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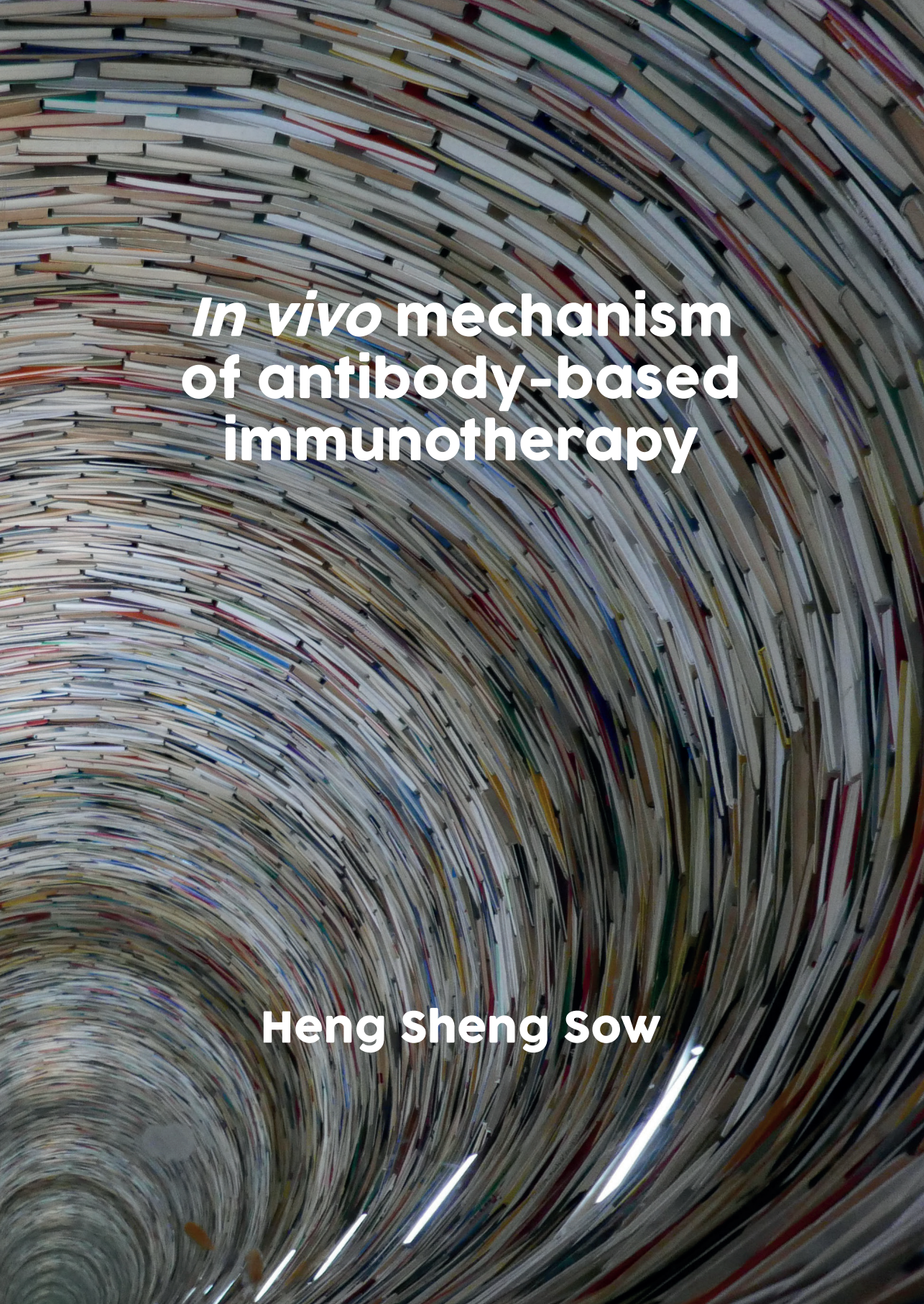

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CHAPTER 1.

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 resonses and long-lasting remissons 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

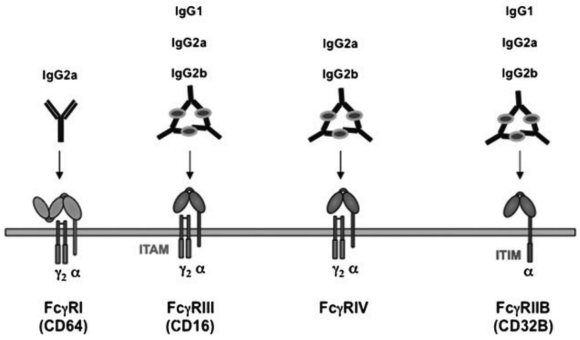
Unlike T cell development in thymus, the development and selection of naïve B cell repertoire occurs in the bone marrow and are subsequently recruited into the germinal centres within the spleen (22) and lymph nodes (23). Here, B cells can encounter soluble antigen or antigen presented on MHC II either 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 Fcγ receptors (FcγR) 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 FcγRs 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).

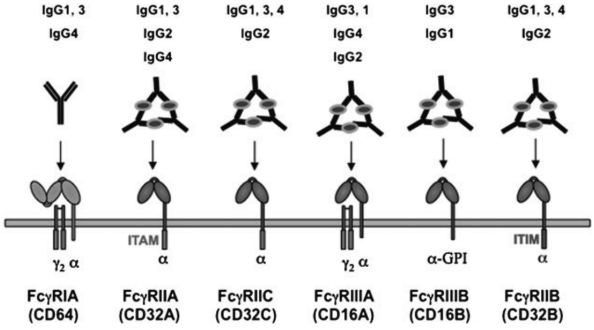
In mice, there are 3 activating FcγR receptors (FcγRI, FcγRIII and FcγRIV) associated with a common signal transduction subunit, the FcR γ-chain, which contains an immunoreceptor tyrosine-based activation motif (ITAM). There is one inhibitory FcγR, FcγRIIb, which contains an immunoreceptor tyrosine-based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

(A) Mouse Fc receptors



Expression:				
Monocytes/ Macrophages	+	+	+	+
NK cell	-	+	-	-
Neutrophil	-	+	+	+
Dendritic cell	+/-	+	-	+
B cells	-	-	-	+

(B) Human Fc receptors



Expression:						
Monocytes/ Macrophages	+	+	+	+	-	+/-
NK cell	-	-	+	+	-	-
Neutrophil	(+)	+	+	-	+	+/-
Dendritic cell	+	+	-	-	-	+
B cells	-	-	-	-	-	+

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 ablity to bind monomeric IgG2a (high affinity FcγRI) or IgG in the form of mmune complexes (low affinity FcγRIII, FcγRIV, FcγIIB). The activating FcγR I, III and IV signal via ITAM motifs located in the associated dimer of the common FcR γ-chain, while the inhibiting FcRIIB signals via an intracellular ITIM motif. (B) The mouse FcγRI, FcγRIII ,FcγRIV, and FcγR IIB are orthologue to human FcγRIA, FcγRIIA/IIC, 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 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).

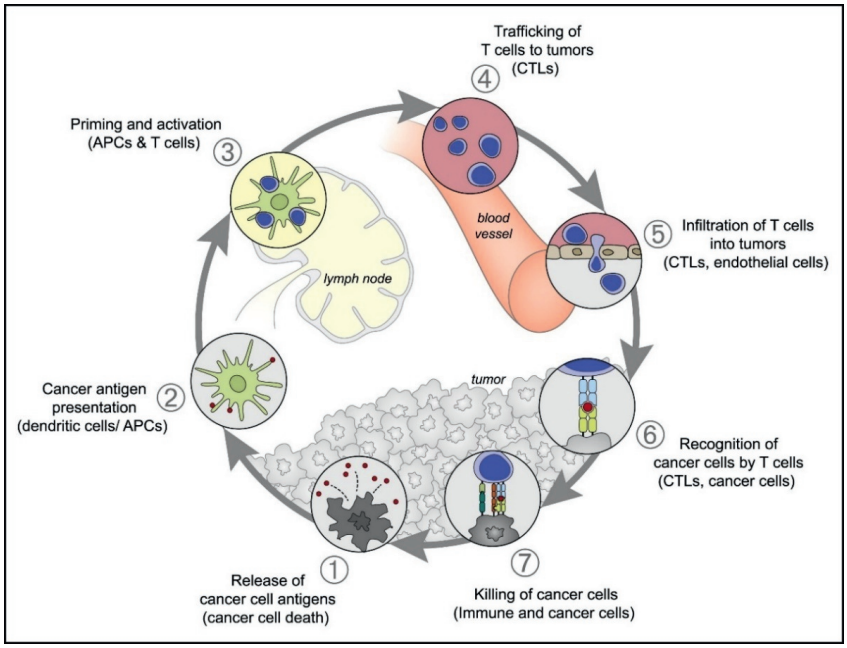


Figure 2. The cancer immunity cycle. The initiation of stepwise events of an antitumor responses. First, the released of tumor associated antigens are captured by antigen-presenting cells (APCs) (Step 1 and Step 2). Next, the captured antigens are loaded on MHCI and MHCII and presented by APCs to T cells 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 targetingT 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. IFNγ, IL-2 and TNFα) (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 IFNγ 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-β1, -β2, TGF-β3) 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 ($FcR\gamma^{-/-}$, 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.

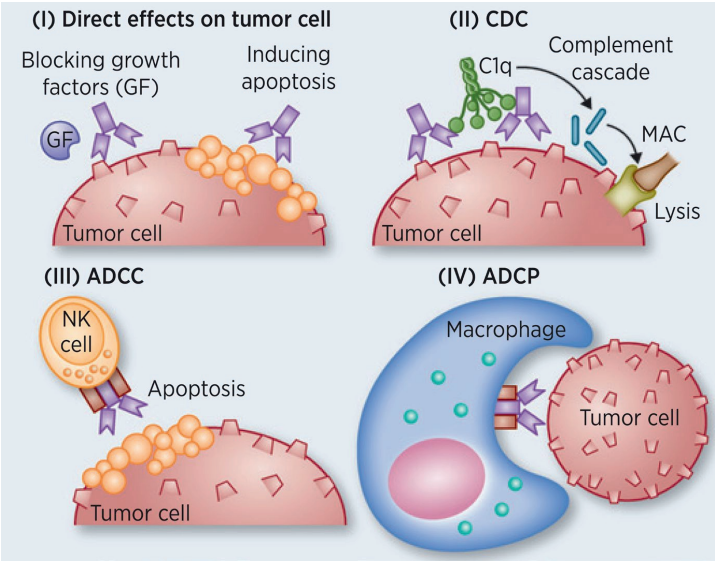


Figure 3. Mechanisms of action of tumor targeting monoclonal antibodies. (I) Tumor targeting mAbs can have a direct antitumor effect by inhibiting/triggering possible downstream signalling events that lead to target cell dead. (II) mAbs can trigger complement-dependent cytotoxicity (CDC) to kill the 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 mA (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).

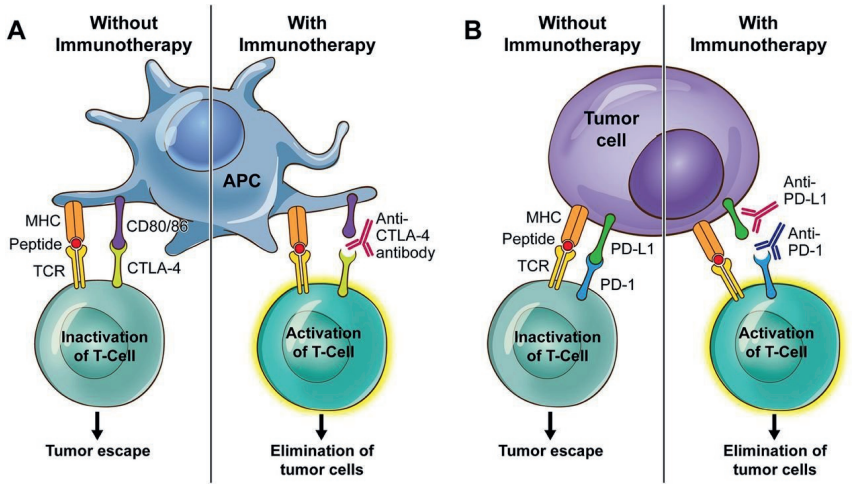


Figure 4. Targeting CTLA-4 and PD-1. (A) Binding of CTLA-4 on APC to CD80/86 on T cells downregulates T-cell activation, this can be abolished with anti-CTLA-4 mAb. (B) Antitumor immune reaction can be provoked with anti-PD-1 blocking mAb which blocks the binding of PD-1 on T cells to PD-L1 on tumor 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 elicites 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 subtitutes a serine residue with the proline in the hinge region) to abolish FcγR binding. This results in reduced Fc-mediated killing of $PD\text{-}1^+$ immune effector cells while retaining its blocking function of the PD-1(160) (Figure 5).

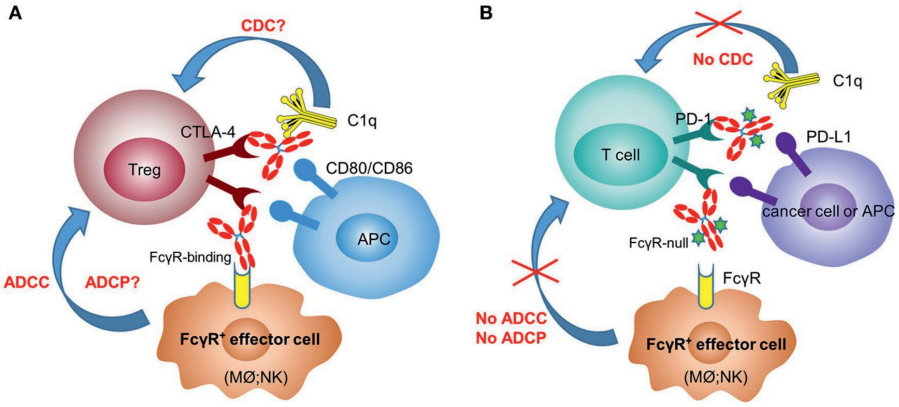


Figure 5. Anti-CTLA-4 and anti-PD-1 mAbs have differential FcγR-binding requirement for optimal therapeutic activity. (A) The additional therapeutic efficacy of anti-CTLA-4 mAb is achieved by depleting intratumoral regulatory T cells (Treg) through the interaction of activating FcγR on immune effector cells. (B) Interaction to activating FcγR is detrimental to the antitumor efficacy of anti-PD-1 mAb due to the elimination of $PD\text{-}1^+$ 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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CHAPTER 2.

FcγR interaction is not required for effective anti-PD-L1 immunotherapy but can add additional benefit depending on the tumor model

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Sjef, ik kan bijna alles muriniseren
Cor, je bent fantastisch !

Abstract

Immunomodulatory antibodies blocking interactions of co-inhibitory receptors to their ligands such as CTLA-4, PD1 and PD-L1 on immune cells have shown impressive therapeutic efficacy in clinical studies. The therapeutic effect of these antibodies is mainly mediated by re-activating antitumor T cell immune responses. Detailed analysis of anti-CTLA4 antibody therapy revealed that an optimal therapeutic efficacy also requires binding to Fc receptors for IgG, FcγR, mediating depletion of intratumoral regulatory T cells. Here, we investigated the role of Fc binding in anti-PD-L1 antibody therapy in the MC38 C57BL/6 and CT26 BALB/c colon adenocarcinoma tumor models. In the MC38 tumor model, all IgG subclasses anti-PD-L1 showed similar therapeutic efficacy when compared with each other in either wild-type mice or in mice deficient for all FcγR. In contrast, in the CT26 tumor model, anti-PD-L1 mIgG2a, the IgG subclass with the highest affinity for activating FcγR, showed stronger therapeutic efficacy than other IgG subclasses. This was associated with a reduction of a myeloid cell subset with high expression of PD-L1 in the tumor microenvironment. This subclass preference for mIgG2a was lost in C57BL/6 x BALB/c F1 mice, indicating that the genetic background of the host may determine the additional clinical benefit of the high affinity antibody subclasses. Based on these data, we conclude that FcγR are not crucial for anti-PD-L1 antibody therapy but might play a role in some tumor models.

Introduction

Expression of programmed death ligand 1 (PD-L1), the ligand for programmed death 1 (PD-1), plays a paramount role in suppressing antitumor responses in human cancer patients. Because of the clinical success of PD-L1 blocking antibodies (1-6), Atezolizumab, Avelumab and Durvalumab have been approved by the U.S. Food and Drug administration (FDA) for the treatment of various advanced cancers.

Several lines of evidence suggest that the main effect of anti-PD-L1 mAb is abolishing suppression of on-going antitumor T cell responses through blocking PD-L1 on the tumor or tumor infiltrating myeloid cells from interacting with PD-1 on T cells (5, 7-9). Theoretically, this mechanism should be FcγR independent. Nevertheless, it has been suggested that antibody subclass, and subsequent FcγR binding might contribute to the therapeutic efficacy (10). For example, many studies have demonstrated an essential role for activating FcγR in the antitumor activity of mAbs targeting the co-inhibitory receptor C TLA-4 (11-13). Binding of the Fc part of anti-CTLA4 mAb to FcγR resulted in the depletion of regulatory T cells by antibody-dependent effector mechanisms within the tumor.

In the clinic, Atezolizumab and Durvalumab are engineered human IgG1 anti-PD-L1 mAbs, with a modification in their Fc-domain to prevent antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (14, 15). On the other hand, Avelumab has a wild-type human IgG1 Fc region which has been shown *in vitro* to engage FcγR expressing cells to induce ADCC. Since PD-L1 is expressed on tumor cells and tumor infiltrating immune cells, it might be that anti-PD-L1 mAb with the capability to engage FcγR influences the antitumor responses not only by blocking PD1-PD-L1 signalling pathway but also by depleting PD-L1 expressing tumor cells and/or tumor associated myeloid cells. Conversely, Fc-mediated effector mechanisms might be detrimental to the immune response due to the depletion of PD-L1^{+} immune effector cells.

In this study, we investigated the effect of the use of different mAb IgG subclasses on the efficacy of anti-PD-L1 in two mouse models of colorectal cancer (MC38 on C57BL/6 and CT26 on-BALB/c background). For this, we replaced the constant regions of anti-mouse PD-L1 mAb (rat IgG2a; clone MIH5) with different mouse constant regions to vary the binding to mouse FcγR. Our data suggest that FcγR are not critical for anti-PD-L1 mAb therapy but depending on the genetic background, the therapeutic efficacy of anti-PD-L1 could be enhanced by using the IgG subclass with the highest affinity for activating FcγR.

Materials and Methods

Generation of murinized anti-PD-L1 mAb

From the anti-mouse PD-L1 rat IgG2a producing Hybridoma line MIH-5, mRNA was isolated. This RNA was used as a template with the Clonetech SMARTer RACE cDNA 5'RACE kit to synthesize total cDNA. In the next step, the heavy and light chain variable regions encoding DNA sequences of the anti-mouse PD-L1 rat IgG2a cDNA were amplified using the following primers: Rat IgG2a Heavy chain: CH1: gcaccaaggtggacaagaaaattg (m&r_CH1IgG1_2a_& mIgG2b), Heavy chain CH2: acgtgtgttgtggtagacattagc

(Rat_IgG21_GSP1), Hinge region: ccaagggaatgcaatccttgt (Rat_IgG2a Hinge R), Light chain: gtcagcccaagtccactcccacactca (Rat lambdaR2); gtggacactgcaaatcccaccaa (Rat Lambda R1); gtggagactacccagcctttcaa (Rat Lambda R1 diff); tcagcccaagtccactcccacactca (Rat Lambda R2).

The resulting single PCR fragments of the variable regions of both the heavy and light chain were analysed by DNA sequencing. This sequence was codon optimised for expression in human cells. On the basis of this information the heavy and light chain variable region encoding DNA fragments were assembled from GBLOCKS (Integrated DNA Technologies, Leuven, Belgium). The anti-mouse PD-L1 heavy chain variable region encoding DNA fragment was cloned in the expression vectors pFUSE_CHIg_mG1, pFUSE_CHIg_mG2a and pFUSE_CHIg_mG3 to express the anti-mouse PDLI mIgG1, mIgG2a and mIgG3 heavy chain respectively. The light chain variable region encoding DNA fragment was cloned in pFUSECLIg mk to express the anti-mouse PD-L1 Kappa Light chain. Unfortunately, these vectors showed low expression of the proteins when transfected into HEK293 cells and therefore the antibody encoding sequences were cloned into the expression vector pCDNA3.1(A) which showed good transient expression in these human cells. In addition, an anti-PD-L1 mIgG2a antibody was generated with a D256A mutation in the Fc domain of mIgG2a engineered according to the protocol as described in Liu et al (16).

Mice

C57BL/6 and BALB/c mice were purchased from Charles River (L'Arbresle, France) and housed in the animal facility of the Leiden University Medical Center (LUMC). FcγRI/II/III/IV deficient C57BL/6 mice were generated in the transgenic mouse facility of the LUMC (17). These transgenic mice were bred in house and routinely checked for their genotype by PCR. All mice were housed in individually-ventilated-cage (IVC) systems under specific pathogen-free conditions and used at 8-12 weeks of age.

The health status of the animals was monitored over time. Animals tested negative for all agents listed in the FELASA (Federation of European Laboratory Animal Science Associations) guidelines for SPF mouse colonies (18). All mouse studies were approved by the animal ethics committee of the LUMC. Experiments were performed in accordance with the Dutch Act on Animal Experimentation and EU Directive 2010/63/EU ('On the protection of animals used for scientific purposes').

Syngeneic tumor models and tumor therapy

CT26 (kindly provided by Mario Colombo, Milano) and MC38 tumor cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Lonza) supplemented with 8% Fetal Calf Serum (Greiner), 25µM 2-mercaptoethanol and 100 IU/ml penicillin/streptomycin (Gibco). Cell lines were mycoplasma and MAP-tested before the start of experiments. The tumor cells ($2.5x10^5$ for MC38/MC38 PD-$L1^{-/-}$ and $1x10^5$ for CT26/ CT26 PD-$L1^{-/-}$) were injected subcutaneously into 8-12week-old mice in 100µl of PBS. PD-L1 deficient MC38 and CT26 cell lines were generated using CRISPR/Cas9 technology as described in Kleinovink et al 2017(9). 200µg of therapeutic antibody was injected intraperitoneally at day 6, 9 and 12 after tumor inoculation. Once palpable tumors were present, tumor size was measured twice per

week, using a caliper. Mice were sacrificed when tumors reached a size of 100mm^2 because of ethical reasons.

Re-challenge tumor studies

After an 80 days tumor-free period, mice with complete regression of MC38 and CT26 tumors, as well as naïve control mice, were re-inoculated subcutaneously in the left flank with either 2,5x10^5 MC38 or 1x10^5 CT26 tumor cells. Once palpable tumors were present, tumor size was measured twice per week, using a caliper. Mice were sacrificed when tumors reached a size of 100mm^2 because of ethical reasons.

Flow cytometry

Tumors were harvested into 1 ml of non-supplemented IMDM media in 24-well plates and manually minced into small pieces with scalpels, incubated with 2.5 mg/mL Liberase TL (Roche) for 20 minutes at 37ºC and single-cell suspensions were made using 70-µm cell strainers (BD Biosciences). FcR were blocked with 10% normal mouse serum and anti-mouse CD16/CD32 antibody (2.4G2). Cell surface markers were stained using the following antibodies: CD8α (clone 53-6.7), CD4 (clone L3T4), CD3ε (clone 145-2c11), CD11b (clone M1/70), F4/80 (clone BM8), CD45.2 (clone 104), Ly6G (clone 1A8), Ly6C (clone HK1.4), PD-L1 (clone MIH5). Dead cells were excluded based on 7-AAD staining (Invitrogen).

For assessing the binding of murinized anti-PD-L1 mAb to mouse PD-L1, PD-L1 expressing MC38 or B16F10 tumor cells (pre-treated with 20U/ml mIFNγ for 24hour) were pre-incubated for 15min with titrated murinized anti-PD-L1 mAb or serum collected from anti-PD-L1 mAb treated mice prior to staining with PE-labelled anti-PD-L1 mAb (clone MIH5). Cells were analysed by flow cytometry for PE labeling, which was blocked by the presence of unlabelled murinized anti-PD-L1 mAb. Analysis were performed using LSRII cytometer (BD) using FacsDIVA software (BD) and FlowJo Software (Tree Star).

