapeutic effect of the combination therapy was analysed. In **chapter 5**, the impact of the tumor immunogenicity in a breast cancer mouse model on the outcome of anti-neu mAb therapy was investigated. Using a transplantable tumor cell line (TUBO) derived from a BALB/c-NeuT primary mammary cancer, the TUBO tumor outgrowth and tumor infiltrating T cells in non-syngeneic (WT BALB/c) and syngeneic (BALB/c-NeuT) recipient mice was compared. The role of pre-existing tumor infiltrating T cells in anti-neu mAb therapy was analysed in this model. In **chapter 6**, a MMTV-NeuT congenic strain by backcrossing the MMTV-BALB/c-NeuT mouse strain into C57BL/6 background was generated using a speed congenics approach and compared its tumor immune microenvironment with that of the parental tumor bearing NeuT-BALB/c mice. Their potential value for studies of the role of FcγR in anti-neu antibody therapy is evaluated. Finally, in **chapter 7**, the results obtained in this thesis are summarised and discussed.

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