Surface plasmon resonance

Surface plasmon resonance (SPR) measurements were carried out on a IBIS MX96 (IBIS technologies) as described (19). Biotinylated mouse FcγRI (50086-M27H-B-50), FcγRIIb (50030-M27H-B-50) and FcγRIV (50036-M27H-B-50) were purchased from SinoBiologicals; biotinylated mouse FcγRIII was not available, therefore His-tag conjugated FcγRIII (Sino-Biologicals, 50326-M08H-50) was used. All FcγR were spotted using a Continuous Flow Microspotter (Wasatch Microfluidics) onto a single SensEye G-streptavidin sensor (Senss, 1–08–04–008) allowing for binding affinity measurements of each antibody to all FcγR simultaneously on the IBIS MX96 (IBIS technologies) as described (19). The biotinylated FcγR were spotted in duplicate and in 3-fold dilutions, ranging from 30 nM to 1 nM for FcγRI, FcγRIIb and FcγRIV in PBS 0.075% Tween-80 (VWR, M126–100mL), pH 7.4. For the His-tagged FcγRIII, biotinylated anti-His IgG1 (GenScript, A00613) was spotted in duplicate and 3-fold dilution onto the sensor and 100 nM his-FcγRIII (equally diluted in PBS 0.075% Tween-80, pH 7.4) was loaded onto the sensor before each antibody injection. Antibodies were then injected over the IBIS at 1.5 dilution series starting at 8.8 nM until 795.915 nM in PBS in 0.075% Tween-80. Regeneration after every sample was carried out with acid buffer (100 nM

H3PO4, 0.075% Tween 80, pH 1.5). Calculation of the dissociation constant (KD) was done by equilibrium fitting to R_{max}=500. In the case of FcγR III, anti-His association and dissociation curves were subtracted before calculation of IgG-binding affinity using SPRINT 1.9.4.4 software (IBIS technologies). Analysis and calculation of all binding data was carried out with Scrubber software version 2 (Biologic Software, Campbell, Australia).

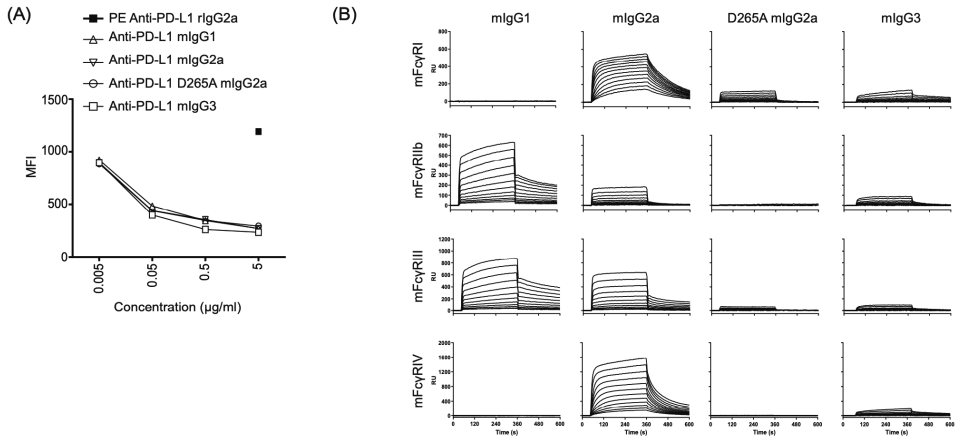
Statistical analyses

Data was analysed using Prism 7.0 (GraphPad Software). Statistical significance was calculated using the two-way ANOVA and Mann Whitney non-parametric test. Statistical significance was defined as $p < 0.05$. Tumor survival data was analysed with the Kaplan-Meier method and the log-rank (Mantel-Cox) test.

Results

Characterization of murinized anti-mouse PD-L1 IgG monoclonal Antibody

In order to determine the role of FcγR in anti-PD-L1 mAb therapy, the variable region of the rat anti-mouse PD-L1 mAb (clone MIH5, rat IgG2a) was fused to the different mouse immunoglobulin heavy chain constant regions by molecular cloning and protein was purified from HEK293 cells transfected with the expression vectors encoding the recombinant murinized immunoglobulins. These included mIgG1, mIgG2a, mIgG3 and a mIgG2a containing the D265A mutation (IgG2a D265A) which has been reported to abrogate binding to FcγR and strongly reduces complement activity (20, 21). We also assessed whether the murinized mAbs bound to the same epitope on PD-L1 as the parental anti-PDL1 rat IgG2a mAb. PD-L1 expressing MC38 tumor cells were pre-incubated with respective concentrations of purified murinized anti-PD-L1 mAb and then stained with fluorescently labelled anti-PD-L1 rat IgG2a. Each of these murinized mAbs showed equivalent binding to PD-L1 expressing MC38 tumor cells (Fig. 1A), suggesting that the murinized anti-PD-L1 and parental anti-PD-L1 mAbs recognize the same epitope on PD-L1 and that the different IgG subclasses bind with the same affinity. In addition, the binding affinity of the Fc portion of each mAb to mouse FcγRI, II, III, and IV was measured using surface plasmon resonance (Fig. 1B). Consistent with literature, mIgG1 bound with low affinity to FcγRIII and FcγRII, mIgG2a bound with high affinity to FcγRI, intermediate affinity to FcγRIV and low affinity to FcγRIII, mIgG3 did not bind to any FcγR. The mIgG2a D265A showed only residual binding to FcγRI (Fig.1 B). The calculated K_D values are shown in Fig.1C.



(C)

mFcγR	mIgG1	mIgG2a	D265A mIgG2a	mIgG3
I	no binding	16nM	387nM	>1500nM
II	226nM	>1400nM	no binding	>2100nM
III	250nM	409nM	>4500nM	>2300nM
IV	no binding	49nM	no binding	>2800nM

Figure 1. Characterization of murinized anti-PD-L1 mAb. A, Antibody competition binding assay. PD-L1 expressing MC38 tumor cells were pre-incubated with the depicted concentrations of murinized anti-PD-L1 mAbs followed by 5 µg/ml PE-labelled anti-PD-L1 rat IgG2a (clone MIH5) antibody. Single black square dot represents PE labelled anti-PDL1 rat IgG2a binding without competition. Cells were analysed by flow cytometry for PE labeling. B, Surface plasmon resonance analysis of murinized anti-PD-L1 antibody binding to mouse FcγRs. C, Calculated K_D values for IgG- mouse FcγRs interactions shown in B.

Therapeutic efficacy of murinized anti-PD-L1 IgG Monoclonal Ab is FcγR independent in MC38 tumor model

The MC38 colon adenocarcinoma syngeneic model on C57BL/6 background is highly immunogenic and it has been demonstrated to be sensitive to anti-PD-L1 mAb monotherapy(9, 22). The therapeutic efficacy of the murinized anti-PD-L1 mAbs was analysed in this tumor model. Strong inhibition of tumor growth (Fig. 2A), increased survival rates (Fig. 2B) as well as long-term immune memory (Fig.S1A) were observed after i.p. administration of each of the murinized anti-PD-L1 mAbs (Fig. 2A and B). We did not observe significant differences in delay of tumor outgrow and long-term survival between mice treated with anti-PD-L1 mAb of different IgG subclasses including the D265A mIgG2a, indicating that FcγR engagement was not required for the optimal therapeutic efficacy of anti-PD-L1 mAb. To confirm this, we studied the therapeutic efficacy of IgG1 and IgG2a anti-PD-L1 in MC38 tumor bearing C57BL/6 mice deficient for the ligand binding chains of all four FcγR (FcγRI/II/II/IV$^{-/-}$ mice) which show normal innate and adaptive immunity (17). In MC38 tumor bearing FcγRI/II/II/IV$^{-/-}$ C57BL/6 mice, the therapeutic efficacy of the murinized anti-PD-L1 IgG1 and IgG2a anti-PD-L1 antibodies was similar, resulting in cure in most of the treated mice (Fig. 2C and D) as

well as strong memory response (Fig. S1B). Collectively, our data demonstrate that interactions with FcγR are dispensable for the therapeutic efficacy of anti-PD-L1 IgG in the MC38 tumor model.

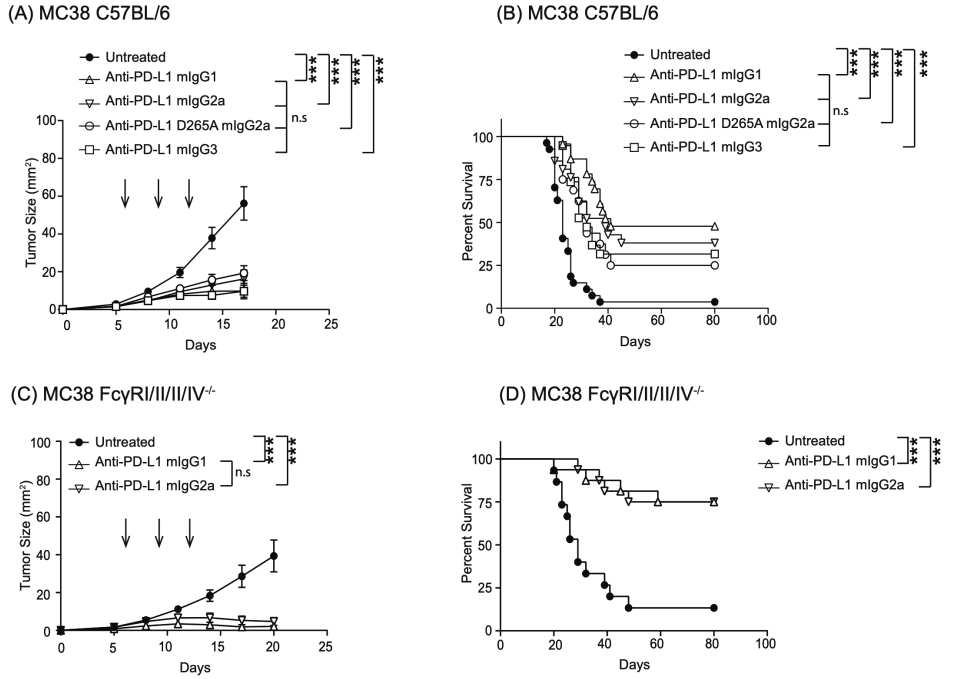


Figure 2. Antitumor activity of anti-PD-L1 antibodies in MC38 tumor model is Fc independent. A, Wild type C57BL/6 mice with established MC38 tumors were treated with the designated antibody. Data are presented as mean of tumor size mm^2 ± SEM. B, Same as A except data are presented as Kaplan-Meier survival curve. C and D, Same as A and B respectively, except FcγR I/II/III/IV$^{-/-}$ C57BL/6 mice were used. For the mean of tumor size, statistical significance between groups was determined by two-way ANOVA (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Logrank test was used to determine the statistical significance of the survival. Pooled data of two independent experiments, 15 to 16 mice per group.

Anti-PD-L1 IgG2a is more effective than anti-PD-L1 IgG1 and D265A mIgG2a in CT26 tumor model

As FcγR were dispensable for the therapeutic efficacy of anti-PD-L1 antibody in MC38 tumor model, we aimed to test if this is a general feature of this treatment modality, by verifying this in another tumor model. For this, we selected the CT26 colon adenocarcinoma syngeneic model on BALB/c background which is also known to be immunogenic and hence highly responsive to anti-PD-L1 mAb monotherapy (9, 23). When CT26 tumor bearing BALB/c mice were treated with murinized anti-PD-L1 mAb, all mIgG subclasses including D265A mIgG2a again effectively delayed tumor outgrowth resulting in prolonged survival (Fig. 3A and B) and formation of antitumor memory response (Fig. S1C). However, in contrast to the MC38 tumor model, overall survival rate of mice treated with anti-PD-L1 mIgG2a was significantly higher compared to mice treated with anti-PD-L1 mIgG1 or D265A IgG2a (Fig.3B). To establish that

this was not caused by differences in antibody clearance we measured the serum levels of circulating murinized anti-PD-L1 mAb in CT26 tumor bearing BALB/c mice using flow cytometry. We found that serum levels of the four murinized anti-PD-L1 mAb were similar. Thus, the higher efficacy of the murinized anti-PD-L1 mIgG2a in the CT26 tumor model cannot be explained by differences in drug exposure (Fig. S2).

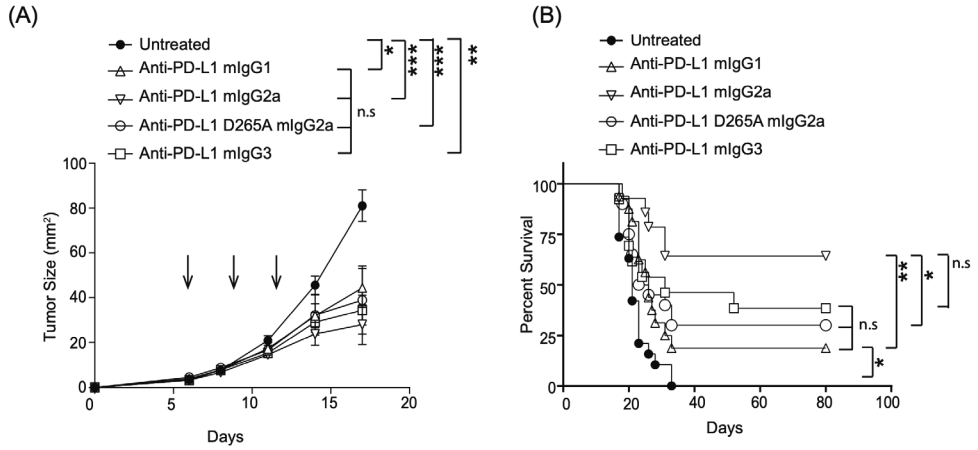


Figure 3. Anti-PD-L1 IgG2a is more effective than anti-PD-L1 IgG1 and D265A mIgG2a in CT26 tumor model. A, Wild type BALB/c mice with established CT26 tumors were treated with the designated antibody. Data are represented as mean of tumor size mm^2 ± SEM. B, Same as A except data are represented in Kaplan-Meier survival curve. For the mean of tumor size, statistical significance between groups was determined by two-way ANOVA (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Logrank test was used to determine the statistical significance of the survival. Pooled data of two independent experiments, total 15 to 16 mice per group.

Additional therapeutic effect of anti-PDL1 mIgG2a in a CT26 PD-L1$^{-/-}$ tumor model

CT26 tumor expresses PD-L1 (9), suggesting that the effect of anti-PD-L1 therapy may be a direct elimination of tumor cells mediated by FcγR on innate effector cells. Compared to mIgG1, mIgG2a binds with higher affinity to activating FcγRs resulting in more effective induction of downstream effector mechanisms such as antibody-dependent cellular phagocytosis (ADCP) or ADCC. In order to examine whether the difference in efficacy between IgG1 and IgG2a was solely due to Fc-mediated effector function of anti-PD-L1 mIgG2a directed against PD-L1 expressing tumor cells, we made use of a PD-L1$^{-/-}$ CT26 tumor model to compare the efficacy of anti-PD-L1 mIgG2a and mIgG1. Although PD-L1$^{-/-}$ CT26 tumor bearing mice treated with anti-PD-L1 mIgG1 or mIgG2a showed significant inhibition of tumor growth (Fig. 4A), improved long-term survival was observed only in mice treated with anti-PD-L1 mIgG2a but not mIgG1 (Fig. 4B). Our result suggests that the treatment of PD-L1$^{-/-}$ tumor can also benefit from the use of the mIgG2a subclass of anti-PD-L1 antibody. The additional therapeutic effect might be mediated through ADCC or ADCP of PD-L1 expressing non-tumor cells.

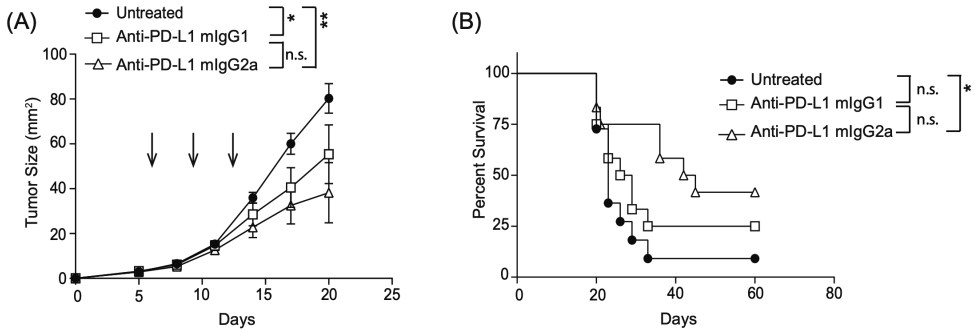


Figure 4. Antitumor activity of anti-PD-L1 mIgG1 and mIgG2a antibodies in CT26 PD-L1$^{-/-}$ tumor bearing mice. A, Wild-type BALB/c mice with established PD-L1$^{-/-}$ CT26 tumors were treated with the designated antibody. Data are represented as mean of tumor size mm^2 ± SEM. B, Same as A, except data are represented as Kaplan-Meier survival curve. Statistical significance of the mean tumor size was determined by two-way ANOVA (* P < 0.05; ** P < 0.01; *** P < 0.001). Logrank test was used to determine the statistical significance of the survival. Pooled data of two independent experiments, 12 mice per group.

Anti-PD-L1 mIgG2a but not mIgG1 modulates the tumor infiltrating myeloid cell subsets in the CT26 tumor microenvironment

Because PD-L1 is not only expressed on tumor cells but also on tumor infiltrating immune cells, we evaluated the relative expression of PD-L1 on multiple immune cell populations within the MC38 and CT26 tumor microenvironment. High levels of PD-L1 expression on F480^{hi} Ly6C^{lo} myeloid cells was observed as compared to F4/80^{int} Ly6C^{hi}, F4/80^{lo} Ly6C^{lo} myeloid cells, CD3 T lymphocytes, granulocytes (Ly6G$^+$) and CD45-negative cells (Fig. 5A). Based on the differences in PD-L1 density, we hypothesized that anti-PD-L1 mAb therapy could result in selective modulation of myeloid cell subsets. Therefore, we compared the impact of murinized anti-PD-L1 mIgG1 and mIgG2a on the frequency of immune cells in the tumor microenvironment of both tumor models. Administration of anti-PD-L1 mAb resulted in an increased frequency of CD4$^+$ and CD8$^+$ T cells in both CT26 and MC38 tumor (Fig. 5B). In MC38 tumors, the frequency of myeloid cell subsets between mice treated with murinized IgG1 or IgG2a anti-PD-L1 mAb and untreated mice was similar. In constrast, in CT26 tumors ,anti-PD-L1 mIgG2a treatment resulted in reduction of F480^{hi} Ly6C^{lo} cells, whereas the mIgG1 subclass did not affect this myeloid subset (Fig. 5C). Therefore, we hypothesized that the additional antitumor effect of anti-PD-L1 mIgG2a in the CT26 tumor model is due to its potential capability to directly target tumor-associated myeloid cells.

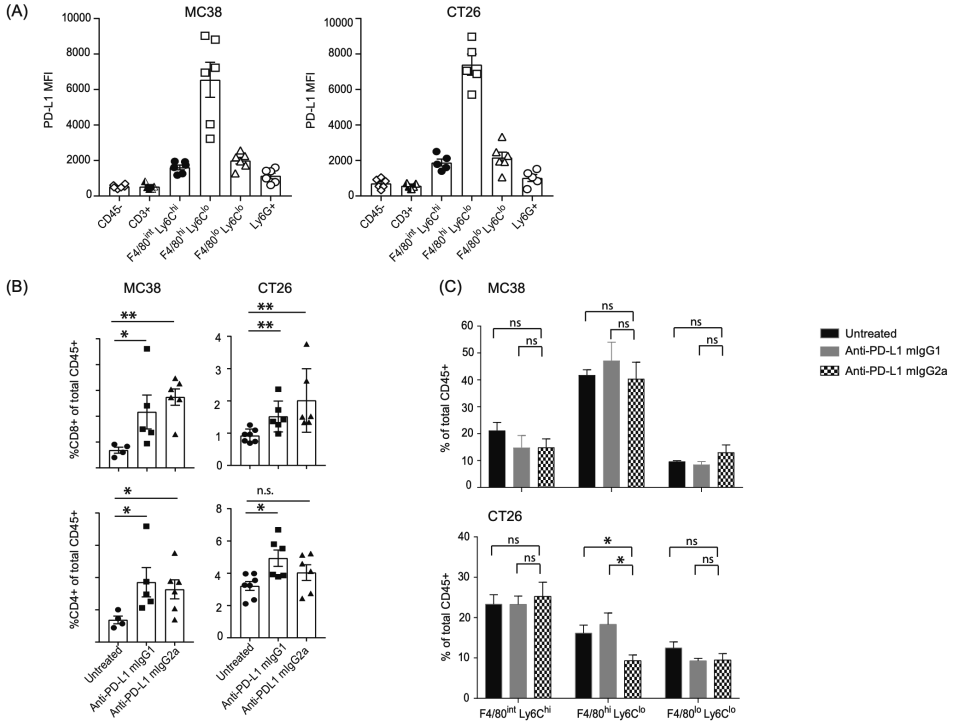


Figure 5. Anti-PD-L1 mIgG2a mAb modulates myeloid subsets in CT26 but not MC38 tumor. A, Untreated MC38 or CT26 tumors were harvested at Day 14 and analysed for PD-L1 expression pattern on indicated cell populations. B, Mice with established MC38 or CT26 tumores were treated with murinized anti-PD-L1 mIgG1 or mIgG2a. Tumors were harvested at Day 14 and analysed for the percentages of T lymphocytes. C, Same as B, except the tumors were analysed for the percentages of F4/80^{int} Ly6C^{hi}, F4/80^{hi} Ly6C^{low}, and F4/80^{low} Ly6C^{low} myeloid subsets. Bars represent mean ± SEM. Pooled data of two independent experiments, 5 - 6 mice per group. Statistical significance was determined by Mann Whitney non-parametric test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Similar therapeutic efficacy of IgG1 and IgG2a murinized anti-PD-L1 antibodies in C57BL/6 x BALB/c F1 mice

We next analysed whether the genetic background (BALB/c versus C57BL/6) of the mice or tumor cell intrinsic differences (MC38 versus CT26) determined the difference in therapeutic efficacy of anti-PD-L1 mIgG1 and mIgG2a. For this, C57BL/6 x BALB/c F1 mice (strain CB6F1/J) were inoculated with either MC38 or CT26 tumor cells and treated with anti-PD-L1 mIgG1 or mIgG2a. In both tumor models, no difference in survival was observed between mice treated with anti-PD-L1 mIgG2a or mIgG1 (Fig. 6A and B) indicating that the genetic background of the mice but not tumor cell intrinsic differences played a paramount role in the therapeutic efficacy of these antibodies.

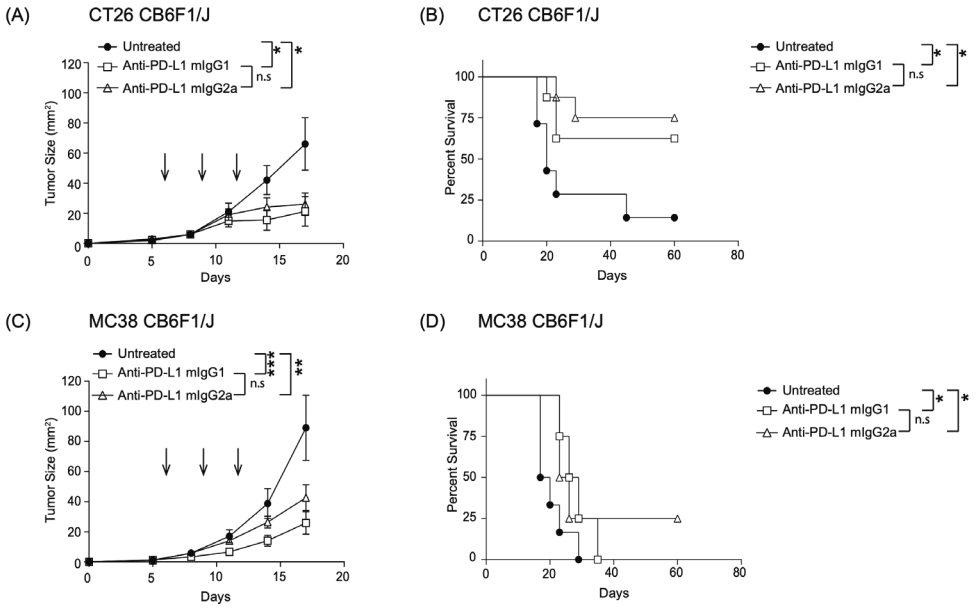


Figure. 6. Therapeutic efficacy of IgG1 and IgG2a murinized anti-mouse PD-L1 antibodies in CB6F1/J F1 mice. A, CB6F1/J mice with established CT26 tumors were treated with the designated antibody. Data are represented as mean of tumor size $mm^2 \pm$ SEM. B, Same as A except data are represented in Kaplan-Meier survival curve. C and D, Same as A and B respectively except MC38 tumor model was used. Statistical significance of the mean tumor size was determined by two-way ANOVA (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Logrank test was used to determine the statistical significance of the survival. Data from one experiment, 8 mice per group.

Discussion

It has been proposed that the underlying mechanism of tumor rejection in anti-PD-L1 antibody therapy is reactivation and increase of intratumoral T cells as the consequence of blocking PD1-PD-L1 inhibitory activity. It has recently become apparent, however, that the therapeutic efficacy of several other immunomodulatory antibodies such as anti-OX40, GITR, and CTLA4 mAb can also be attributed to the activation of Fc mediated pathways (11, 13, 24, 25). In contrast, two preclinical studies have revealed that interaction with its Fc part is detrimental to the therapeutic efficacy of anti-PD-1 mAb, by facilitating macrophages to deplete $PD1^+$ effector T cells (10) or to remove this mAb from T cells (26). This illustrates that it is important to understand the impact of the interaction with FcγR on the therapeutic efficacy of any immunomodulatory antibody. Here, we investigated the role of FcγR in the antitumor effect of PD-L1 blocking antibody. From our studies with the MC38 and CT26 syngeneic colon adenocarcinoma models, we conclude that the therapeutic effect of anti-PD-L1 mAb is predominantly based on FcγR independent blocking of PD-L1. This is conflicting with a recent study showing a minor enhancement of therapeutic efficacy of anti-PD-L1 mIgG2a over other IgG subclasses in MC38 tumor model (10). This might be caused by differences in experimental conditions as some of the genetically modified mouse strains and anti-PD-L1 mAb used in their study are different from ours.

On the other hand, in the subcutaneous CT26 tumor model, anti-PD-L1 mIgG2a elicited stronger therapeutic effect and survival compared to anti-PD-L1 mIgG1, mIgG3 and mIgG2a D265A. We observed that this enhanced antitumor efficacy correlated with the reduction of a tumor-infiltrated myeloid subset in CT26 tumor. For this tumor model, an association between an elevated number of myeloid cells and the increased magnitude of their immunosuppressive tumor microenvironment was reported while elimination of these cells can lead to strong antitumor responses (27, 28). Preferential reduction of this myeloid subset by anti-PD-L1 mIgG2a may be due to its high levels of surface PD-L1 expression compared to other immune and non-immune cells, similar to that proposed for anti-CTLA4 mAb, which preferentially depletes high CTLA4 expressing regulatory T cells (12). As mIgG2a (but not IgG1) can also efficiently activate complement (29, 30), we cannot exclude that the higher efficacy of IgG2a anti-PD-L1 in the CT26 tumor model is caused by the activation of complement pathways. The potential involvement of various Fc-mediated effector functions towards myeloid cell eradication and how the eradication of a myeloid subset in the CT26 tumor microenvironment augment subsequent adaptive immune responses will need further investigation.

We observed a comparable therapeutic efficacy of anti-PD-L1 mIgG1 and mIgG2a in CT26 tumor bearing CB6F1 mice, offspring of a cross between BALB/c and C57BL/6 mice, suggesting that the stronger antitumor efficacy of anti-PD-L1 mIgG2a is strain-dependent. Although multiple *in vitro* and *in vivo* data indicate that human Fc polymorphisms can influence the therapeutic activity of anti-CD20 (rituximab), anti-CD52 (alemtuzumab), anti-Her2 (trastuzumab, pertuzumab) and anti-EGFR (cetuximab), in mice two polymorphisms are reported, one in FcγRII and one in FcγRIII, but no difference in IgG binding was observed (31). Nevertheless, polymorphism in the Fc region of IgG has been found in mice. Whereas most inbred mouse strains including BALB/c express IgG2a, C57BL/6 mice express an allelic variant of that, named IgG2c (32). To the best of our knowledge, it is not known whether there is any difference in functionality between IgG2c and IgG2a. However, we cannot exclude the possibility that IgG2a is immunogenic in C57BL/6 mice and induces an immune response decreasing its efficacy over time.

The objective response rate with approved anti-PDL1 mAb as monotherapy is ~20% in urothelial carcinomas (6, 33, 34), ~15% in non-small-cell lung cancer (NSCLC) (35, 36) and ~30% in Merkel cell carcinoma (2, 37). Anti-PD-L1 mAb therapy is very effective but so far has only be tested in a restricted number of specific cancer types. However, in animal studies, antitumor activity of anti-PD-L1 treatment varies across a range of syngeneic tumor models (38). Thus, evaluation of the role of different tumor types and genetic background in the additional effect of anti-PD-L1 mIgG2a may have clinical implications, because the results might guide design and development of more effective anti-PD-L1 mAb for cancer therapy. To date, antibodies targeting PD-L1, including Atezolizumab, Avelumab and Durvalumab have been approved by FDA for treating various cancers. Unlike Atezolizumab and Durvalumab which are engineered human IgG1 mAbs to avoid Fc interaction, Avelumab has a wild-type human IgG1 Fc region. Although results of *in vitro* studies have suggested that Avelumab is capable of inducing ADCC of tumor cells (39-41), as far as we know, no evidence from clinical results has been published to support this. As previously reported, PD-L1 expression on immune but not tumor cells in the tumor microenvironment was significantly

associated with higher response to PD-L1 blocking antibodies(3, 6), indicating that blocking of PD-L1 on immune cells with antibodies (Atezolizumab and Durvalumab) is critical for the effectiveness of this therapy. Hence, it remains to be evaluated whether Avelumab has an additional therapeutic benefit in patients whose tumors are infiltrated by PD-L1$^+$ myeloid cells as our study in the CT26 model suggests. A study by Boyerinas et al. 2015 (41) showed very low levels of Avelumab mediated *in vitro* lysis of PD-L1$^+$ PBMC subsets of patients receiving Avelumab, but this does not rule out the possibility that PD-L1$^+$ immune effector cell subsets in the tumor microenvironment would be subject to some degree of depletion. The concerns related to toxicity versus clinical benefits of Avelumab have to be defined in larger clinical studies. Overall, our work suggests that anti-PD-L1 mAbs work primarily by blocking PD-L1 and binding to FcγR is no prerequisite for their antitumor activity. However, depending on the tumor model, interaction with FcγR can potentially enhance the therapeutic efficacy of anti-PD-L1 mAb.

Supplementary Figures

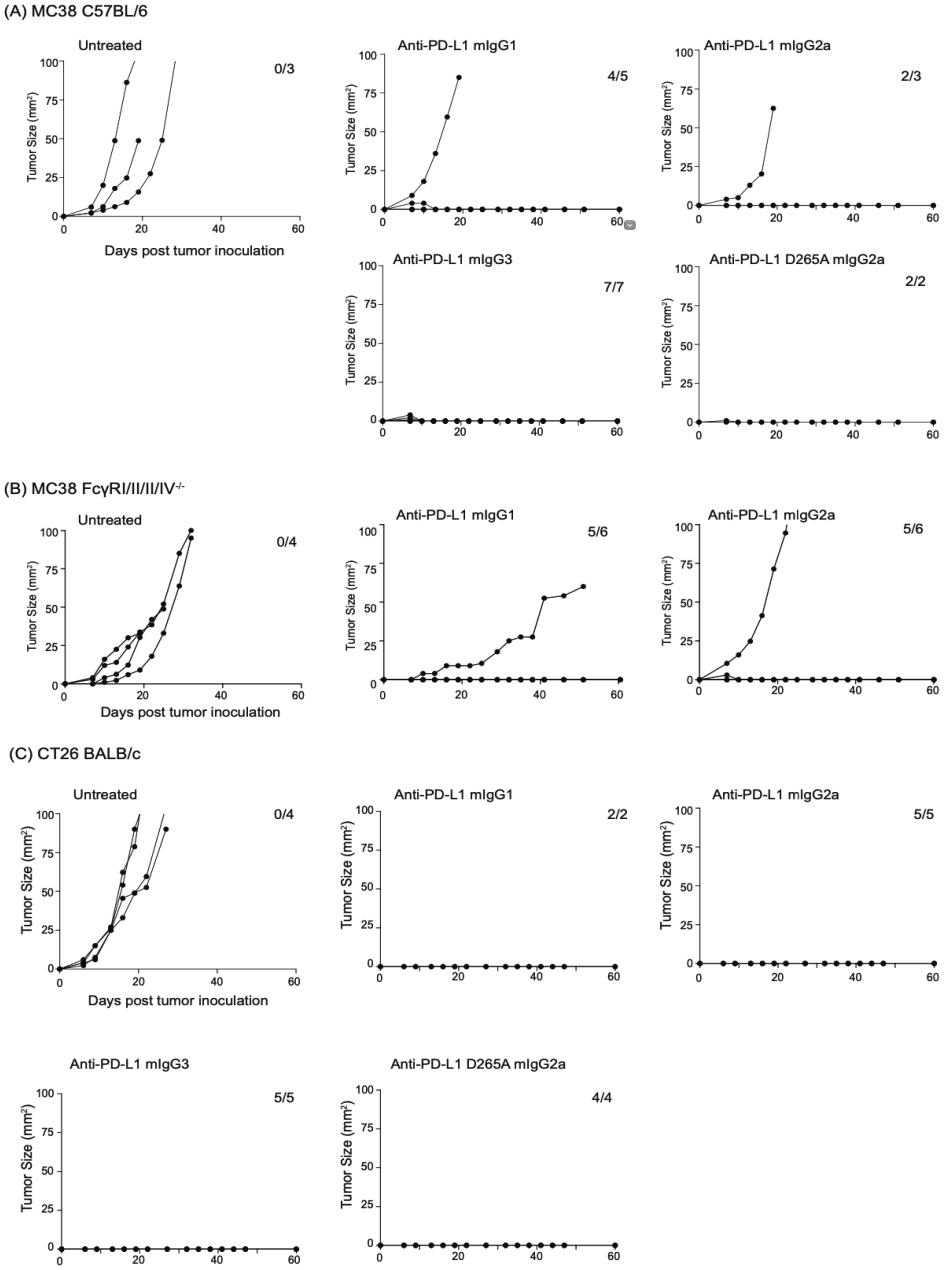


Figure S1. Protection against MC38 and CT26 tumor re-challenge–upon effective anti-PD-L1 antibody therapy is FcγR independent. A, C57BL/6 mice; B, FcγR I/II/III/IV$^{-/-}$ C57BL/6 and C, BALB/c with either complete MC38 or CT26 tumor rejection after anti-PD-L1 antibody therapy were re-challenged with 2.5x10^5 MC38 or 1x10^5 CT26 tumor cells at day 80 and these mice were followed for tumor outgrowth in the next 60 days.

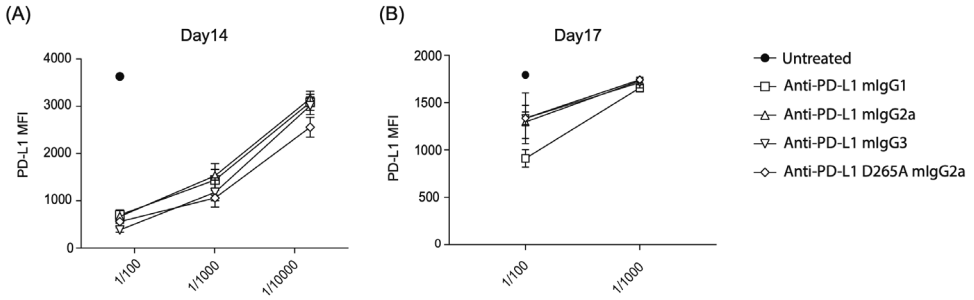


Figure S2. No difference in the serum levels of murinized anti-PD-L1 mAb in tumor bearing mice. Serum levels of murinized anti-PD-L1 mAb at Day 14 (A) and 17 (B) in CT26 tumor bearing BALB/c mice. PD-L1 expressing B16F10 tumor cells were pre-incubated with respective dilutions of mouse serum drawn from anti-PD-L1-treated CT26-tumor bearing mice, followed by PE-labelled anti-PD-L1 ratIgG2a (clone MIH5) antibody. Cells were analysed by flow cytometry for PE labeling, which was blocked by the presence of unlabelled anti-PD-L1-antibodies.

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3

CHAPTER 3.

Combined inhibiton of TGF-β signalling and the PD-L1 immune checkpoint is differentially effective in tumor models

Heng Sheng Sow, Jiang Ren, Marcel Camps, Ferry Ossendorp, and Peter ten Dijke.

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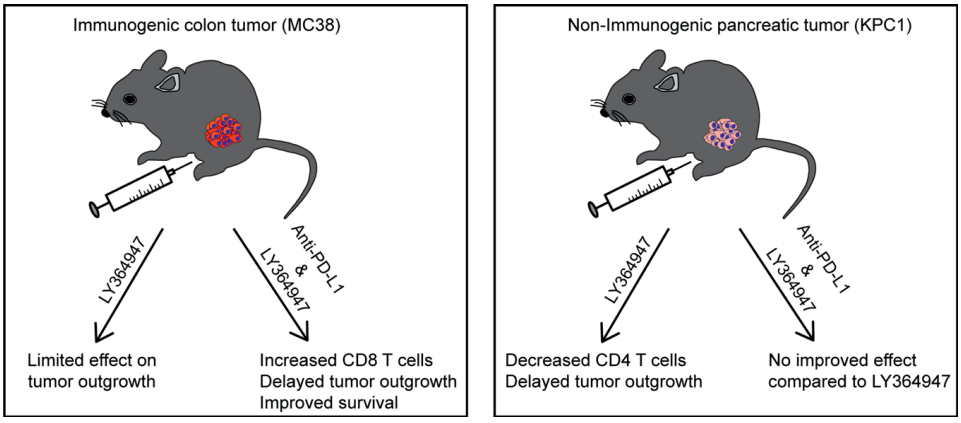
Is ball
Hahaha, Marcel heeft pijn in zijn benen
TGFb is fun, but Peter is funnier
Ik heb pijn in mijn benen

Abstract

Antibodies blocking the programmed death-ligand 1 (PD-L1) have shown impressive and durable responses in clinical studies. However, this type of immunotherapy is only effective in a subset of patients and not sufficient for rejection of all tumor types. In this study, we explored in two mouse tumor models if the antitumor effect can be enhanced by the combined blockade of PD-L1 and TGF-β, a potent immunosuppressive cytokine. The effect of anti-PD-L1 mAb and TGF-β type I receptor small molecule kinase inhibitor (LY364947) was evaluated in the highly immunogenic MC38 colon adenocarcinoma and poorly immunogenic KPC1 pancreatic tumor model. In the MC38 tumor model, LY364947 monotherapy did not show any antitumor effect, whereas treatment with anti-PD-L1 mAb significantly delayed tumor outgrowth. However, combination therapy showed the strongest therapeutic efficacy resulting in improved long-term survival compared with anti-PD-L1 mAb monotherapy. This improved survival was associated with an increased influx of $CD8^+$ T cells in the tumor microenvironment. In the KPC1 tumor model, LY364947 did not enhance the antitumor effect of anti-PD-L1 mAb. Despite this, delayed KPC1 tumor outgrowth was observed in the LY364947 treated group and this treatment led to a significant reduction of $CD4^+$ T cells in the tumor microenvironment. Together, our data indicate that an additive anti-tumor response of dual targeting PD-L1 and TGFβ is dependent on the tumor model used, highlighting the importance of selecting appropriate cancer types, by in-depth analysis of the tumor microenvironment, which can benefit from combinatorial immunotherapy regimens.

Graphical Abstract



Introduction

Immune checkpoint molecules are gaining prominence as targets for cancer immunotherapy, even demonstrating durable remission of patients with metastasic lesions (1). Last year the Nobel prize for physiology and medicine was awarded to James P. Allison and Tasuku Honjo "for their discovery of cancer therapy by inhibition of negative immune regulation (2)." Antibodies targeting programmed death ligand 1 (PD-L1) such as atezolizumab, avelumab, and durvalumab have received regulatory approval (3-6). Despite of showing remarkable durable remissions, these antibodies only demonstrate its efficacy in a subset of specific cancer types (7). In order to increase the therapeutic efficacy, many on-going preclinical and clinical studies are evaluating anti-PD-L1 mAb in combination with other immunostimulatory agents or cancer-modulating drugs. An important strategy is to down-regulate the immune suppression that is elicited by the tumor microenvironment to allow immunotherapy to be effective.

Transforming growth factor-β (TGF-β) is an immunosuppressive cytokine, which is often produced in large quantities by many cell types in the tumor microenvironment, including tumor cells (8, 9); regulatory T cells (10, 11); and myeloid suppressor cells (12, 13) . TGF-β is well known for its pleiotropic role from initiating to promoting tumor development (14-17) and it has a negative effect on anti-tumor immunity by suppressing the effector functions of several immune effector cells such as neutrophils, macrophages, natural killer (NK) cells, CD8 and CD4 T cells (16, 18-20). Together with other cytokines such as IL-2 and IL-6, TGF-β also induces the generation and recruitment of regulatory T cells to further suppress the antitumor T and NK cell responses (21, 22). Moreover, it is also known for its role in regulating and promoting the accumulation of stiff fibrillary extracellular matrix composed of collagen (23), resulting in hindering drug transport (24) and infiltration of immune cells (25-27) into the tumor. Most importantly, high serum levels of three TGF-β isoforms, TGF-β1, TGF-β2 and TGF-β3 correlate with poor clinical outcome (28-32).

As such, it is plausible that TGF-β inhibition, through reducing immune suppression and decreasing deposition of matrix collagen content, could potentially improve infiltration of activated immune effector cells and delivery of drug into the tumor microenvironment. In this study, we investigated if the treatment of TGF-β receptor 1 selective small molecule kinase inhibitor, termed LY364947 (33), can enhance the antitumor efficacy of anti-PD-L1 mAb in immunogenic (MC38 colorectal tumor) and poorly immunogenic (KPC1 pancreatic tumor) tumor models.

Materials and Methods

Cell culture

All tumor cell lines (RMA, EL-4, KPC1, KPC3, MC38, B16F10, B16OVA, C3 and TUBO) were cultured in Iscove's modified Dulbecco's medium (IMDM) (Lonza) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Greiner), 2 mM L-glutamine (Gibco), 25 μM 2-mercaptoethanol (Merck) and 100 IU/ml penicillin/streptomycin

(Gibco). Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS (Greiner) and 100 U/ml penicillin/streptomycin (Gibco) were used to culture HEK293 cells. All cell lines in our studies were maintained at 37°C, 5% CO_2 in a humidified incubator and were free of mycoplasma

Mice

Wild-type (WT) C57BL/6 female mice were purchased from Charles River (L'Arbresle, France) and maintained under specific pathogen free (SPF) animal facilities of the Central Animal Facility (PDC) of the Leiden University Medical Center (LUMC) and were 8-9 weeks old at the beginning of each experiment. The health status of the animals was monitored over time. Animals tested negative for all agents listed in the FELASA (Federation of European Laboratory Animal Science Associations**)** guidelines for SPF mouse colonies (34). All animal studies were approved by the animal ethics committee of LUMC. Experiments were performed recommendations and guidelines set by LUMC and the Dutch Act on Animal Experimentation and EU Directive 2010/63/EU (Guidelines on the protection of experimental animals').

Syngeneic tumor studies

MC38 colon adenocarcinoma cancer cells ($4x10^5$ cells) were injected subcutaneously into 8-12-week-old mice in 100µl of phosphate buffered saline (PBS). 200µg of anti-PD-L1 mAb (clone MIH5) was injected intraperitoneally at day 6, 8 and 11 after inoculation. LY364947 was purchased from Selleckchem and dissolved in DMSO to make final concentration of 20mg/ml. 10mg/kg of LY364947 was injected intraperitoneally at day 6, 8 and 11 and once every three days after cancer cell inoculation. The KPC1 pancreatic cancer cell line was generated from $Kras^{LSL\text{-}G12D/+}$, $Trp53^{LSL\text{-}R172H/+}$, Pdx1-Cre (KPC) mice and was a gift from Thorsten Hagemann (Queen Mary University of London). The tumor cells ($1x10^5$ cells) were injected subcutaneously into 8-12-week-old mice in 100µl of PBS. At day 9,11 and 14 post tumor inoculation, mice were injected intraperitoneally with 200µg of anti-PD-L1 mAb (clone MIH5). For the LY364947 or combination group, mice received 10mg/kg of LY364947 (intraperitoneally) at day 9 and once every day post tumor inoculation. All tumors were measured twice weekly using calipers. Mice were sacrificed when tumors reached a size of $100mm^2$ to avoid unnecessary suffering. Both cell lines were mycoplasma and MAP-tested before the start of tumor studies.

Flow cytometry

Harvested Tumors were manually minced into small pieces with scalpels before incubating with 350 µg/mL Liberase TL (Roche) for 20 minutes at 37ºC and filtered through a 70µm cell strainers (BD Biosciences) to obtain single cell suspension. The cells were subjected to ACK lysis (5 minutes) before staining with 10% normal mouse serum and anti-mouse CD16/CD32 antibody (clone 2.4G2) to block FcγRs. Single-cell suspensions of tumor-infiltrating lymphocytes were stained using the following antibodies: CD8α (clone 53-6.7), CD4 (clone L3T4), CD3ε (clone 145-2c11), CD11b (clone M1/70), F4/80 (clone BM8), CD45.2 (clone 104), Ly6G (clone 1A8), PD-L1 (clone MIH5). LAG-3 (C9B7W), CTLA-4 (9H10). 7-AAD

staining (Invitrogen) were used to exclude dead cells. All stained cells were analyzed on a LSRII cytometer (BD) and data analysis was performed with FlowJo Software (Tree Star).

mTGF-β1 ELISA

Briefly, tumor cell lines were cultured in 24-well plates in complete IMDM until 80% confluent. Cells were washed twice with PBS and cultured in IMDM supplemented with 1% FBS (not heat-inactivated) for 24 hours at 37°C. Supernatants were collected and stored at-20°C until further analysis. Total mTGF-β1 levels were measured by using a Mouse TGF-β1 duoset ELISA kit according to manufacturer's instructions (R&D Systems #DY1679).

CAGA luciferase reporter assay

To produce conditional medium (CM), MC38, KPC1, KPC3, B16F10 cells were washed two times with PBS at 70-80% confluency and incubated in serum-free DMEM medium for 24 h. CM was then collected and passed through a 0.45 mm Syringe Filter (SLHP033RB, Merck Millipore). HEK293 cells were seeded at approximately 5×10^4 cells per well into a 24-well plate. The next day, cells in each well were co-transfected with 0.1μg TGF-β/Smad-inducible $(CAGA)_{12}$ luciferase transcriptional reporter construct, which encodes 12 repeats of the AGCCAGACA sequence, identified as Smad3/Smad4-binding element in the human PAI-1 promoter (35), and 0.08 μg β-galactosidase construct (driven by cytomegalovirus promoter) using five time of polyethyleneimine in quantity. After overnight incubation, HEK293 cells were starved with serum free medium. Eight hrs later, serum free media were removed and replaced by CM. A TGF-β treatment (5 ng/ml, 8420-B3, R&D SYSTEMS) was also performed that served as a standard. After another overnight incubation, luciferase and β-galactosidase activities were measured. The luciferase activity was normalized based on the β-galactosidase activity. Representative experiments indicating the mean and standard deviation of triplicate values are shown.

Western blot

Approximately 2.5×10^5 of MC38 and KPC1 cells were plated in 6-well plate in complete medium and incubated overnight at 37°C. The next day, the complete medium was replaced with 0.2% FBS medium and further incubated at 37°C for eight hrs. Cells were then treated with 1 μg/ml of LY364947 for 30min before stimulating with 5 ng/ml of TGF-β3 for 2 hours. Cells were lysed in Radioimmunoprecipitation assay buffer (RIPA) sampler buffer (50 mM Tris–HCl (pH 8.0) with 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate) containing complete™ protease inhibitor Cocktail (11697498001, Roche). Protein concentration was determined using DC™ Protein Assay Kit (5000111, Bio-Rad). Equal amount of protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane (IPVH00010, Merck Millipore). Membrane was probed with Phospho-SMAD2 antibody (36) (homemade) and GAPDH antibody (AB2302, Merck Millipore). The chemiluminescent signal was detected using the Clarity™ Western ECL Substrate (1705060, Bio-Rad) and visualized using the ChemiDoc™ Imaging Systems (17001402, Bio-Rad).

***In vitro* cell proliferation assay**

MC38 and KPC1 cells were plated in 96-well plate, 2×10^3 cells/well approximately, and incubated overnight. Cells were treated with vehicle control, or 1 μg/ml of LY364947, or 5 ng/ml of TGF-β3, or LY364947 and TGF-β3 combination. The cell proliferation was determined by CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) (G5421, Promega BioSciences) following the manufacturer's protocol. Absorbance was measured at 490 nm over 5 consecutive days using VICTORX Multilabel Plate Reader (2030-0050, Perkin Elmer). Each group was evaluated in five repeats, and a cell growth curve was plotted.

Statistical analyses

Data was analysed using Prism 7.0 GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, United States). To determine statistical significance between two groups, an unpaired Student's t-test was performed. Significance between more than two groups was evaluated by one-way ANOVA. Kaplan-Meier method and the log-rank (Mantel-Cox) test were used to determine statistical differences in the survival of mice.

Results

Colorectal and pancreatic cancer cells produce high level of mTGF-β1

In order to select mouse tumor models to investigate the therapeutic efficacy of combining TGF-β inhibitor and anti-PD-L1 mAb, we measured mTGF-β1 production by various mouse tumor cell lines. As illustrated in Figure 1A, ELISA analysis revealed that both pancreatic (KPC1) and colorectal (MC38) cancer cell lines produce high levels of latent mTGF-β1 protein. Using a transcriptional reporter assay, we observed that MC38 but not KPC1 secreted elevated amounts of active mTGF-β (Fig 1B). Due to the high level of production of latent and/or active mTGF-β, MC38 and KPC1 were selected for *in vivo* analysis. We first evaluated the TGF-β/SMAD2 response and efficacy of the small molecule inhibitor LY364947 targeting the TGF-βRI serine/threonine kinase activity in both cell lines when cultured *in vitro*. TGF-β potently stimulated the phosphorylation of SMAD2 (pSMAD2) in MC38 and KPC1 cell lines and this was blocked by LY364947 (Fig. 1C). Despite these inhibitory effects, the proliferation of tumor cells remained unaffected by LY36947 and/or TGF-β treatment (Fig. 1D). Next, the effect of LY364947 treatment *in vivo* was determined by investigating intra-tumoral levels of pSMAD2 after i.p. injection of LY364947 in mice bearing either established MC38 or KPC1 tumors (Fig 1D). Histology analysis using phospho-SMAD2 antibody revealed strong phosphorylation of SMAD2 in control DMSO and, 1 and 4 hrs post LY364947 treated MC38 and KPC1 tumors. Decreased TGF-β-induced SMAD2 phosphorylation was observed in 8hrs post LY364947 treated tumors and this inhibitory effect of LY364947 appeared to last longer in MC38 than KPC1 tumors.

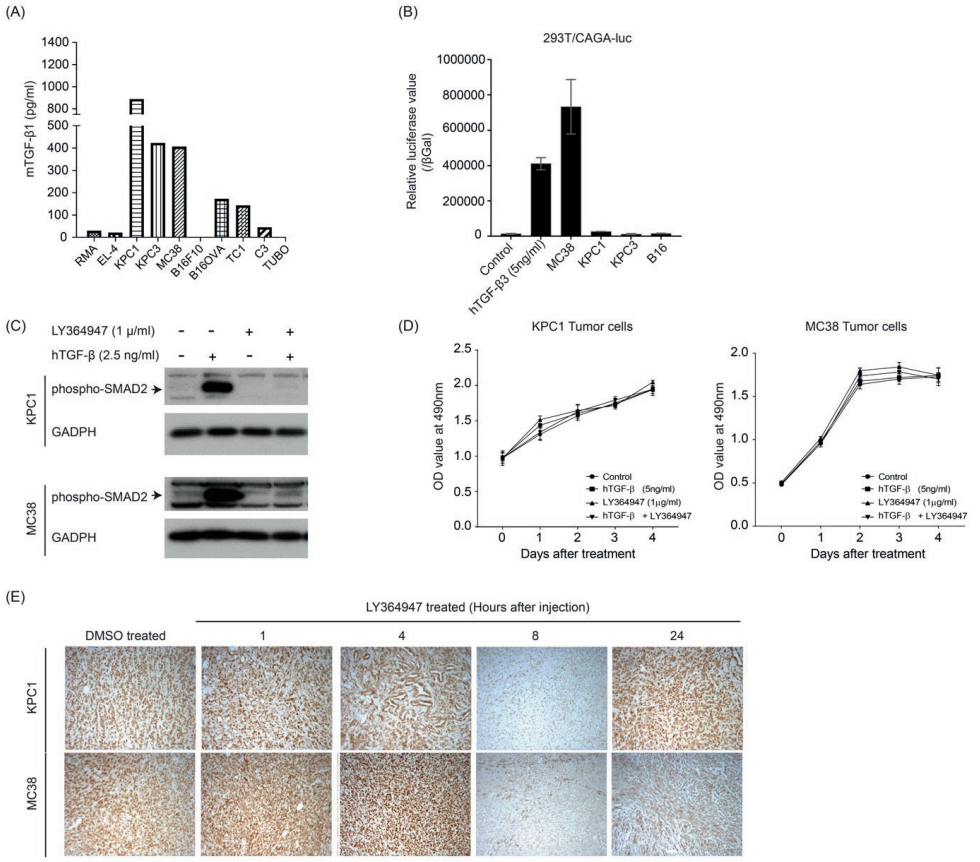
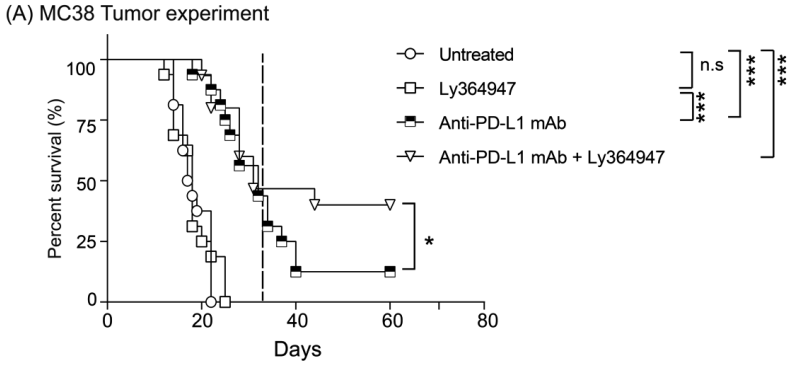


Figure 1. Production level of TGF-β by various preclinical mouse tumor models and the potency of LY364947 to inhibit TGF-β mediated cellular SMAD2 phosphorylation. Latent (A) and active (B) TGF-β in the conditioned media of cancer cell lines was assessed by TGF-β1 ELISA and CAGA-luciferase reporter assay, respectively. (C) Immunoblotting of phospho-Smad2 of KPC1 and MC38 tumor cell line after TGF-β and/or LY364947 treatment. GAPDH was measured as loading control. (D) Effect of the TGF-β and/or LY364947 on the proliferation of KPC1 and MC38 tumor cell lines. (E) Established MC38 or KPC1 tumor bearing C57BL/6 mice were administrated with LY364947 or DMSO, respectively. 1, 4, 8 and 24 hours after the injection, mice were sacrificed and tumors were analysed by immune-histochemical staining for phosphor-SMAD2.

TGF-β kinase inhibitor LY364947 improves therapeutic efficacy of anti-PDL1 mAb

The MC38 colon adenocarcinoma syngeneic model on C57BL/6 background is highly immunogenic and it has been demonstrated to be sensitive to anti-PD-L1 immune checkpoint monotherapy (37, 38). To test if LY364947 boosts the antitumor effect of anti-PD-L1 mAb, we examined the anti-tumor effect of these treatments on subcutaneously growing MC38 tumors in immune competent C57BL/6 mice. As shown in Fig 2A, LY364947 induced little therapeutic effect whereas treatment with anti-PDL1 mAb or combination therapy significantly delayed tumor outgrowth, leading to prolonged overall survival. Beyond day 31, the survival rate of mice treated with combination therapy showed significantly higher survival rate than mice received anti-PD-L1 mAb (Fig. 2A). These data suggest that the blockade of TGF-β

receptor activity enhances the anti-tumor immunity of anti-PD-L1 mAb therapy leading to improved overall long-term survival in immunogenic MC38 tumor model (Fig. 2B).



(B) Long-term survival (%)

Untreated	Ly364947	Anti-PD-L1 mAb	Combination
0/16 (0%)	0/16 (0%)	2/16 (12.5%)	6/16 (37.5%)

Figure 2. LY364947 improves anti-PDL1 mAb therapy. (A) MC38 tumor bearing mice were treated with 200 ug anti-PDL1 mAb i.p. (MIH5; Day 8, 10 and 13) and/or 10mg/kg TGF-β receptor kinase inhibitor i.p. (LY364947; Day 8, 10, 13 and every three days). Data presented as Kaplan-Meier survival curves with a total of 16 animals per group. Dashed line represents Day 31. Logrank test was used to determine the statistical significance of the survival. (C) Percentage of mice bearing subcutaneous MC38 treated with indicated regimens that rejected the tumor and survived tumor-free-long-term. Data compiled from 2 independent experiments, 16 mice per group.

Effect of combination therapy on the MC38 tumor microenvironment

To investigate the mechanism of action of anti-PDL1 mAb and LY364947 in the MC38 tumor model, we first analysed the impact of the therapies on the frequency of immune cells in the tumor microenvironment of MC38 tumors by flow cytometry. As shown in Fig. 3A, treatment with combined therapy of LY364947 and anti-PDL1 mAb led to a higher frequency of tumor infiltrating $CD3^+$ T cells. $CD8^+$ (Fig.3B, Fig.S1A) but not $CD4^+$ (Fig. 3C) T cells were accountable for the higher frequency to tumor infiltrating T cells. Moreover, blockade of TGF-β had no effect on the frequency of $Foxp3^+$ $CD4^+$ T cells (Fig.S2A). Frequencies of $F4/80^+$ macrophages (Fig. 3D) and $Ly6G^+$ granulocytes (Fig. 3E) were not significantly affected by LY364947 and combination therapy. Our results support previously reported studies which show that the combination of TGF-β and PD-L1 blockade increased the percentages of $CD8^+$ T effector cells in the tumor bed (27) which correlates with the improved tumor eradication of the combination treatment.

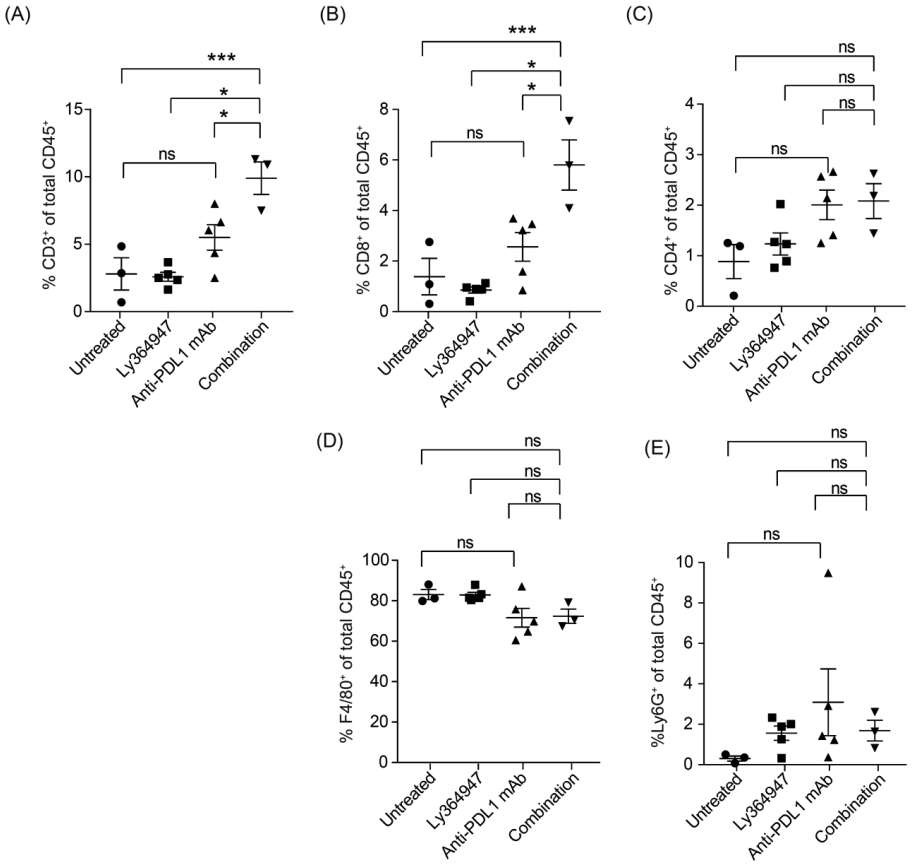


Figure 3. Combining TGF-β receptor kinase inhibitor LY364947 with anti-PDL1 mAb modulates infiltration of T cells in the tumor. Mice with established MC38 were treated with anti-PD-L1 mAb (200ug; Day 8, 10 and 13) and /or LY364947 (10mg/kg; Day 8, 10, 13, and 14). Tumors were harvested at Day 15 and anlysed for the percentages of CD3 (A) CD8 (B) and CD4 (C) T lymphocytes by flow cytometry. Bars represent mean ± SEM. Statistical significance was determined by one-way ANOVA (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

TGF-β inhibitor delays KPC1 pancreatic tumor outgrowth

Unlike the MC38 colorectal tumor model which is known to have high mutational load (39), the KPC tumors, which are derived from the KPC transgenic mouse strain which drives pancreatic ductal adenocarcinoma (PDA) tumorigenesis by expression of a combination of strong oncogenes, is a poorly immunogenic tumor due to a low mutational burden (40). To investigate the potential checkpoint inhibitors in this PDA model, we examined the expression of Programmed cell death protein 1 (PD-1), Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and Lymphocyte-activation gene 3 (Lag-3) on T cells within the KPC tumor microenvironment. In KPC tumor, the infiltrating T cells were predominantly $CD4^+$ and majority of them expressed PD-1 (Fig. 4A). These data suggest that blockade of PD-1/PD-L1 may improve antitumor immunity in KPC1 tumor model.

We therefore tested the effect of anti-PD-L1 mAb and LY364947 on KPC1 pancreatic tumor outgrowth. Treatment with anti-PD-L1 mAb did not impact tumor outgrowth. In contrast, treatment with LY364947 or combination therapy significantly reduced tumor outgrowth as compared to untreated group (Fig. 4B). This suggest that anti-tumor effect was most likely elicited by blocking of TGF-β signalling pathway. Moreover, flow cytometric analysis revealed a decrease of total $CD3^+$ T cells, particularly $CD4^+$ T cells (Fig. 4C; Fig. S1B) but no detectable decrease of $Foxp3^+$ $CD4^+$ T cells (Fig. S2B). No reduction was observed of granulocytes and macrophages (Fig 4C).

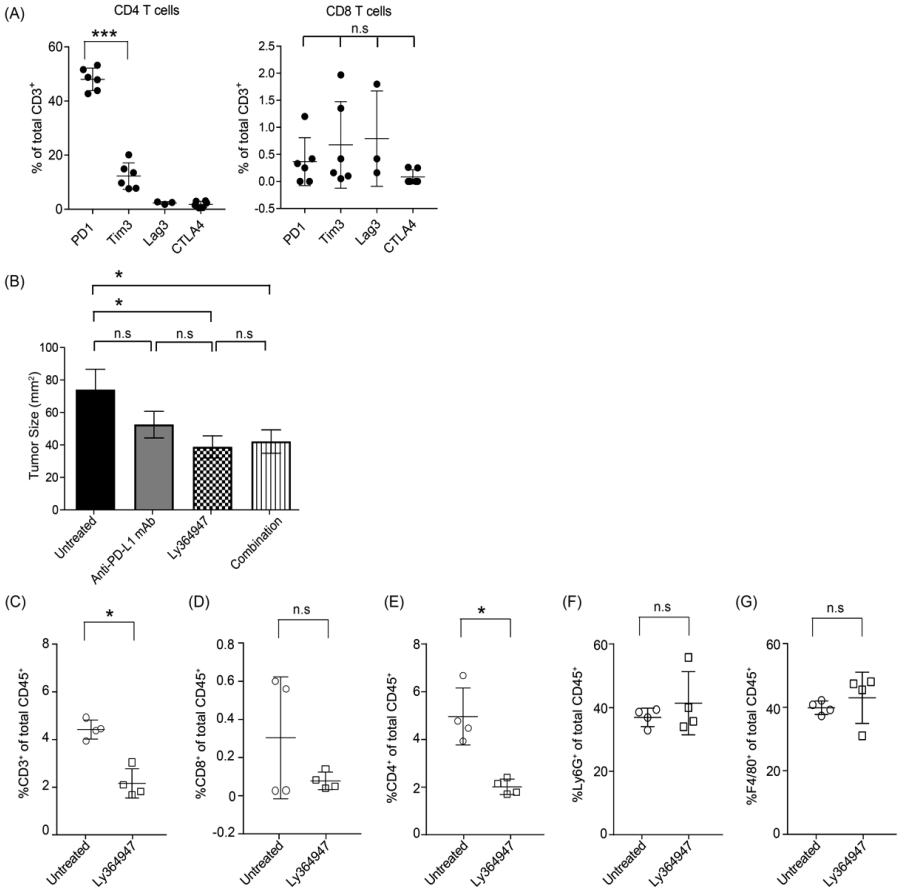


Figure 4. Antitumor effect of LY364947 in KPC1 tumor model. (A) Established KPC1 tumors were harvested at Day 17 and analysed for the percentages of PD-1^+; $Tim3^+$; $Lag3^+$; $CTLA4^+$ $CD4^+$ or $CD8^+$ T cells. (B) KPC1 tumor bearing mice were treated with 200ug anti-PDL1 mAb (MIH5; Day 8, 10 and 13) and/or 10mg/kg TGF-β inhibitor (LY364947; Day 8 and once every day). Data are represented as mean of tumor size mm^2 ± SEM at Day 23. Statistical significance was determined by two-way ANOVA (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Data from one experiment, 8 mice per group. (B) Mice with established KPC1 were treated with LY364947 (10mg/kg; Day 10 to 15). Tumors were harvested at Day 16 and analysed for the percentages of (C) $CD3^+$, (D) $CD8^+$, (E) $CD4^+$ T lymphocytes, (F) $Ly6G^+$ granulocytes and (G) $F4/80^+$ macrophages. Statistical significance was determined by Student's t-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Discussion

Experimental tumor models are essential preclinical step for the development and evaluation of cancer immunotherapy strategies. From our studies with the MC38 and KPC1 tumor models, one key finding that emerged is that tumor immunogenicity is a dominant feature predicting responsiveness to dual targeting of TGF-β signalling and PD-L1. In an immunogenic MC38 tumor model, blocking PD-L1 significantly delayed MC38 tumor outgrowth. However, combination LY364947 with anti-PD-L1 mAb further improved overall survival versus anti-PD-L1 mAb monotherapy. The antitumor activity of this combination therapy is consistent with the findings of multiple recent studies using immunogenic tumor models which demonstrated the improvement of anti-PD-L1 mAb when it is combined TGF-β receptor kinase inhibitor galunisertib (41, 42). In all studies investigating the therapeutic efficacy of galunisertib in the colon adenocarcinoma model, galunisertib was injected at high amounts (from 75mg/kg to 800mg/kg) and frequent intervals. This might explain the limited antitumor effect of LY364947 (10mg/kg) monotherapy on MC38 tumor outgrowth observed in our study. Nonetheless, we show that the anti-tumor activity of the combination therapy is associated with higher levels of tumor infiltrating $CD8^+$ T cells. This observation is in agreement with the finding of Mariathasan et al. (27) who demonstrated that the main mechanism of action of TGF-β is to increase T-cell infiltration into MC38 tumor. Together, these data suggest that co-administration of TGF-β and PD-L1 blocking agents may provide a subset of colorectal cancer patients a more favourable outcome.

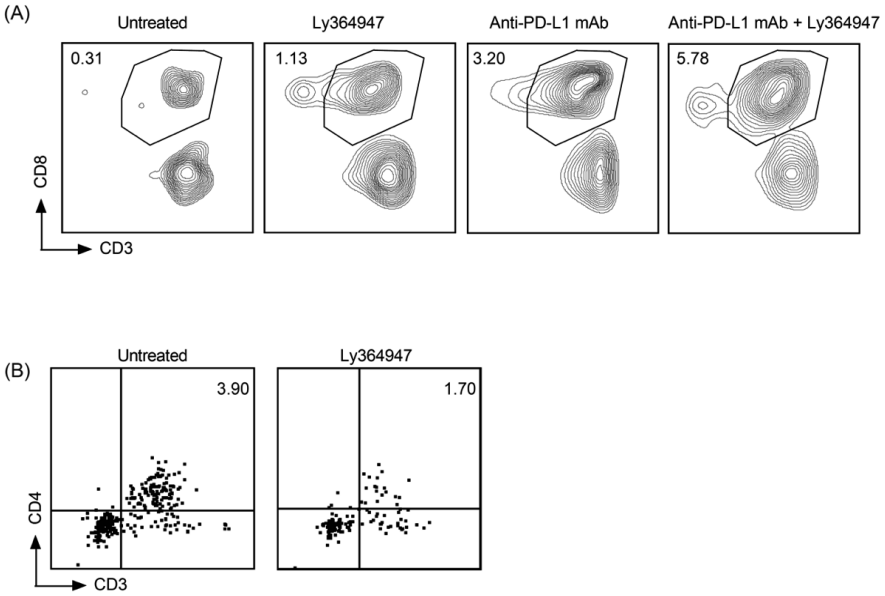
On the other hand, a combined effect of anti-PD-L1 mAb and LY364947 was not observed in poorly immunogenic KPC1 tumor model, blocking of TGF-β resulted in significant reduction of KPC1 tumor outgrowth in contrast to the anti-PD-L1 mAb treated group which was not effective in this model. This lack of antitumor efficacy is similar to the lack of responses observed in KPC tumor bearing animal treated with anti-PD1 and/or anti-CTLA-4 mAb (43). The limited effect of immune checkpoint inhibitors in this tumor model may due to the low mutational burden and absence of potential neoepitopes derived from tumor mutations (40). This model is reminiscent to most human pancreatic cancers with similar low number of mutations (44). For this reason, KPC pancreatic tumor model has a high potential of translational relevance for examining therapeutic efficacy of anti-PD-L1 mAb and LY364947. However, a small cohort of pancreatic cancer patients have been shown to have a relatively high mutational burden (45, 46), this may have an impact on the therapeutic efficacy of anti-PD-L1 mAb and LY364947. Therefore, study with an alternative pancreatic tumor cell line such as Pan02 (derived from PDAC tumor induced by implanting 3-methyl-cholanthrene in the pancreas of C57BL/6 mice)(47) that has a higher mutational burden may help address this question.

More evidence is emerging that targeting TGF-β can elicit beneficial effects in halting the pancreatic tumorigenic process. In a study by Principe and colleagues (48), global loss of TGF-β signaling protected against pancreatic tumor development via inhibition of tumor-associated fibrosis, stromal TGF-β1 production, and restoration of anti-tumor $CD8^+$ T cells responses. Here we showed that the treatment with LY364947 alone of established subcutaneous KPC1 tumor decreases the relative amount of $CD4^+$ T cells within the tumor

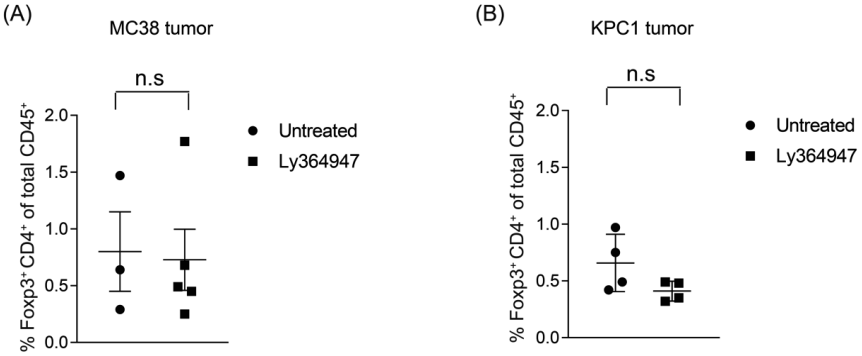
microenvironment. The potential role of $CD4^+$ T cells in promoting pancreatic tumorigenesis has been reported by Alam et al, who showed that the p38 MAP kinase inhibitor induced reduction in the percentage of $CD4^+$ TILs producing tumor necrosis factor (TNF)α, Retinoic acid-related orphan receptor (RORγt, interferon (IFN)γ and interleukin (IL)-17 was associated with improved survival in KPC tumor bearing animals. Significant delay in pancreatic intraepithelial neoplasia (PanIN) was reported in spontaneous pancreatic tumor model KC mice that received weekly CD4 depleting antibodies (49). Although TGF-β might also be expected to reduce the regulatory $CD4^+$ T cells (Tregs) cell population (11, 50), our data suggest that the numbers of Tregs are not strongly affected by LY364947 and therefore future investigations are warranted to reveal the subsets of CD4 T cells that are affected by the TGF-β inhibitor as this would guide the development of therapeutic strategies to target specific tumor promoting $CD4^+$ T cells in pancreatic tumor.

Clinical studies with galunisertib (LY2157299 monohydrate) have demonstrated its safety and potential antitumor activity (51-53). It is currently under clinical development in combination with checkpoint inhibitors in patients with non-small cell lung carcinoma (NSCLC), hepatocellular carcinoma (HCC) (NCT02423343), or pancreatic cancer (NCT02734160). In addition, there is an ongoing phase I/II study of galunisertib in combination with the anti-PD-1 antibody nivolumab in participants with advanced refractory solid tumors and in recurrent or refractory non-small cell lung cancer or hepatocellular carcinoma (metastatic and/or unresectable; NCT02423343). Anti-PDL1 mAb therapy is very effective but not all patients respond to this as single agent. The objective response rate with approved anti-PD-L1 mAb as monotherapy is ~20% in urothelial carcinomas (54-56), ~15% in non-small-cell lung cancer (NSCLC) (57, 58) and ~30% in Merkel cell carcinoma (5, 6). Targeting TGF-β pathway inhibition represents an attractive strategy to enhance immune checkpoint blockade. Indeed, a recent study has shown that lack of response to atezolizumab (anti-PD-L1 mAb) in metastatic urothelial cancer patients was associated with active TGF-β signalling in peritumoral stroma and especially in patients with T cells excluded from the tumor parenchyma (27). However, it is unclear whether lack of responsive to PD-L1 checkpoint blockade is also correlated with active TGF-β signalling in other patients of different tumor types. Furthermore, even though the combination of TGF-β blockade and checkpoint inhibitor has been demonstrated in multiple preclinical studies, their therapeutic efficacy varies across a range of syngeneic tumor (27, 41, 42, 59-61). Together, our studies indicate that adequate immune phenotyping of the various tumor models is critical for both rational model selection and data interpretation. This is critical as TGF-β has a diverse and profound effects on the immune system and therefore knowledge of the mechanisms by which TGF-β interferes in different tumor models may improve the current TGF-β based immunotherapeutic approaches for specific tumor types.

Supplementary Figures



Supplementary Figure 1. Flow cytometric analysis of T cells in the tumor microenvironment. (A) Representative plots show the frequency of $CD8^+$ T cells (plotted against the frequency of $CD45^+$ cells) in MC38 tumor. (B) Representative plots show the frequency of CD4 T^+ cells (plotted against the frequency of $CD45^+$ cells) in KPC1 tumor.



Supplementary Figure 2. No detectable reduction of tumor infiltrating $Foxp3^+$ $CD4^+$ T cells upon treatment with LY364947. Flow cytometry analysis of frequency of $Foxp3^+$ $CD4^+$ T cells in (A) MC38 and (B) KPC1 tumors. Statistical significance was determined by Student's t-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

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CHAPTER 4.

High Fcγ-receptor expression on intratumoral macrophages enhances tumor-targeting antibody therapy

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TA99 is mIgG2c, okay!!!
Hreinn
This world is crazy
Hreinn
Not good
Hreinn

Abstract

Therapy with tumor-specific antibodies (Abs) is common in the clinic but has limited success against solid malignancies. We aimed at improving the efficacy of this therapy by combining a tumor-specific Ab with immune-activating compounds. In this study, we demonstrate in the aggressive B16F10 mouse melanoma model that concomitant application of the anti-TRP1 Ab (clone TA99) with TLR3-7/8 or -9 ligands, and IL-2 strongly enhanced tumor control in a therapeutic setting. Depletion of NK cells, macrophages, or $CD8^+$ T cells all mitigated the therapeutic response, showing a coordinated immune rejection by innate and adaptive immune cells. FcγRs were essential for the therapeutic effect, with a dominant role for FcγRI and a minor role for FcγRIII and FcγRIV. FcγR expression on NK cells and granulocytes was dispensable, indicating that other tumoricidal functions of NK cells were involved and implicating that FcγRI, -III, and -IV exerted their activity on macrophages. Indeed, $F4/80^+$ $Ly6C^+$ inflammatory macrophages in the tumor microenvironment displayed high levels of these receptors. Whereas administration of the anti-TRP1 Ab alone reduced the frequency of these macrophages, the combination with a TLR agonist retained these cells in the tumor microenvironment. Thus, the addition of innate stimulatory compounds, such as TLR ligands, to tumor-specific Ab therapy could greatly enhance its efficacy in solid cancers via optimal exploitation of FcγRs.

Introduction

Tumor-targeting Ab therapy is an established treatment in cancer, and Abs such as anti-Her2/neu (ErBb2) (trastuzumab, pertuzumab), anti-CD20 (rituximab), and anti-EGFR (cetuximab) are commonly used in the clinic. There are several means by which tumor-targeting Abs can mediate tumor cell killing. The Abs for solid tumors, anti-Her2 (ErBb2) and anti-EGFR, interfere directly with cell signaling by blocking these receptors on the cell surface, resulting in induction of apoptosis. Interaction of the Fc part of Abs with complement can result in the induction of complementdependent cell-mediated phagocytosis and complement-dependent cell-mediated cytotoxicity (1). Another important mechanism is the activation of innate effector cells, such as NK cells, macrophages, and neutrophils, through binding of the Fc region of the Ab to FcγR. FcγR binding initiates downstream effector functions directed toward tumor cells, including Ab-dependent cell-mediated cytotoxicity, Abdependent cell phagocytosis (ADCP), and trogocytosis (2, 3) as well as innate effector functions like release of cytokines and chemokines that can alter the tumor microenvironment (4, 5).

So far, all Food and Drug Administration–approved tumortargeting Abs act via interference with cell signaling (6, 7). To what extent additional activation of innate effector cells contributes to their therapeutic efficacy is difficult to determine in cancer patients. However, several studies have demonstrated a correlation between FcγR polymorphisms influencing the affinity of IgG binding and the therapeutic efficacy of anti-EGFR, anti-HER2/neu, and anti-CD20 Abs (6, 8–10). These data strongly argue for FcγR contribution, in addition to cell signaling interference, to the mode-of-action of these tumor-targeting Abs.

TA99 is a widely used tumor-targeting mouse IgG2a mAb that binds to the protein Trp1 (gp75) expressed at the cell surface of B16F10 tumor cells. Trp1 is not a signaling protein, making it an optimal model to study the role of Ab downstream effector functions. Several publications demonstrated the efficacy of the TA99 Ab in a prophylactic setting, as the treatment started at the same day as injection of the tumor cells (11–15). All these studies demonstrated a crucial role of FcγRs. The clearance of tumor cells, most likely by ADCP, required macrophages that express all activating FcγR (14, 16, 17) or neutrophils expressing FcγRIII and FcγRIV (18). In contrast, application of the TA99 Ab in a therapeutic setting, after a solid tumor is established, had no impact on tumor outgrowth (19). Interestingly, a combination of TA99 with TLR4 agonists or IL-2 with extended half-life had some therapeutic effect, suggesting that there is room for improvement of TA99-based therapy (20–22).

In preclinical studies, TLR agonists are applied in tumor therapy, as they stimulate both adaptive and innate immune responses. It has been shown that they are effective in many mouse models both in combination with other treatments and as monotherapy to induce tumor clearance (23–26). Moreover, a recent phase I clinical trial showed that a TLR8 agonist improved treatment with anti-EGFR Ab (27).

In this study, we investigated the synergy between TA99 with the innate stimulating compounds imiquimod, CpG, poly(I:C), agonistic CD40 Ab, and IL-2 in a therapeutic setting of B16F10 melanoma. We observed a significant increase in survival of mice treated with the combination of TA99, a TLR ligand, and IL-2 compared with untreated mice or mice treated with TA99 alone. The therapeutic effect of this combination depended on $CD8^+$ T cells, NK

cells, and macrophages and activating FcγR. The addition of imiquimod and IL-2 increased the relative proportion of inflammatory macrophages with high expression of FcγR in the tumor microenvironment and, most likely, thereby making the tumor-targeting TA99 Ab more efficacious in elimination of tumor cells.

Materials and Methods

Mice

Mice were housed and all experiments were performed at the specific pathogen–free animal facilities of the laboratory animal facility of the Leiden University Medical Center (LUMC). The health status of the animals was monitored over time. Animals tested negative for all agents listed in the Federation of European Laboratory Animal Science Associations guidelines for specific pathogen–free mouse colonies (28). All mouse studies were approved by the animal ethics committee of the LUMC. Experiments were performed in accordance with the Dutch Act on Animal Experimentation and European Union Directive 2010/63/EU ("on the protection of animals used for scientific purposes"). C57BL/6J mice, both female and male, were purchased from Charles River Laboratories. All FcγR knockout (KO) mice were generated in the transgenic mouse facility of the LUMC: FcγRI 2/2 mice (29), FcγRIII/IV$^{-/-}$ mice (30), and the novel generated FcγRIII/IV$^{fl/fl}$ (Supplemental Fig. 1). FcγRI/III/IV$^{-/-}$ were generated by crossbreeding FcγRI$^{-/-}$ and FcγRIII/IV$^{-/-}$ mice. The EIIaCre deleter strain (n > 20 on C57BL/6J background) was a kind gift of Dr. H. Westphal. The Flp deleter strain C57BL/6-Tg(CAG-lpe)36Ito/ItoRbrc, was purchased from The Jackson Laboratory (Bar Harbor, ME). The hMRP8-Cre strain was provided by Dr. O. Sohnlein as a kind gift of Dr. E. Pasague´, and the Ncr1-Cre strain was a kind gift of Dr. V. Sexl. Both strains were backcrossed into C57BL/6 background. Mice were checked for their genotype by PCR. All mice were used in the age range of 8–12 wk.

Generation of novel conditional FcγRIII/IV$^{fl/fl}$ mouse

For the generation of the conditional FcγRIII/IV double KO mouse (FcγRIII/IV$^{fl/fl}$) on C57BL/6 background, a single LoxP site was introduced in the first intron of the FcγRIII gene by microinjection of a homologous LoxP site containing single-stranded oligodeoxynucleotide, CRISPR single guide RNA, and Cas9 mRNA in the fertilized oocytes of a previously generated FcγRIV$^{fl/fl}$ mouse strain (Supplemental Fig. 1A) (30). The sequence of single guide RNA homologous to intron 1 of FcγRIII gene is 5′-GTTCCAATTCCAGGCCAACC-3′ (underlined position indicates the double strand break). The sequence of single-stranded oligodeoxynucleotide with LoxP site (bold) and NheI restriction site (underlined) is 5′-TGATAGGAGAATCAGGAGTTCAAGGTTAGCTTCAACTATAGTGCGTTCCAATTCC AGGCCGCTAGCA**TAACTTCGTATAGCATACATTATACGAAGTTAT**AACCTGGC TTTGTGAGACCCTGTGAAAAAAAGAGATTTCTAGAGCTGGGTGTGGTGATCG-3′. The obtained offspring were backcrossed two generations into C57BL/6J background and subsequently intercrossed, and mice homozygous for the LoxP site in intron 1 of the FcγRIII gene and the two LoxP sites in the FcγRIV gene were crossed with C57BL/6 hMRP8-Cre or C57BL/6 Ncr1-Cre transgenic mice, resulting in neutrophil-specific and NK cell–specific FcγRIII/IV$^{-/-}$ mice, respectively. The deletion was confirmed by specific PCR (Supplemental

Fig. 1B, 1C). The sequences of the set of primers used were as follows: F3_cr_AB forward: 5′-GGTTTAGAAACCGGGGAGAG-3′, F3_cr_AB reverse: 5′-GACAGCCTTTTCAGGGACTG-3′; F4_geno_null forward: 5′-TAGACTAAAGGTCATGTGTGATC-3′, F4_geno forward: 5′-GGAGGCCAGAAGACTTTTA-3′, F4_geno reverse: 5′-GGAACTGTTTTGGAAGGATT-3′; Mrp8 forward: 5′-CGGTCGATGCAACGAGTGATGAGG-3′, Mrp8 reverse: 5′-CCAGAGACGGAAATCCATCGCTCG-3′; Ncr1 forward: 5′-GACCATGATGCTGGGTTTGGCCCAGATG-3′, Ncr1 reverse: 5′-ATGCGGTGGGCTCTATGGCTTCTG-3′. Neutrophil- and NK cell–specific FcγRIII/IV$^{-/-}$ mice developed normally and showed normal breeding characteristics. There were no evident effects on the homeostasis of the immune system, and the abundance of neutrophils and NK cells in these crossed mice was comparable to that of wild type (WT) mice (Supplemental Fig. 1D). The cell type–specific absence of FcγRIII and FcγRIV on the surface of immune cells was confirmed by flow cytometry using FcγRIII- and FcγRIV-specific Abs (Supplemental Fig. 2).

TA99 tumor therapy

C57BL/6J, FcγRI$^{-/-}$, FcγRIII/IV$^{-/-}$, FcγRI/III/IV$^{-/-}$, Ncr1-Cre × FcγRIII/IV$^{fl/fl}$, hMRP8-Cre × FcγRIII/IV$^{fl/fl}$, and control FcγRIII/IV$^{fl/fl}$ mice were s.c. injected at the left flank with 100,000 B16F10 cells that had been passaged twice after thawing. Mice were treated by i.p. injections of 200 μg of TA99 (mouse IgG2c isolated from TA99 hybridoma) Ab at day 5 and 7 in combination with topical application of 62.5 mg Aldara imiquimod cream (Aldara, 3M Health Care) containing 5% imiquimod at the right flank on shaved skin at day 4 and 11. Mice were anesthetized for few hours with xylazine and ketamine to prevent them from licking the cream. Alternatively, the other TLR ligands, CpG (50 μg oligodeoxynucleotide 1826; InvivoGen) or 50 μg poly(I:C) (Hiltonol; Oncovir) were applied by injecting the mice s.c at the right flank. Mice were injected i.p. with recombinant human IL-2 (6×10^5 IU; Novartis, Breda, the Netherlands) at day 11 and 12 after tumor transplantation.

In the combination of agonistic anti-CD40 Ab (clone FGK) and TA99, C57BL/6J mice were injected i.p. with 200 μg of TA99 at day 5, 10, and 15 and with 80 μg of anti-CD40 i.p at day 8, 13, and 18.

Analysis of tumor-infiltrating immune cells

C57BL/6J mice were injected with 100,000 B16F10 cells. When tumors were around 27 mm^3 in size, mice were treated with imiquimod cream. One day later, mice were injected i.p. with 200 μg of TA99. At day 2, mice were euthanized, and tumors were harvested. The tumor was cut in small pieces and incubated with 0.2 μg Liberase (Roche) for a half hour at 37°C. Single-cell suspensions were made by mincing the tumor through a cell strainer (BD Biosciences). Cells were resuspended in staining buffer (PBS plus 0.5% BSA plus 0.05% sodium azide) and incubated with Abs to Ly-6C (FITC, clone HK1.4), Ly-6G (Alexa Fluor 700, clone 1A8), F4/80 (PE or PE-Cy7, clone Bm8), CD86 (allophycocyanin, clone IT2.2), NK1.1 (PE-Cy7 or PE, clone PK136), CD45 (PE, clone 30-F11), FcγRI (a647, clone X54-5/7.1), FcγRIII (FITC, clone 275003 or AT154-2), FcγRIIb (a647, clone Ly-17.2), CD11b (PB), FcγRIV (FITC, Clone 012), CD4 (FITC, clone RM4-4), CD8 (A700, clone 53-6.7),

CD69 (allophycocyanin, clone H1.2F3) and CD3 (Pacific Blue, clone 17A2) and aminoactinomycin-D. The Abs were acquired from BD Biosciences, BioLegend/Sony, R&D Systems, Sino Biological, or eBioscience. The FcγRIIb-specific Ab was conjugated with an Alexa Fluor 647 labeling kit from Bio-Rad. FcγRIII-FITC of the clone AT154-2 was a kind gift of M.C. Southampton (31).

For intracellular cytokine analysis, splenocytes or tumor cells were washed and stained for cell surface markers as described above. Next, cells were fixed and permeabilized using the intracellular cytokine staining kit (BioLegend) according to the manufacturer's protocol. Next, cells were stained for IFN-γ using the mAb XMG1.2.

Immune cell depletion

C57BL/6J mice were injected i.p. with 100 μg of the mouse IgG2a (PK136) Ab to deplete NK cells and 50 μg of the rat IgG2b anti-CD8 (2.43) and anti-CD4 (GK1.5) to deplete $CD8^+$ and $CD4^+$ T cells, respectively, 2 and 5 d after tumor transplantation and then every 6 d onwards. Mice were injected i.v. and i.p. with 200 μl of control and clodronate liposomes (Liposoma B.V.) at day 3 and then every 5 d onwards. Blood was analyzed to monitor the depletion.

Statistics

Survival between groups was compared using the Kaplan–Meier curves and the log-rank (Mantel–Cox) test. Comparison of individual groups was done by one-way ANOVA or Student t test. All p values <0.05 were considered significant.

Results

Synergistic therapeutic effect of TA99 Ab with imiquimod and IL-2 in B16F10 melanoma

Two subsequent injections of the tumor-targeting mAb TA99 (anti-Trp1/gp75, mouse IgG2a isotype) at day 5 and 7 after transplantation of B16F10 melanoma cells did not induce a therapeutic effect. To improve its therapeutic efficacy, we added immune stimuli to create a therapeutic window. A combination of TRL7/8 agonist imiquimod in the form of Aldara cream application on the shaved skin, IL-2, and TA99 induced a significant increase in survival of B16F10-bearing mice compared with mice treated with TA99 alone or imiquimod/IL-2 or untreated mice (Fig. 1A, 1B). Imiquimod and IL-2 were administered at distant sites and were not targeted directly into the tumor microenvironment, indicating that systemic activation of adaptive and innate immune cells improves the tumor-targeting TA99 Ab therapy. In a subsequent experiment, it was evident that imiquimod provided the strongest support to TA99 because removal of IL-2 but not imiquimod still significantly improved survival (Supplemental Fig. 3). However, addition of IL-2 to this protocol seemed to further boost the combination and was thus included in further studies (Supplemental Fig. 3).

To investigate whether imiquimod cream was unique in this therapy improvement or whether other TLR agonists improved Ab therapy as well, we replaced imiquimod with CpG or poly(I:C), which are agonists of TLR7/8 and TLR3, respectively. Systemic delivery through

s.c injection on a distant site from the tumor injection of these compounds induced a similar effect as imiquimod (Fig. 1C). Similar findings were recently reported with the use of TA99, poly(I:C), and a stabilized form of IL-2 (32). Furthermore, the therapeutic efficacy of TA99 was strongly improved by combination with an agonistic anti-CD40 Ab, a well-known stimulator of innate and adaptive immunity. Addition of anti-CD40 Ab resulted not only in delayed tumor outgrowth but also significantly more long-term survival of animals (Fig. 1D). Thus, activating stimuli of the adaptive and innate immune cells in general can improve tumor-directed Ab therapy.

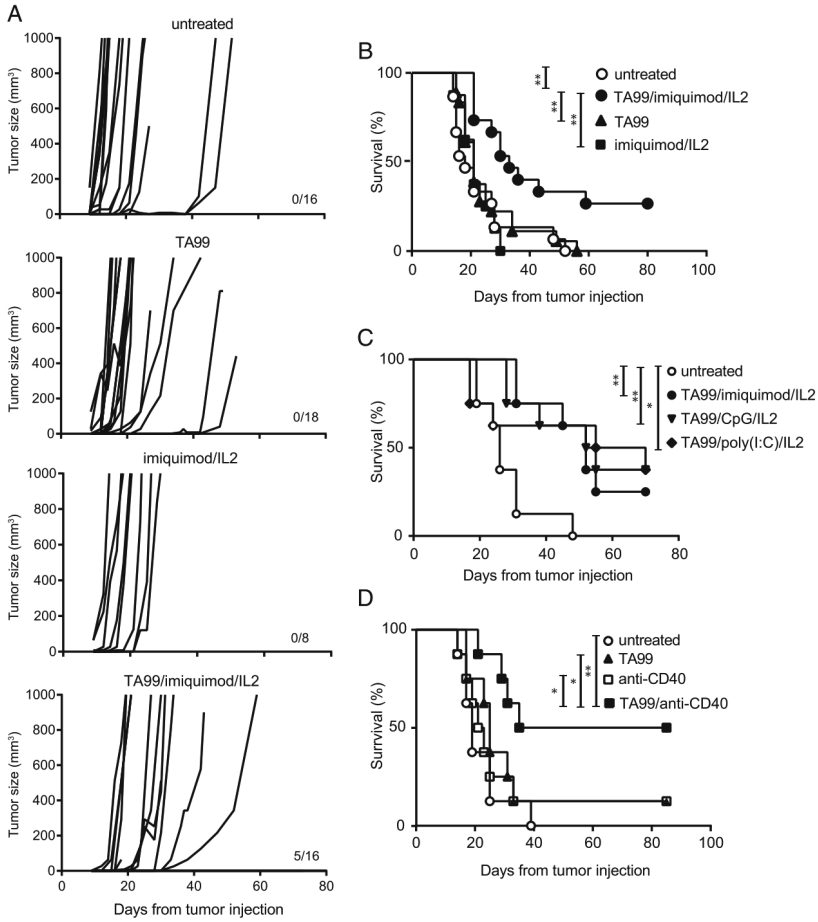


Figure 1. Imiquimod cream and IL-2 enhances the therapeutic efficacy of TA99 in B16F10 mouse model of melanoma. (A) Tumor growth curves of B16F10 melanoma treated with TA99 alone or combinations of imiquimod/IL-2 or TA99/imiquimod/IL-2. Numbers in each graph represent fraction of surviving mice at the end of the experiment at day 80. Compiled data from two independent experiments with similar outcomes are depicted (16–18 mice per group, except for imiquimod/IL-2, n = 8). (B) Kaplan–Meier survival curves of data shown in (A). (C) Kaplan–Meier survival curves of B16F10 melanomas treated with either TA99/imiquimod/IL-2 or in which imiquimod was replaced by CpG or poly(I:C). Experiment performed once, seven to nine mice per group. (D) Kaplan–Meier survival curves of B16F10 melanomas treated with TA99 in combination with agonistic CD40 Ab. Experiment performed three times with similar outcomes, seven to nine mice per group. *p < 0.05, **p 0.01, log-rank analysis.

The therapeutic efficacy of the combination of imiquimod, IL-2, and TA99 Ab depends on CD8$^+$ T cells and FcγRs

To unravel the underlying mechanisms of the synergistic effect of imiquimod, IL-2, and TA99, the combination therapy was tested in mice either depleted for CD8$^+$ or CD4$^+$ T cells or deficient for all three activating FcγRs. Depletion of CD8$^+$ T cells clearly mitigated the effect of the combination therapy, as significantly less survival in CD8$^+$ T cell–depleted mice was observed compared with nondepleted mice. In contrast, CD4$^+$ T cell depletion had no significant effect on survival (Fig. 2A, 2B). The depletion efficiency was monitored on blood cells and reached 100% in all mice (Supplemental Fig. 4A). The combination therapy failed to induce increased survival in B16F10-bearing mice lacking the three activating receptors FcγRI, -III, and -IV (Fig. 2B). In conclusion, in TA99/imiquimod/IL-2 combination therapy, both adaptive immune cells and activating FcγR were essential.

To understand which FcγRs on which immune cells were involved in the mechanism of action, several FcγR KO mouse strains were analyzed. In mice deficient for FcγRI, the therapeutic effect was completely abrogated (Fig. 3A). The treatment effect was also lost in the majority of mice constitutively lacking FcγRIII and FcγRIV, but, still, some mice controlled the melanoma (Fig. 3B). Together, these data suggested that FcγRI was the dominant receptor in this system. It is well documented that FcγRI is expressed exclusively on mononuclear myeloid cells, FcγRIV is expressed on mononuclear myeloid cells and neutrophils, and FcγRIII is expressed on all myeloid cells and NK cells (33). Thus, our observations suggested that FcγRI-expressing mononuclear cells play a dominant role in TA99/imiquimod/IL-2 combination therapy with a minor role for FcγRIII and FcγRIV in yet unidentified innate immune cells.

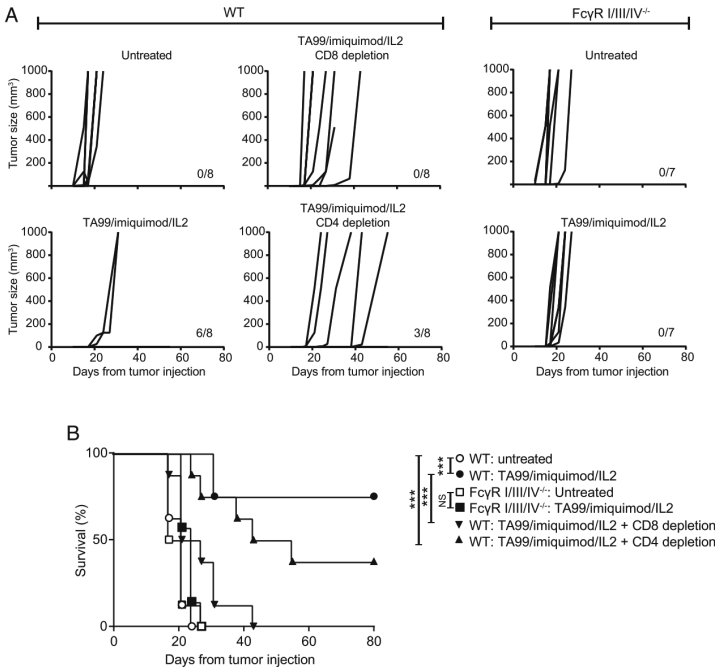


Figure 2. The therapeutic efficacy of TA99/imiquimod/IL-2 combination therapy depends on FcγR and CD8$^+$ T cells. (A) Tumor growth curves of B16F10 melanoma in WT mice (left panels) or in FcγRI/III/IV$^{-/-}$ mice (right panels). WT mice were also depleted for CD4$^+$ or CD8$^+$ T cells before the start of the TA99/imiquimod/IL-2 treatment (middle panels). Numbers in each graph represent fraction of surviving mice at the end of the experiment at day 80. Experiment was performed twice with similar outcomes, seven to eight mice per group. (B) Kaplan–Meier survival curves from data shown in (A). $p < 0.001$, log-rank analysis.

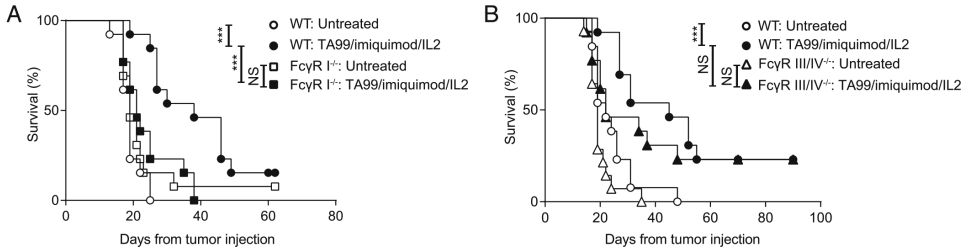


Figure 3. TA99/imiquimod/IL-2 combination therapy is abrogated in FcγRI$^{-/-}$ mice and diminished in FcγRIII/IV$^{-/-}$ mice. (A) Kaplan–Meier survival curves of B16F10 melanoma in WT mice and FcγRI$^{-/-}$ mice. Mice were treated with TA99/imiquimod/IL-2. (B) Kaplan–Meier survival curves of B16F10 melanoma in WT mice and FcγRIII/IV$^{-/-}$ mice. Mice were treated with TA99/imiquimod/IL-2. For both experiments, 12–14 mice per group from two comparable experiments were included. ***$p < 0.001$, log-rank analysis.

FcγRIII-independent role of NK cells in the combined therapy

NK cells only express FcγRIII and are capable of killing tumor cells via Ab-dependent cell-mediated cytotoxicity (34). Because FcγRIII and -IV were involved in the combination therapy and imiquimod is known to activate NK cells (23), we tested the influx and activation of NK cells in the tumor microenvironment by flow cytometry. NK cells were present in B16F10 tumors, and 3 d after imiquimod application, the proportion of NK cells seemed to be higher (Fig. 4A, 4B). Importantly, a significantly higher proportion of CD69-expressing activated NK cells were observed in B16F10 tumors (Fig. 4A, 4B), confirming previous data (23). To better understand the contribution of NK cells to the combination therapy, NK cells were depleted with anti-NK1.1 Abs during the therapy (Fig. 4C). Efficient depletion was confirmed on blood samples (Supplemental Fig. 4B). The survival was significantly lower compared with survival of nondepleted mice, but the therapeutic effect was not completely gone (Fig. 4C), demonstrating a minor contribution of NK cells. To determine whether this role of NK cells was FcγRIII dependent, we made use of mice deficient for FcγRIII exclusively on NK cells: Ncr1-Cre × FcγRIII/IV$^{fl/fl}$ mice (Supplemental Figs. 1, 2). NK-specific FcγRIII deficiency had no effect on the therapeutic efficacy of the combination therapy (Fig. 4D), and we concluded that other effector mechanisms of NK cells were involved. Alternate effector mechanisms of NK cells include release of cytokines, and imiquimod application, indeed, systemically induced IFN-γ release by NK cells and also led to higher frequencies of IFN-γ producing NK cells in the local tumor environment (Fig. 4E). In summary, our data indicate that NK cells contribute to the therapeutic effect of the combination therapy independent of FcγRIII.

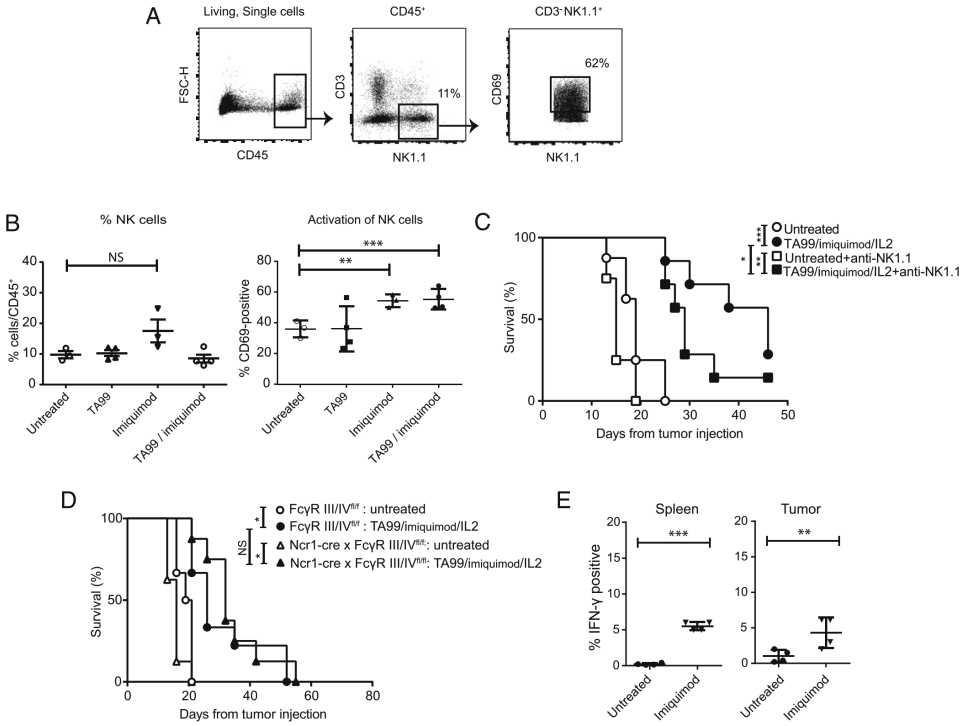


Figure 4. NK cells contribute to the efficacy of TA99/imiquimod/IL-2 therapy independent of FcγRIII. (A) B16F10 tumors were harvested from mice and enzymatically dispersed before analysis by flow cytometry. Gating strategy is shown for NK cells. (B) Tumor infiltration frequency and activation status (CD69 expression) of NK cells were determined by flow cytometry. Each symbol represents one mouse, seven to nine mice per group. Experiment performed twice with comparable outcomes. Means and SEM shown. $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA. (C) Kaplan–Meier survival curve of treated B16F10 melanoma in WT mice depleted for NK cells (anti-NK1.1). Experiment performed twice with similar outcomes, seven to nine mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, log-rank analysis. (D) Kaplan–Meier survival curve of B16F10 melanoma in mice lacking FcγRIII in NK cells (Ncr1-Cre x FcγRIII/IV$^{fl/fl}$ mice) and FcγRIII/IVfl/fl control mice. Experiment performed once, seven to nine mice per group. Log-rank analysis. $^{*}p < 0.05$. (E) Expression of IFN-γ by NK cells from spleen and tumor in WT mice determined by flow cytometry. Means and SEM shown. $^{**}p < 0.01$, $^{***}p < 0.001$, Student t test.

Improvement of anti-Trp1 therapy by imiquimod is mediated by mononuclear cells but not neutrophils

In addition to NK cells, other innate immune cells expressing activating FcγR were present in the microenvironment of B16F10 tumors. To get insight in the dynamics of these cells in response to the treatment, the immune infiltrates of tumors were analyzed by flow cytometry 3 d after the start of treatment. Comparisons of single treatment allowed us to determine the intrinsic effect of imiquimod. No gross changes were observed in the frequencies of total immune cells (CD45$^+$ cells) or total myeloid cells (CD11b$^+$ cells) (Fig. 5A). We then examined myeloid cell subsets and discerned inflammatory macrophages (CD45$^+$CD11b$^+$F4/80$^+$Ly-6C$^+$), stationary macrophages (CD45$^+$CD11b$^+$F4/80$^+$Ly-6C$^-$), and neutrophils (CD45$^+$CD11b$^+$F4/80$^-$Ly-6G$^+$). Frequencies of neutrophils seemed to decrease after Ab or

imiquimod administration, but these differences were not statistically significant (Fig. 5B). Importantly, imiquimod did enhance the proportion of inflammatory macrophages at the expense of stationary macrophages (Fig. 5B). The proportion of these inflammatory macrophages decreased after therapy with TA99 alone (Fig. 5B), most likely also decreasing downstream effector functions of TA99. Importantly, application of imiquimod corrected these decreased frequencies and resulted in sustained high proportion of these myeloid immune cells (Fig. 5B).

We then analyzed the FcγR expression levels by these three different myeloid cell subsets in the tumor and found that inflammatory macrophages displayed much higher levels of all four FcγRs than stationary macrophages and neutrophils (Fig. 5C). Neutrophils only expressed FcγRIII and FcγRIV, and we determined the role of these two activating receptors on neutrophils by using hMRP8-Cre × FcγRIII/IV$^{fl/fl}$ mice, which selectively lack FcγRIII and FcγRIV on this myeloid subset (Supplemental Fig. 2). Neutrophil-specific FcγR deficiency had no effect on survival (Fig. 5D), indicating that neutrophils did not play a FcγR-dependent role in the combination therapy. Together, these results implied an important contribution of mononuclear cells, most likely inflammatory macrophages, and their activating FcγR to this Ab combination therapy.

Knowing that imiquimod retained high proportions of inflammatory macrophages in the tumor environment (Fig. 5B) and that these myeloid cells displayed the highest FcγR levels of all myeloid subsets (Fig. 5C), we were interested in gaining insight into the differentiation direction of these cells. Application of imiquimod strongly enhanced the expression of CD86 on inflammatory macrophages, whereas the expression levels of this T cell costimulatory ligand did not alter on other myeloid subsets (Fig. 6A). These data suggested that imiquimod either polarized tumor-resident macrophages toward M1-like phenotypes or promoted an influx of M1-like macrophages. Of note, imiquimod boosted the presence of activating FcγRs, including the involved high-affinity FcγRI (Fig. 2A), in the tumor environment. To confirm the essential role of macrophages during combination therapy, we depleted them using clodronate liposomes (Fig. 6B, 6C). Tumor outgrowth was clearly hastened in TA99- and imiquimod-treated mice after macrophage depletion and percentages of long-term surviving mice were lower compared with mice with control liposomes. Efficiency of macrophage depletion was validated during the experiment (Supplemental Fig. 4C).

In summary, our data indicate that systemic application of immune stimuli improve tumor-targeting Abs in a therapeutic setting of established melanoma. This combination therapy depends on adaptive immune cells (CD8$^+$ T cells), innate lymphocytes (NK cells), and activating FcγR on intratumoral inflammatory macrophages.

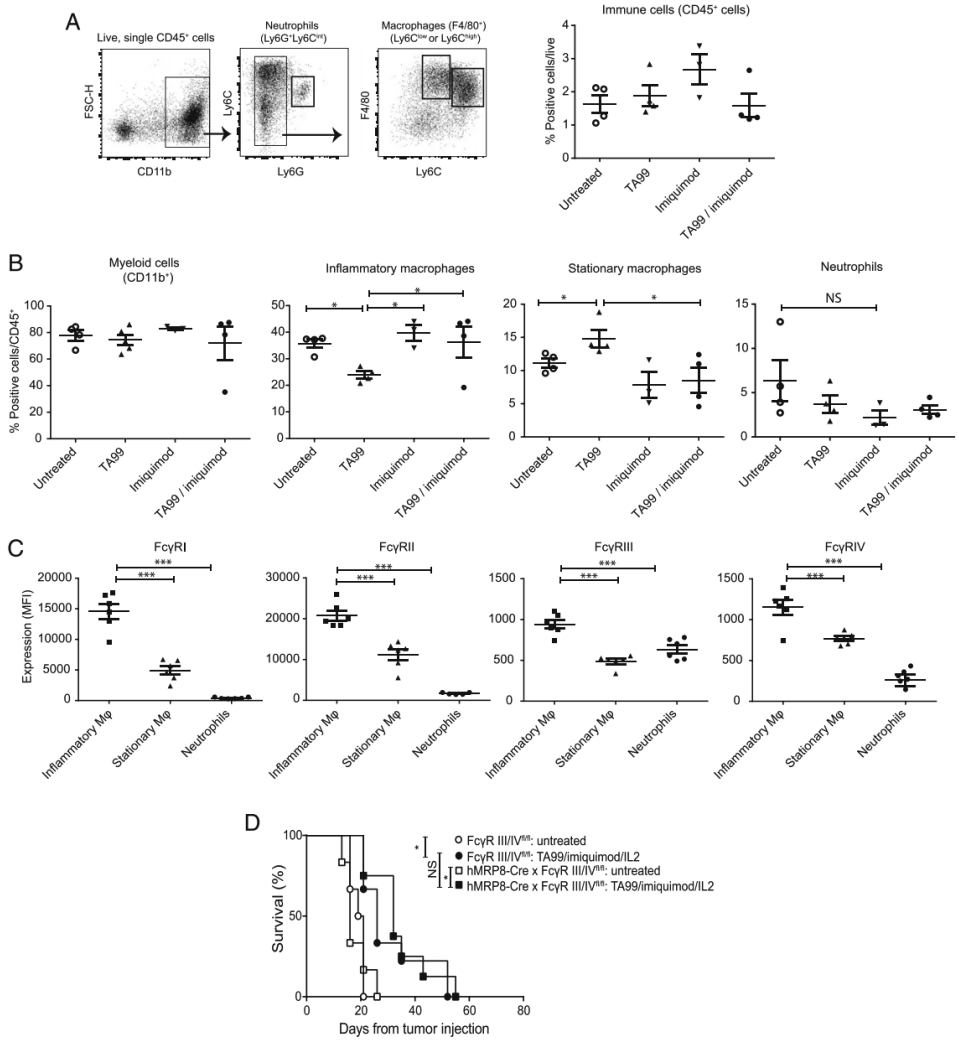


Figure 5. Imiquimod sustains the infiltration of highly FcγR-positive inflammatory macrophages. (A) B16F10 tumors were harvested from mice and enzymatically dispersed before analysis by flow cytometry. Gating strategy is shown for myeloid cells, and frequencies of all $CD45^+$ cells out of live cells are shown. (B) B16F10 melanomas were treated with imiquimod (3d before) or with TA99 (2d before) or a combination of both. Frequencies of intratumoral total myeloid cells ($CD11b^+$ out of all $CD45^+$ cells) and the myeloid subsets "inflammatory macrophages" ($CD45^+$ $CD11b^+$ $F4/80^+$ $Ly\text{-}6C^{high}$), stationary macrophages ($CD45^+$ $CD11b^+$ $F4/80^+Ly\text{-}6C^{low}$), and neutrophils ($CD45^+$ $CD11b^+$ $Ly\text{-}6G^+$ $Ly\text{-}6C^{int}$) were determined. Means and SEM from one representative experiment out of two is shown. *$p < 0.05$, one-way ANOVA. (C) Expression levels of FcgRI, FcgRII, FcgRIII, and FcgRIV on tumorinfiltrating myeloid subsets. Means and SEM are shown from one out of two similar experiments. ***p , 0.001, one-way ANOVA. (D) Kaplan–Meier survival curve of B16F10 melanoma in mice that selectively lack FcgRIII and IV on neutrophils (hMRP8Cre 3 FcgRIII/IV2/2 mice) and FcgRIII/IVfl/fl control mice. Experiment performed twice with similar outcomes, shown are six to eight animals per group. *$p < 0.05$, log-rank analysis.

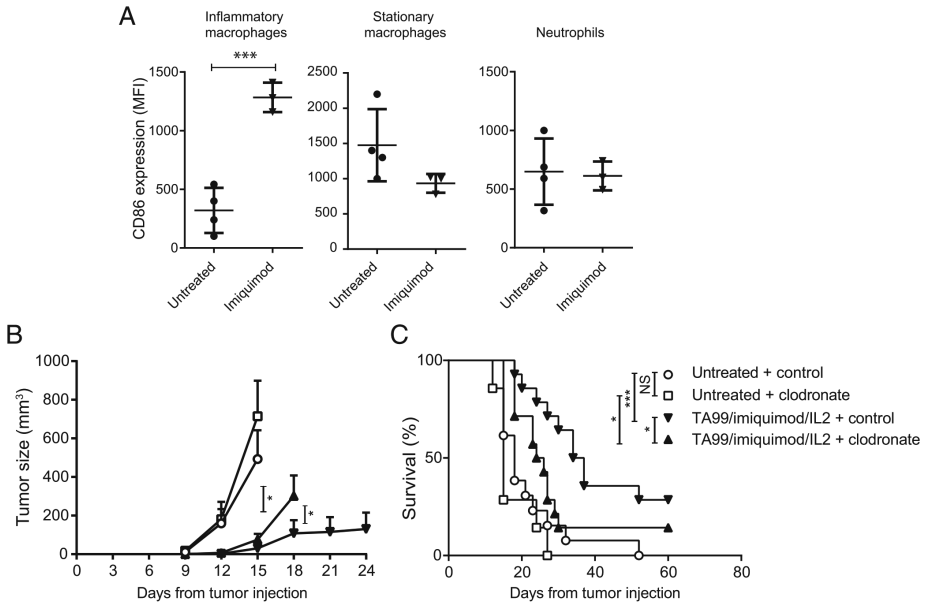


Figure 6. Macrophages are indispensable for TA99/imiquimod/IL-2 therapy in B16F10 melanoma model. (A) Expression of CD86 by tumor-infiltrating inflammatory macrophages, stationary macrophages, and neutrophils in WT mice measured by flow cytometry. Means and SEM shown from four animals per group. ***$p < 0.001$, Student t test. (B) Average tumor growth curves and (C) Kaplan–Meier curves of B16F10-bearing WT mice treated with TA99/imiquimod/IL-2 in the presence of clodronate or control liposomes. Average growth curves in (B) were terminated at the moment the first mouse tumor reached its maximum size of 1000 mm^3. Results are shown from two pooled experiments, 14–16 mice per group. One-way ANOVA (B) and log-rank analysis (C). *$p < 0.05$, ***$p < 0.001$.

Discussion

In this study, we report on combination therapy based on tumor-targeting Abs. Addition of several TLR agonists or agonistic CD40 improved the therapy of established B16F10 melanoma. The increased therapeutic efficacy depended on CD8 T cells and FcγR expression on macrophages, with a minor FcγR-independent role of NK cells. Addition of imiquimod cream resulted in a relative increase of inflammatory macrophages that highly express FcγRs in the tumor microenvironment.

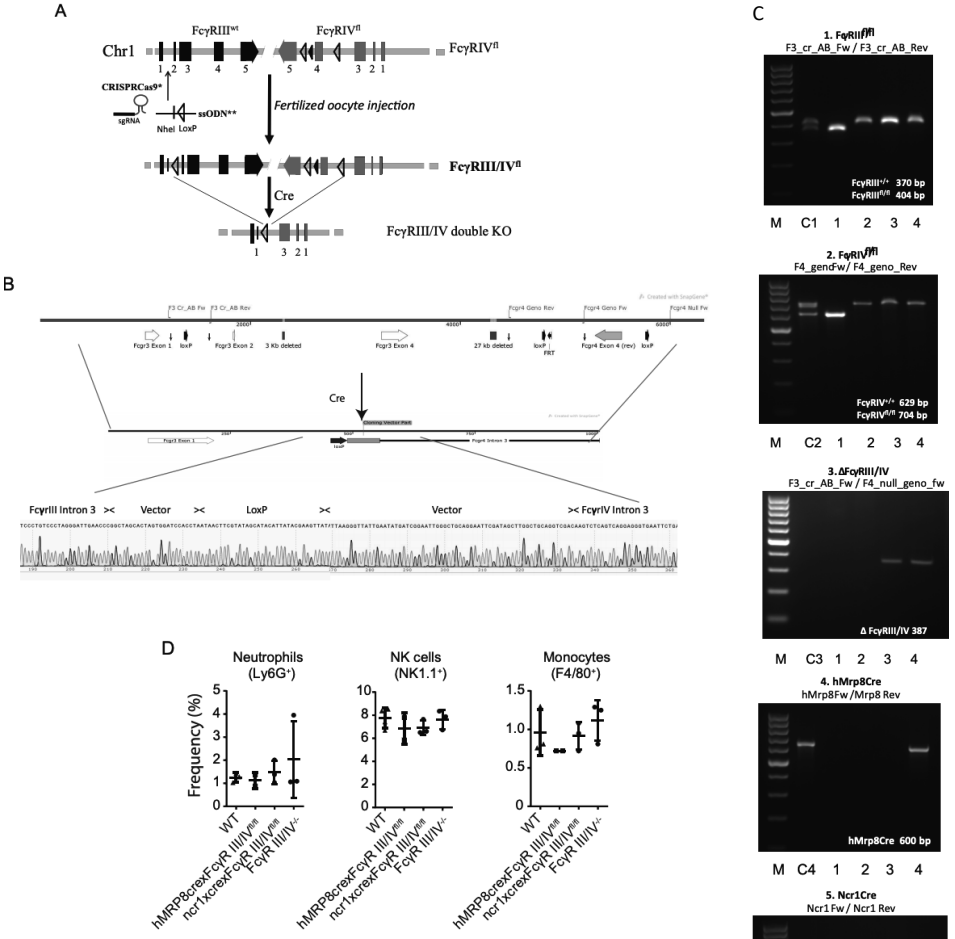
In all studies investigating the role of FcγR in TA99 therapy in the B16F10 model, the Ab was injected on the same day as the tumor was transplanted. Different laboratories reported contradictory results from their analysis of the role of activating FcγR using different panels of $Fc\gamma R^{-/-}$ mice and FcγR-blocking Abs. Some of these differences between the studies can probably be attributed to variations in experimental conditions, like location of the tumor and production process of the TA99 Ab. When B16F10 was seeded in lung (13, 15) and liver (14), a prominent role for FcγRI was found (13) together with FcγRIV (14) or FcγRIII (15). Lehmann et al. (17) showed that the location of the tumor determines what macrophage subset mediates ADCP of tumor cells. The s.c. transplanted B16F10 model required Ly-$6C^{high}$ inflammatory

macrophages, whereas CCL2-independent but CSF2-dependent stationary lung macrophages were required for the treatment of tumors in the lung. Importantly, our study was performed in a therapeutic setting with established s.c. growing B16F10 tumors and provides new insight in the FcγR dependencies. In this study, we show that treatment with TA99 alone of an s.c. transplanted solid B16F10 tumor decreases the relative amount of Ly-6C$^+$ macrophages within the tumor microenvironment, whereas in the combination therapy of TA99 and imiquimod cream, this cellular subset remained constant. Lehmann et al. (17) showed that the influx of Ly-6C$^+$ monocytes depends on CCL2, and others demonstrated that imiquimod induces CCL2-mediated migration of plasmacytoid dendritic cells into the tumor microenvironment (35), which could as well fit our results, as clodronate predominantly depletes macrophages in the spleen and liver, which would thus prevent recruitment to the tumor. This suggests that imiquimod can induce migration of CCL2-attracted myeloid cells into the tumor microenvironment, which is in concordance with a previous study that showed a requirement of CCL2-signaling for TA99-dependent killing of tumor cells in an s.c. tumor setting. These authors showed that the CCL2-mediated effect was dependent on FcγRI and FcγRIV (17), which is in agreement with our study.

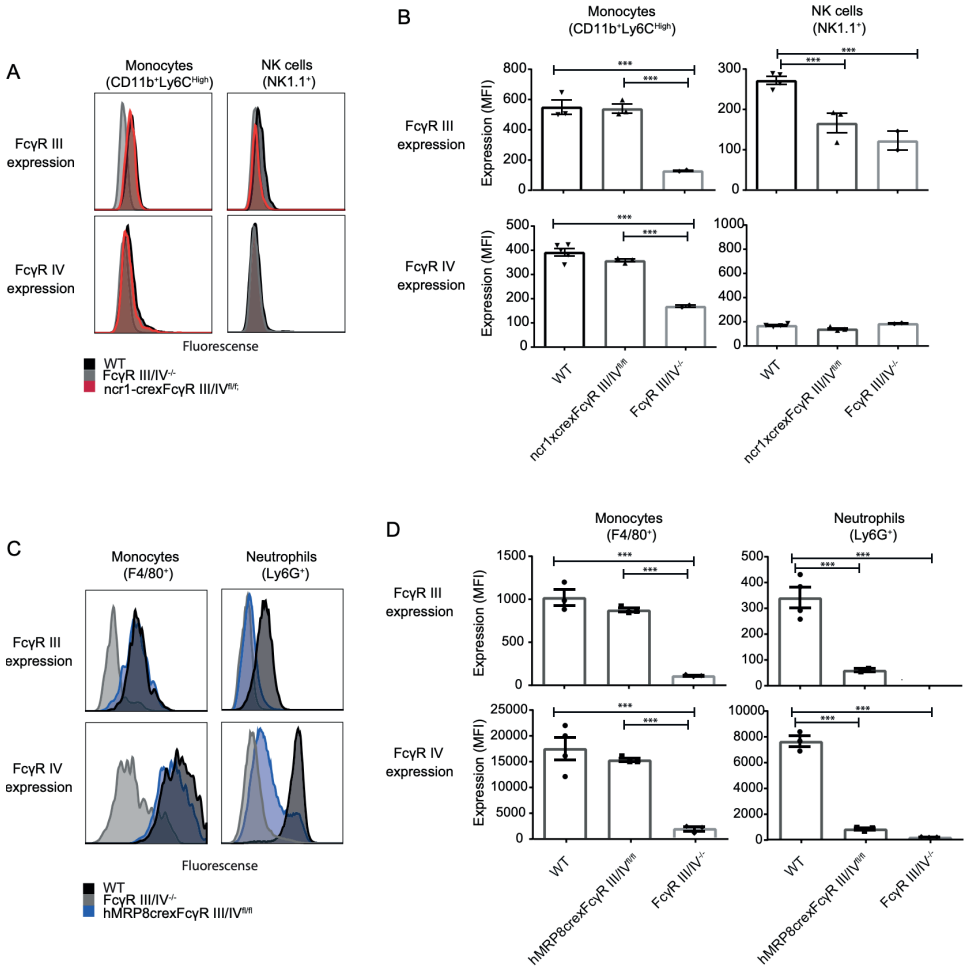
In a variety of tumor-targeting Ab monotherapy models, it has been shown that CD8$^+$ T cells are required (36–40). CD8 T cells were, however, not involved in most TA99 monotherapy of B16F10 melanoma in a prophylactic model (41). More effective combination therapies based on TA99, in which immune stimulating factors such as stabilized IL-2 (Fc–IL-2), TLR4 agonist, peptide vaccination, or anti–PD-L1 were supplemented, controlled tumor outgrowth in the therapeutic setting and depended on CD8$^+$ T cells (20, 22). The induction of adaptive immunity depended on cross-presentation by host APCs of Ab/Ag complexes, as treatment was abrogated in mice with impaired cross-presentation capacity (22). As our study also showed the same need for CD8$^+$ T cells, cross-presentation is most likely an underlying mechanism in our therapeutic protocol. In a previous study, in which the combination of imiquimod cream with IL-2 was examined in the RMA/S tumor model, we showed induction of tumor-specific CD4$^+$ and CD8$^+$ T cell responses. Because the RMA/S tumor contains a defect in presenting tumor Ags to CD8$^+$ T cells, only NK cells and CD4$^+$ T cells contributed to the therapeutic efficacy in that model (23). In our current study, CD8$^+$ T cell responses were effective because of the presentation of tumor Ags by MHC class I molecules on the B16F10 melanoma cells. A partial role for CD4 T cells and NK cells was observed in the B16F10 model (Figs. 2A, 2B, 4C, respectively). The contribution of NK cells did not seem to be related to their FcγRIII, as cell lineage-specific KO mice responded equally well to therapy. CD16-mediated immune effector functions seem to be, nonetheless, much weaker in mice compared with humans (42). IFN-γ production by NK cells was apparent in the tumor environment (Fig. 4E), and this cytokine is pivotal for cytostatic effects in tumors, enhancing Ag presentation and upregulation of immune receptors, such as FcγR and CD86 on macrophages (43, 44). In both these studies, imiquimod and IL-2 were systemically administered at the opposing flank of the B16F10 melanoma. Future investigations need to reveal whether the therapeutic effect is stronger when the TLR ligand is applied topically, as clinical application of an imiquimod cream administered topically on a melanoma showed increased migration of T cells into the tumor (45). Importantly, the combination of imiquimod with IL-2 seems to strongly promote T cell activation and induction of tumor-specific immunity.

In conclusion, we have shown that a combination therapy of TA99 and TLR agonist significantly increased survival of B16F10 tumor–bearing mice and that this depended on FcγRs, macrophage recruitment, and $CD8^+$ T cells. This demonstrated that, even with a highly aggressive solid tumor, strong therapeutic effects can be achieved by a tumor- targeting Ab when combined with immune stimulating compounds.

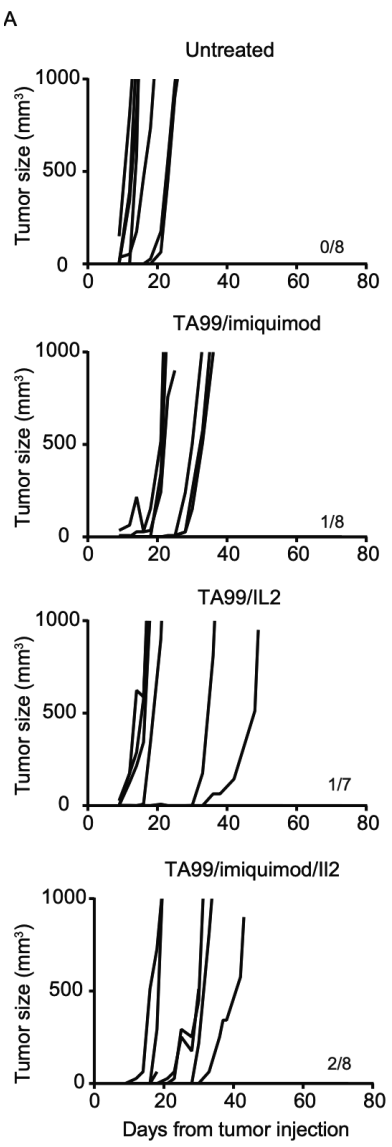
Supplementary Figures

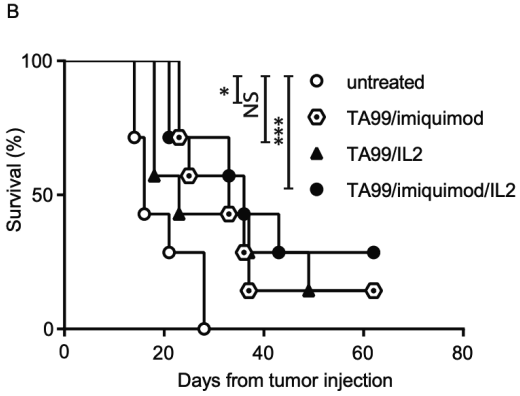


Supplementary Figure S1. Generation and genotyping of a floxed FcγRIII/IV allele using CRISPRCas9. (A) The genomic structure of FcγRIII/FcγRIV gene cluster and indicated CRISPR sgRNA targeting intron one and the ssODN containing the loxP site and additional NheI restriction site. The FcγRIII/IVfl allele and the genomic structure after Cre mediated recombination resulting in the deletion of a 33.7 kb DNA fragment between the most distal LoxP sites. Closed rectangles: exons. EC1 and EC2: extra cellular domain1 and 2 encoding exons; TM/C: transmembrane and cytoplasmic domain encoding exon. Open triangles: LoxP sites; closed triangles: FRT sites. sgRNA and ssODN sequences are given in Material and Methods. (B) Schematic presentation of the FcγRIII/IV locus. Exons are presented as open arrows, LoxP sites as large closed arrows and one Frt site as small closed arrow. Plasmid vector sequences remaining from the cloning procedure presented as gray rectangle. At the bottom: Core sequence of a unique 387 bp PCR fragment flanking the remaining LoxP site and spanning a 33.7 kb deletion in FcγRIII/IV KO cells. (C) Genotyping by PCR as shown on agarose gels. Headings indicate the used primer pairs and the identified gene. At the bottom right of each panel are shown the size of the PCR fragments. Genomic DNA was isolated from the spleens of 1: C57BL/6; 2: FcγRIII/IV$^{fl/fl}$; 3: Ncr1CrexFcγRIII/IV$^{fl/fl}$; 4: hMrp8CrexFcγRIII/IV$^{fl/fl}$. Control DNA was isolated from C1: FcγRIII$^{fl/+}$; C2: FcγRIV$^{fl/+}$; C4: hMrp8Cre^{+} ; C5: Ncr1Cre^{+} . C3: H_20 control. M: 100bp ladder. (D) Shows frequencies of neutrophils (CD11b^{+} F4/80^{-} Ly6G^{+}), NK cells (CD3^{-} NK1.1^{+}) and monocytes (CD11b^{+} F4/80^{+}) in spleens of the indicated mouse strains.

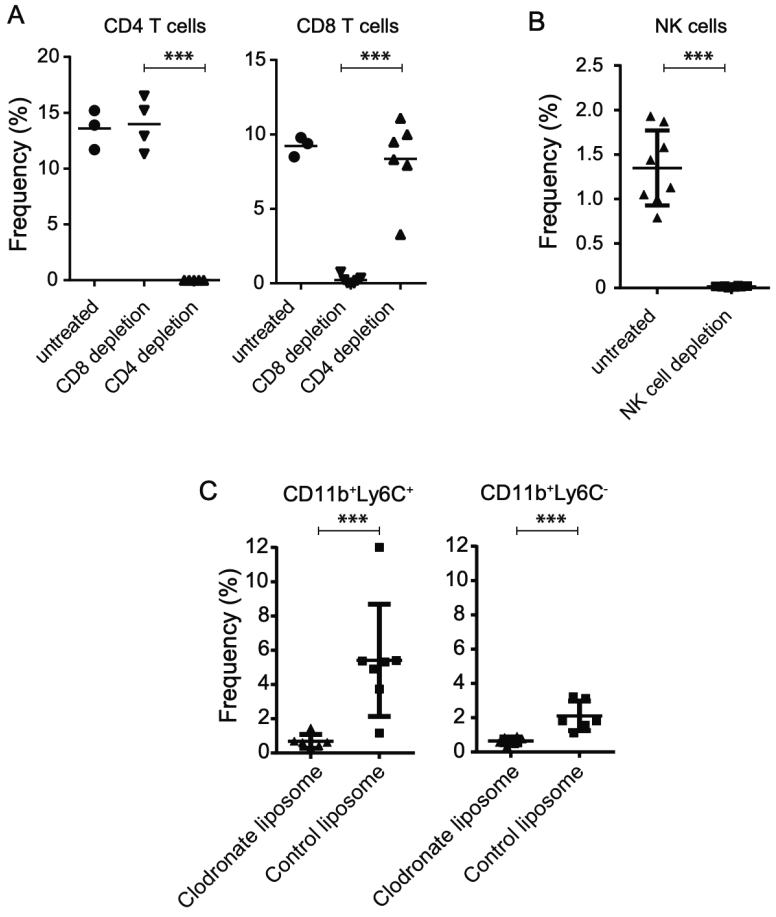


Supplementary Figure S2. Expression analysis of FcγRs in the conditional knockout mice. A-B. Expression of FcγRIII (stained with monoclonal antibody clone AT154-2) and FcγRIV measured by flow cytometry on monocytes and NK cells in blood of WT C57BL/6, Ncr1-cre x FcγRIII/IV$^{fl/fl}$ and FcγRIII/IV$^{-/-}$ mice. Overlapping histograms (A) and average mean fluorescence (MFI) depicted in bar graphs for four animals (B). Geometric mean fluorescence and SEM are depicted. One way Anova, (***p<0.001).C-D. Expression of FcγRIII (stained with monoclonal antibody clone AT154-2) and FcγRIV measured by flow cytometry on monocytes and neutrophils in bone marrow of WT C57BL/6, hMrp8-cre x FcγRIII/IV$^{fl/fl}$ and FcγRIII/IV$^{-/-}$ mice. Overlapping histograms (C) and average mean fluorescence (MFI) depicted in bar graphs for four animals (D). Overlapping histograms (C) and average mean fluorescence (MFI) depicted in bar graphs for four animals (D). Geometric mean fluorescence and SEM are depicted. One way Anova, (***p<0.001). All experiments were repeated twice with similar outcome.





Supplementary Figure S3. Separate contributions of imiquimod and IL-2 to the combination treatment. (A) Tumor growth curves of B16F10 melanoma in WT mice treated with TA99/imiquimod, TA99/IL-2 or the combination of these three components. Numbers at the bottom right represent fraction of surviving mice at the end of the experiment at day 80. (B) Kaplan-Meier survival curves of experiment shown in A, Log-rank analysis. (NS: not significant; *p<0.001).



Supplementary Figure S4. Confirmation of *in vivo* depletion studies. (A) Frequencies of $CD4^+$ and $CD8^+$ T cells in blood (out of $CD45^+$ cells) after injection of depleting antibodies to CD4 or CD8 (2 and 7 days before). Shown are 3-8 animals per group, one way Anova (***$p<0.001$). (B) Frequencies of NK cells in blood (out of $CD45^+$ cells) after injection of depleting antibodies to NK1.1 (2 and 7 days before). Shown are 8 animals per group, one way student t-test (***$p<0.001$). (C) Frequencies of monocytes in the blood (out of $CD45^+$ cells) after administration of clodronate liposomes or control liposomes. Two populations of monocytes were analysed: $CD11b^+$ $Ly6C^+$ and $CD11b^+$ $Ly6C^-$.

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CHAPTER 5.

Immunogenicity of rat-neu+ mouse mammary tumors determines the T cell-dependent therapeutic efficacy of anti-neu monoclonal antibody treatment

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TUBO or not TUBO,
that's the question
You've got to use the milk

Abstract

The use of Trastuzumab (Herceptin), a monoclonal antibody (mAb) targeting HER2/neu, results in an increased median survival in $Her2^+$ breast cancer patients. The tumor mutational burden and the presence of tumor infiltrating lymphocytes (TILs) clearly correlate with response to trastuzumab. Here, we investigated if the immunogenicity of the transplantable rat-neu^+ tumor cell line (TUBO) derived from a BALB/c-NeuT primary tumor is associated with the response to anti-neu mAb therapy. We compared the TUBO tumor outgrowth and tumor infiltrating T cells in isogenic (BALB/c-NeuT) and non-isogenic (WT BALB/c) recipient mice. Furthermore, therapeutic efficacy of anti-neu mAb and the contribution of T cells were examined in both mouse strains. The outgrowth of untreated tumors was significantly better in BALB/c-NeuT than WT BALB/c mice. Moreover, tumor infiltrating T cells were more abundantly present in WT BALB/c than BALB/c-NeuT mice, showing that the TUBO tumor was more immunogenic in WT BALB/c mice. In TUBO tumor bearing WT BALB/c mice, anti-neu mAb therapy resulted in an increase of tumor infiltrating T cells and long-term survival. When T cells were depleted, this strong anti-tumor effect was reduced to an outgrowth delay. In contrast, in TUBO tumor bearing BALB/c-NeuT mice, treatment with anti-neu mAb resulted only in tumor outgrowth delay, both in the presence and absence of T cells. We concluded that in immunogenic tumors the response to anti-neu mAb therapy is enhanced by additional T cell involvement compared to the response to anti-neu mAb in non-immunogenic tumors.

Introduction

Overexpression of oncogenic Her2 protein occurs in 15-20% of breast cancers and is associated with highly aggressive disease. Trastuzumab, a humanized IgG1 monoclonal antibody targeting Her2, is the standard therapy for Human epidermal growth factor receptor 2 (HER2/Erbb2/neu) overexpressing breast cancer owing to its apparent efficacy in adjuvant and neoadjuvant uses (1, 2). This antibody was designed to disrupt the ligand-independent HER2-HER2 interaction resulting in rapid inhibition of pro-survival signalling pathways, leading to cell cycle arrest of the cancer cells (3, 4). The clinical success of Trastuzumab has paved the way for devising novel Her2 targeting approaches in breast cancer treatment. To date, two other therapeutic agents are available to inhibit her2 mediated signalling, a monoclonal antibody (pertuzumab) and tyrosine kinase inhibitors (lapatinib, neratinib). However, a recent clinical study has shown that Trastuzumab therapy increases the pathological complete response (pCR) in patients more than laptinib (5). This can be attributed to the ability of Trastuzumab to engage the immune system to achieve tumor killing. Several preclinical studies have suggested that innate immune responses are essential to anti-Her2/neu mAb cancer therapies through the recruitment of Fcγ receptor (FcγR) expressing immune cells which can mediate antibody-dependent cellular cytotoxicity (ADCC) (6-8). This is supported by the observation that in patients the efficacy of Trastuzumab correlates positively with the presence of allelic variants of FcγRIII with higher affinity for IgG (9, 10).

In addition, *in vivo* preclinical studies have demonstrated adaptive immune responses being also essential for the therapeutic efficacy of anti-Her2/neu mAb (8, 11). These studies were mostly performed using a transplantable mammary tumor derived from a spontaneous primary tumor of an inbred BALB/c-NeuT transgenic female mouse (H-2^d) (12). Females of these transgenic animals, which are hemizygous for the constitutively activated/mutated rat-Her2 gene (NeuT) under control of the MMTV promoter, develop invasive mammary carcinoma in all ten mammary glands (13). This model recapitulates the anatomical location and pathophysiology observed in human $Her2^+$ breast cancer, thus allowing the evaluation of potential cancer immunotherapies. A transplantable cell line derived from a spontaneous rat-neu^+ mammary tumor has been preferably used in many laboratories for cancer immunotherapy studies, on the basis of a short-latency periods and reproducible. However, instead of transplanting these cells into syngeneic BALB/c-NeuT, WT BALB/c mice are oftenused as the recipient of the tumor cells in the majority of the studies. Using such a transplantable tumor cell line , called TUBO, and WT BALB/c and F1 BALB/c FVB/N-Tg (MMTV-neu) mice as a recipient, findings of Park (8) and Mortenson (14) et al. suggest that adaptive immunity, in particular the role of $CD4^+$ and $CD8^+$ T cells, is essential for the anti-neu mAb-mediated tumor regression. However, these results raise some concerns. As TUBO cells express activated/mutated rat-neu being a foreign antigen in WT BALB/c, this tumor cells potentially triggers anti-rat-neu adaptive immunity which contributes to anti-neu mAb therapy. Therefore, we studied the role of the adaptive T cell immunity in full syngeneic setting by transplanting TUBO cells on BALB/c-NeuT mice. Here, we show an association between tumor immunogenicity and the therapeutic efficacy of anti-neu mAb depending on antigenic differences between the tumor and the recipient mouse strain. We explored whether and to

what extent both $CD8^+$ and $CD4^+$ T cells are involved in the therapeutic effects of anti-neu mAb in both WT BALB/c and BALB/c-NeuT tumor bearing mice.

Materials and Methods

Mice

WT BALB/c female mice were purchased from Charles River (L'Arbresle, France). MMTV BALB/c-NeuT transgenic (12, 13, 15) mice expressing activated rat-neu under the control of MMTV promoter were kindly provided by Karin de Visser (NKI, Amsterdam) and maintained by mating BALB/c-NeuT males with WT BALB/c females. The mice were housed in the SPF animal facilities of the Central Animal Facility (PDC) of the Leiden University Medical Center (LUMC). BALB/c-NeuT transgenic mice were bred in house and routinely checked for their genotype by PCR. All mice were housed in individually-ventilated-cage (IVC) systems under specific pathogen-free conditions and used at 6-12 weeks of age. The health status of the animals was monitored over time. Animals tested negative for all agents listed in the FELASA (Federation of European Laboratory Animal Science Associations) guidelines for SPF mouse colonies (16). All mouse studies were approved by the Central Committee for Animal Research (Centrale Commissie Dierproeven, CCD) and Animal Welfare Body (AWB) (Instantie voor Dierenwelzijn, IvD). Experiments were performed in accordance with the Dutch Act on Animal Experimentation and EU Directive 2010/63/EU ('On the protection of animals used for scientific purposes').

Antibodies

Anti-neu mAb (clone 7.16.4, mIgG2a) hybridoma(17) was kindly provided by Mark I. Greene, University of Pennsylvania. Anti-neu mAb, anti-CD8 mAb (clone 2.43)(18) and anti-CD4 mAb (GK 1.5)(19) were produced and purified at Leiden University Medical Center (LUMC).

Tumor models and treatment

The TUBO tumor cell line (12) (kindly provided by Pier-Luigi Lollini) was cultured in Iscove's modified Dulbecco's medium (IMDM) (Lonza) supplemented with 16% Fetal Calf Serum (FCS) (Greiner), 25µM 2-mercaptoethanol and 100 IU/ml penicillin/streptomycin (Gibco). Cell lines were mycoplasma and MAP-tested before the start of experiments. TUBO Tumor cells ($5x10^5$) were injected subcutaneously in 200 µl of PBS on the right flank of 6 -12 weeks old BALB/c-NeuT or WT BALB/c female mice. TUBO bearing WT BALB/c or BALB/c-NeuT mice were treated with three intraperitoneal injections of 100µg of anti-neu mAb (clone 7.16.4) at day 10, 15 and 20. Tumors were measured 3 times per week with a calliper and the size was calculated by multiplying the tumor diameters in two dimensions. Mice were sacrificed when established tumors reached a size of $100mm^2$.

For the anti-neu mAb treatment efficacy studies using BALB/c-neuT female mice (12), the mammary glands of BALB/c-neuT mice were inspected weekly for tumor appearance from the twelfth week of age. When a cumulative spontaneous tumor burden of $20mm^2$ was reached, the mice were treated with three intraperitoneal injections of 100µg of anti-neu mAb (clone 7.16.4) at day 10, 15 and 20. The spontaneous tumors were measured 3 times per week with a

calliper and their size was calculated by multiplying the tumor diameters in two dimensions. Cumulative tumor burden was calculated as the sum of all individual tumor sizes. Mice were sacrificed when established spontaneous tumors reached a cumulative tumor burden of 15x15mm.

Flow cytometry

Flow cytometry analysis for tumor infiltrating $CD45^+$, $CD4^+$ and $CD8^+$ were performed as previously described(20). In brief, tumors were harvested into 1mL of non-supplemented IMDM media in 24-well plates and manually dissociated into small pieces with scalpels, incubated with 2.5 mg/mL Liberase TL (Roche) for 20 minutes at 37ºC and single-cell suspensions were made using 70-µm cell strainers (BD Biosciences). FcγRs were blocked with 10% normal mouse serum and anti-mouse CD16/CD32 antibody (2.4G2). Cell surface staining was performed using the following antibodies: anti-mouse CD8α (clone 53-6.7), CD4 (clone L3T4), CD3ε (clone 145-2c11)/TCRβ chain (clone H57-597), CD45.2 (clone 104). Dead cells were excluded based on 7-AAD (Invitrogen). Analysis were performed using LSRII cytometer (BD) using FacsDIVA software (BD) and FlowJo Software (Tree Star).

Statistical analyses

Data was analysed using Prism 7.0 (GraphPad Software). Statistical significance was calculated using the Mann Whitney non-parametric test. Statistical significance was defined as $p < 0.05$. Tumor survival data was analysed with the Kaplan-Meier method and the log-rank (Mantel-Cox) test.

Results

Anti-neu mAb therapy improved survival in transgenic BALB/c-NeuT spontaneous tumor model

We first analysed the anti-tumor effect of anti-neu mAb monotherapy in the BABL/c-NeuT female mice, which develop spontaneously invasive mammary tumors around 4 months of age (13, 15, 21). As shown in Fig 1A, administration of anti-neu mAb to spontaneous mammary tumors bearing BALB/c-NeuT mice resulted in significant delay in tumor outgrowth ($p < 0.001$), leading to significant improvement in survival when compared with untreated mice (Fig. 1B) ($p < 0.01$). Our results support previous studies showing the therapeutic effect of anti-neu antibodies *in vivo* (8, 14).

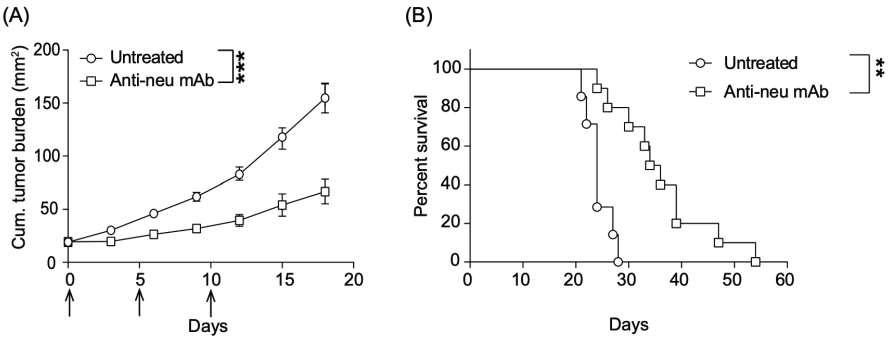


Figure 1. Effective anti-tumor response of anti-neu mAb therapy against spontaneous mammary carcinomas. (A) When cumulative tumors reached an average size of 20 mm^2, NeuT female mice were randomly distributed to the treated and untreated groups. The treatment group was subsequently injected intraperitoneally with 100 µg anti-neu (Day 0, 5, and 10; arrows). Mean tumor size ± SEM is shown. Statistical significance was determined by Mann Whitney non-parametric test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). (B) Data from A presented as Kaplan-Meier survival curves. Logrank test was used to determine the statistical significance of the survival. Pooled data of six independent experiments, 7 to 12 animals per group.

Sub-optimal TUBO tumor take in WT BALB/c recipient mice

Similar to the majority of the oncogene-driven models of cancer in genetically engineered mice (GEMMs) (22, 23), spontaneous mammary tumors driven by activated rat-neu in the MMTV-NeuT mouse model may not harbour high neo-antigen load (24). To evaluate whether the therapeutic efficacy of anti-neu mAb therapy would be stronger when the tumor is more immunogenic, we used a transplantable tumor model, the oncogenic rat-neu (NeuT) expressing tumor cell line (TUBO) established from a spontaneously developed mammary gland tumor of a female BALB/c-NeuT mouse (12). As recipients of the tumor cells, we used either non-syngeneic WT BALB/c mice in which, due to the fact that the rat-neu molecule is a non-self-antigen in WT BALB/c mice, the tumor might be highly immunogenic, or syngeneic transgenic BALB/c-NeuT mice for which the rat-neu is a self-antigen and therefore the tumor might be poorly immunogenic (25).

WT BALB/c and transgenic BALB/c-NeuT female mice were injected subcutaneously with TUBO tumor cells and tumor outgrowth was monitored. All WT BALB/c mice had palpable tumors at around day 8 upon transplantation, however, the growth of established tumors was inconsistent, and some underwent spontaneous regression without therapeutic intervention (Fig. 2A). In contrast, spontaneous regression of established TUBO tumors did not occur in syngeneic BALB/c-NeuT mice (Fig. 2B). These observations suggest that TUBO cells are more immunogenic in WT BALB/c than in BALB/c-NeuT recipient mice. Flow cytometric analysis revealed that there was an increase in percentage of total $CD3^+$ T cells and in $CD8^+/CD4^+$ T cell ratio in TUBO tumors from WT BALB/c mice compared to TUBO tumors from BALB/c-NeuT mice (Fig. 2C and D), suggesting that T cells may contribute to the tumor regression in WT BALB/c mice.

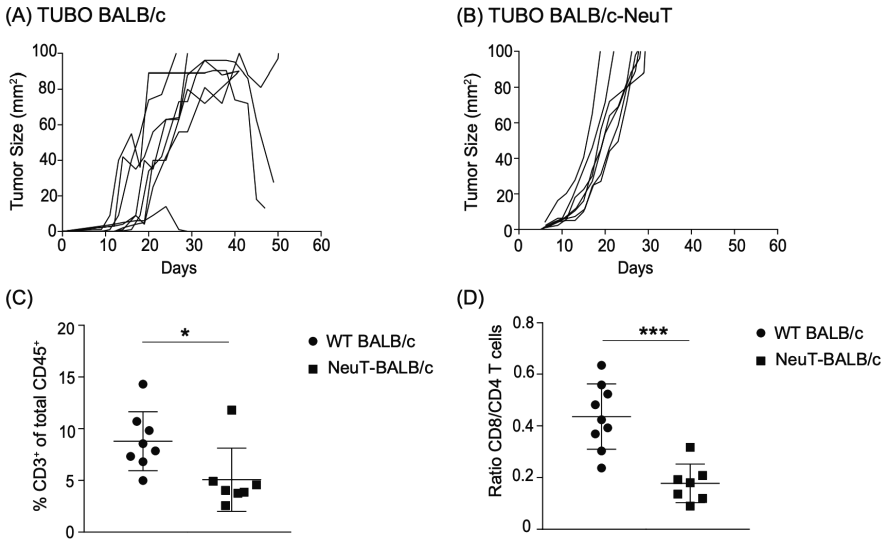


Figure 2. Inconsistent growth of established TUBO tumor in WT BALB/c mice. WT (A) and BALB/c-NeuT (B) mice were injected subcutaneously with $5x10^5$ TUBO tumor cells. Individual tumor growth curves are shown. Tumor cells were injected into WT or BALB/c-NeuT mice, established tumors were harvested on day 15 and analysed with flow cytometry for the percentages of the total $CD3^+$ T cells (C) or the ratio of $CD8/CD4^+$ T cells (D), Data are represented as mean ± SEM. Statistical significance was determined by Mann Whitney non-parametric test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Pooled data of two independent experiments, 7 to 8 animals per group.

Stronger therapeutic efficacy of Anti-neu mAb in TUBO bearing WT than BALB/c-NeuT mice

Based on the difference in immunogenicity of TUBO in WT and BALB/c-NeuT mice, we anticipated different therapeutic responses to anti-neu mAb therapy in WT and BALB/c-NeuT mice. As shown in Figure 3, there was a significant increase in survival of untreated TUBO bearing WT mice compared to untreated TUBO bearing BALB/c-NeuT mice, in keeping with the earlier suggestion that an effective endogenous anti-tumor immune responses against the TUBO tumor is induced in WT but not BALB/c-NeuT mice. Short-term treatment with anti-neu mAb significantly increased the survival of both TUBO bearing WT and BALB/c-NeuT mice. However, the survival of WT BALB/c was significantly higher compared to the survival of BALB/c-NeuT mice. Taken together, our data strongly suggest that the response of established TUBO tumors to anti-neu mAb treatment correlates with the immunogenicity of tumors.

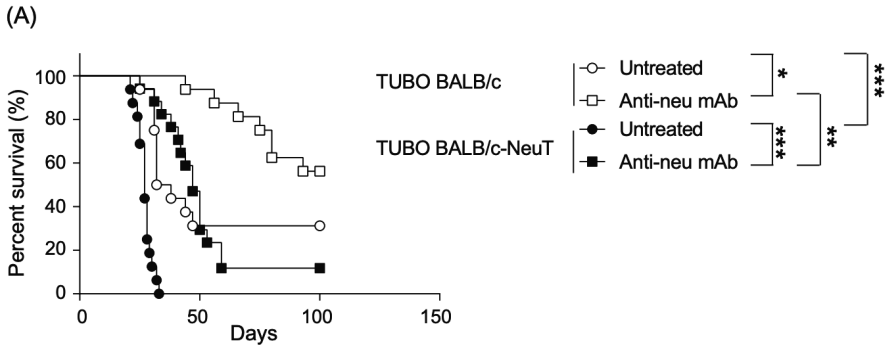


Figure 3. Stronger therapeutic efficacy of Anti-neu mAb in TUBO bearing WT than BALB/c-NeuT mice
(A) WT BALB/c and BALB/c-NeuT female mice were injected subcutaneously with $5x10^5$ TUBO cells and treated intraperitoneally with 100µg anti-neu (Day 10, 15, and 20). Data is presented as Kaplan-Meier survival curves. The mice were followed for tumor outgrowth until day 100 and were sacrificed when the tumor reached 100 mm^2. Logrank test was used to determine the statistical significance of the survival (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Pooled data of two independent experiments, 16 to 17 animals per group.

Anti-neu mAb monotherapy enhances the anti-tumor T cell responses against immunogenic tumors

To investigate the role of T cells in the anti-tumor activity of anti-neu mAb in both WT and BALB/c-NeuT mice, this therapy was tested in mice depleted for $CD4^+$ and $CD8^+$ T cells. Efficiency of T cell depletion was validated during the experiment (Supplemental Fig. 1A and 1B). As expected, depletion of T cells clearly increased the growth of established TUBO tumors in WT BALB/c mice (Fig. 4A). Whereas treatment with anti-neu mAb resulted in strong tumor regression in WT BALB/c mice, tumors relapsed progressively in the absence of T cells, suggesting that T cells are essential for the maximal therapeutic efficacy of anti-neu mAb. In a follow-up experiment, we observed increased $CD4^+$ and $CD8^+$ T cells in the TUBO tumor of WT BALB/c mice treated with anti-neu mAb (Fig. 4B). This increase was not observed in BALB/c-NeuT mice (data not shown). Together, these data indicate that in immunogenic tumors, anti-neu mAb therapy contributes to strong tumor growth inhibition and eradication by improving the anti-tumor T cell responses.

Markedly delayed outgrowth of TUBO tumors was also observed in BALB/c-NeuT mice (Fig. 4C). However, the therapeutic efficacy of anti-neu mAb was not impaired in the absence of $CD4^+$ and $CD8^+$ T cells (Fig.4 C), indicating that the T cells in BALB/c-NeuT mice did not contribute to the therapy in contrast to the T cells in WT BALB/c mice. Together, our results suggest that T cells are responsible for the poor TUBO tumor outgrowth in WT BALB/c mice and that anti-neu mAb monotherapy can enhance the anti-tumor T cell responses to further delay the tumor outgrowth. In contrast, the much weaker anti-neu mAb-mediated anti-tumor effect in TUBO bearing BALB/c-NeuT mice, in which the tumor is less immunogenic, is T cells independent. When T cells were absent in WT BALB/c mice, the anti-tumor response of anti-neu mAb resembled strongly the response in BALB/c-NeuT mice, indicating a T cell-independent tumor-outgrowth inhibition by anti-neu mAb.

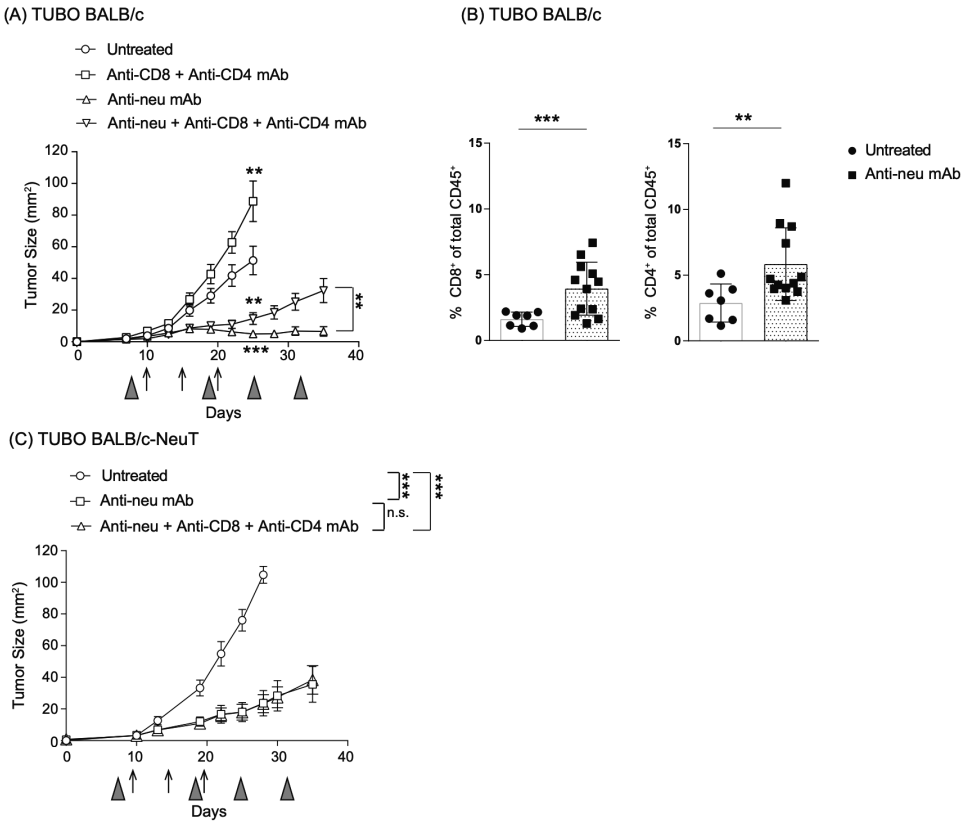


Figure 4. Anti-neu mAb monotherapy enhances the anti-tumor T cell responses to further delay the outgrowth of immunogenic tumors (A) WT BALB/c female mice were injected subcutaneously with $5x10^5$ TUBO cells and treated intraperitoneally with 100µg anti-neu (Day 10, 15, and 20; arrows) and/or 100 µg anti-CD8 (Day 8, Day 18 and weekly; grey triangles) and anti-CD4 mAb (GK1.5; Day 8, Day 18 and weekly; grey triangles). Data from A presented as average tumor outgrowth (mm^2) ± SEM. Statistical significance was determined by Mann-Whitney test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). (B) Tumor bearing WT BALB/c mice were treated with anti-neu mAb on Day 10 post tumor inoculation, tumors were resected on Day 15 and analysed for the presence of $CD4^+$ and $CD8^+$ T cells via flow cytometry. Statistical significance was determined by Mann-Whitney test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Pooled data of three independent experiments. (C) Same as A except BALB/c-NeuT mice were used. Pooled data of two independent experiments, 6 to 9 animals per group.

Discussion

Here we report that the immunogenicity of mouse mammary tumors can have a significant impact on the response to anti-neu mAb immunotherapy. When rat-neu is a non-self-antigen, making the rat-neu^+ tumor highly immunogenic as in WT BALB/c recipient mice, T cells contributed to a more pronounced anti-neu mAb-mediated tumor growth inhibition and eradication of the transplanted tumors than when rat-neu on the tumor is a self-antigen, as in BALB/c-NeuT recipient mice. In the latter mice, the weaker anti-tumor response induced by anti-neu mAb therapy was T cell independent.

Our observation that rat-neu expressing TUBO tumors are highly immunogenic in WT BALB/c mice is in agreement with the finding of Reilly et al. (26) who demonstrated that the minimum tumor cell dose required for tumor outgrowth in 100% of the transplanted animals was 100-fold lower for the neu transgenic mice compared with non-transgenic mice. Our study also confirms previous studies showing that $CD4^+$ and $CD8^+$ T cells can contribute to anti-neu mAb monotherapy to suppress the growth of the TUBO tumor in WT BALB/c immunocompetent mice (8, 14). However, we did not observe a contribution of T cells to the therapeutic efficacy in anti-neu mAb treated TUBO bearing BALB/c-NeuT transgenic mice. An important question raising from these experiments is: why T cells contributed to the therapeutic efficacy of anti-neu mAb in WT BALB/c but not BALB/c-NeuT tumor bearing mice? A simple explanation is that rat-neu is a foreign antigen in normal BALB/c mice, and that the rejection of the TUBO tumor likely involved high avidity rat-neu specific T cells that are strongly, and rapidly activated and expanded after transplantation. This hypothesis is in agreement with our data showing the poor TUBO tumor growth and development in WT BALB/c mice. In contrast, BALB/c-NeuT mice which express rat-neu as self-antigen, harbour lower numbers of self-reactive low-avidity T cells which are more difficult to activate and do not expand effectively (27-29). It has been demonstrated that spontaneous Her2-driven mammary tumorigenesis in BALB/c-NeuT animals is not suppressed by the adaptive immune system (15), suggesting the lack of neo-antigens that can be recognised by T cells.

By depleting $CD8^+$ T cells, Park et al (8) and Stagg et al (11) also noted the importance of $CD8^+$ T cells in anti-neu mAb therapy in TUBO tumor bearing NeuT transgenic animals resulting in complete tumor regression in 20% of the mice . This is not in agreement with our results and it might be due to differences in experimental conditions. Park et al (8) used F1 neu transgenic mice (BALB/c x FVB/N MMTV-neu) as the recipient of TUBO cells. Although these mice are 'tolerized' for rat neu, most likely there are still genetic differences between the TUBO cell line and the F1 BALB/c x FVB/N recipient mouse, which can explain the stronger, partially CD8 T cell dependent, anti-TUBO immune response they report compared to our studies in the fully syngenic BALB/c-NeuT mice. Besides, all other experiments of Park et al (8) showing dependence on adaptive and innate immunity of anti-neu mAb therapy were performed in WT BALB/c or KO BALB/c mice not tolerized for the rat-neu antigen(11). By using other rat-neu^+ tumor cell lines (H2N100, H2N113, and H2N67) derived from the same BALB/cNeuT mice as the TUBO cell line, transplanted onto full syngenic BALB/c-NeuT recipient mice Stagg et al. confirmed the essential role of $CD8^+$ T cells in anti-neu mAb therapy. However, in contrast to Park et al. they observed only a moderate delay in tumor outgrowth and no complete regression in a subset of treated mice., Side by side *in vivo* comparison of the therapeutic efficacy of anti-neu mAb in both the H2N and the TUBO model would be necessary to exclude that differences in immunogenicity between these tumor cell lines underlie the different outcome of the anti-neu mAb therapy experiments of Stagg et al and the experiments described here. Furthermore, Stagg et al (11) used a much more intensive treatment regime (8 times 100 µg anti-neu Ab within a period of 14 days) than we did (3 times 100 µg anti-neu Ab within 10 days). It could be that sustained tumor cell killing by a longer period of treatment leads to induction of a strong inflammatory response activating low avidity anti-neu $CD8^+$ T cells. This might also explain the syngergistic effect of anti-neu mAb and anti-PD-1 mAb (11) in this setting.. Notably, $CD8^+$ T cell dependency has also been reported for the effective

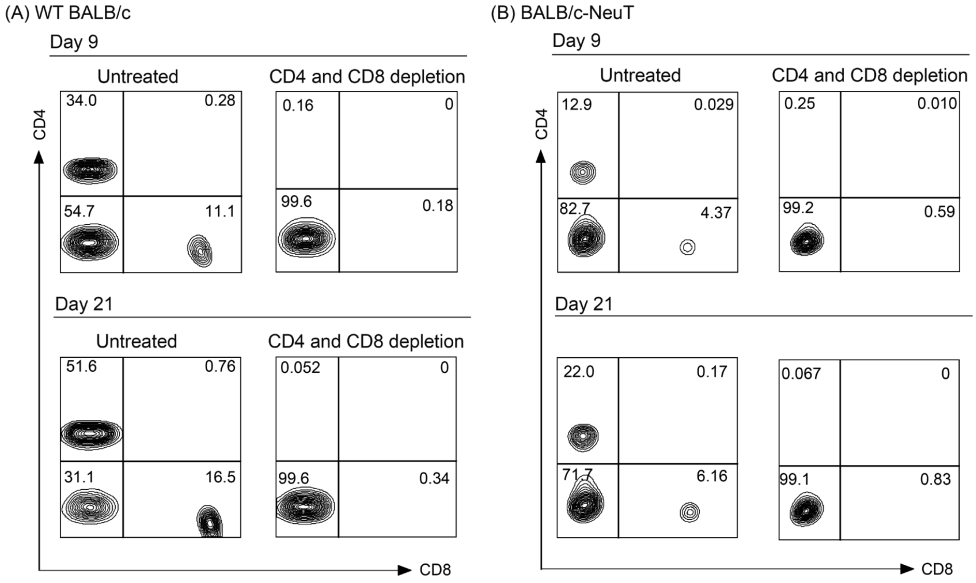
combination therapy of anti-death receptor 5 (DR5) and anti-neu mAb in BALB/c-NeuT mice (30). Since anti-DR-5 mAb triggers mainly apoptosis in tumor cells, this study raises the possibility that the sustained release of danger signals or damage-associated molecular patterns (DAMPs) from tumor cell death could be key at breaking immune tolerance and inducing meaningful $CD8^+$ T cell antitumor immunity in neu-transgenic mice. This might as well explain that neu-specific whole tumor vaccination (26) but not DNA vaccination (12, 31, 32) therapies elicit antitumor CD8 T cell responses in neu transgenic mice.

Although there is an increasing amount of literature supporting that anti-tumor immunity of anti-neu mAb can be enhanced with immunomodulatory agents such as CD73 (33), PolyI:C and CpG (34) in the highly immunogenic TUBO transplanted WT BALB/c mice. Our study suggests that the potential and underlying mechanisms of action of these combination therapies have to be elucidated in low immunogenic setting using transgenic BALB/c-NeuT recipient mice in order to complement the results obtained in WT BALB/c mice. Our result in BALB/c-NeuT mice suggest that direct tumor growth inhibition by blockade of rat-neu signalling is one of the major mechanisms of anti-neu mAb in transgenic NeuT mice. Improved anti-tumor effects could be achieved by the continuation of treatment of these mice with anti-neu mAb, similar to the therapy in breast cancer patients who often receive anti-Her2 mAb over long periods of time (35) or combining it with a tumor targeting agent such as anti-DR5 mAb that induces tumor cell death directly (11). We do not rule out, however, that Fc-mediated effector functions might be involved also in anti-neu mAb therapy. Further investigations are needed to reveal the contribution of FcγR and FcγR expressing immune cells to the anti-tumor efficacy of anti-neu mAb and whether FcγR expression can be modulated in the tumor microenvironment to augment mAb-mediated (36) effector functions for both immunogenic and non-immunogenic tumors. Our study supports the notion that intense immune phenotyping of the various syngeneic tumor models is critical for both rational model selection and data interpretation for clinical translation (37-39). For example, the high immunogenicity of a fully non-self-antigen such as rat-neu in the TUBO tumor bearing WT BALB/c model, will most likely result in overestimation of the potency of combining anti-neu mAb therapy with immunotherapy. On the other hand, using a completely syngeneic model such as MMTV BALB/c-NeuT mice that develop spontaneous mammary tumors or TUBO transplanted in fully syngeneic BALB/c-NeuT mice might result in underestimation of the potential anti-tumor immune response in patients, in which mutations may have led to neo-epitope formation and a certain level of immunogenicity.

Antibody targeting Her2 (trastuzumab, pertuzumab) is among the first of the approved antibodies directed against $Her2^+$ cancer cells. Although these antibodies have become standard of care and improved overall survival of patients with $Her2^+$ breast cancer, heterogeneity exists within Her2-positive tumors, and the overall response rate to anti-Her2 mAb-based therapies remains modest, approximately 26% when used as a single therapy and 40-60% when used in combination with chemotherapy (2, 40, 41). To date, Her2 expression level remains the only suitable marker for patient selection for anti-Her2 based regimen (42, 43). Nonetheless, the relationship between the level of Her2 amplification and efficacy of trastuzumab based therapy in adjuvant treatment remains controversial (44). Two studies (45, 46) showed no correlation and one showed a negative correlation (47) between Her2 amplification and clinical survival. Clearly, beyond Her2 testing, there is a need to identify

more reliable biomarkers to better predict which patients respond to this treatment. Unlike melanoma and lung cancer, the majority of breast cancer has not been considered immunogenic owing to its relatively low mutational load and hence low repertoire of neo-antigens (48, 49). These poorly immunogenic breast tumors are less likely to benefit from cancer immunotherapy. Nonetheless, high mutational burden (50) and an increase of tumor infiltrating lymphocytes (TILs) (51-54) can be found in some $Her2^{+}$ breast cancer patients. Several studies have shown that TILs are significantly associated with improved survival in $Her2^{+}$ breast cancer, as well as better response to anti-her2 mAb therapy (52-55). These observations suggest that host immunity can contribute to the antitumor activity of Her2-targeted agents. Here, we compared the impact of the difference in immunogenicity of the TUBO tumor in non-isogenic WT and isogenic transgenic BALB/c-NeuT mice on the therapeutic efficacy of anti-neu mAb therapy. We observed an increased infiltration of T cells into the untreated established TUBO tumors from WT BALB/c mice compared to untreated tumors in transgenic BALB/c-NeuT mice. The higher immunogenicity of the tumors in WT BALB/c mice correlated with increased efficacy of anti-neu mAb therapy compared to the therapeutic efficacy in BALB/c-NeuT mice. Taken together, our study suggests that selection of $HER2^{+}$ breast cancer patients based on the level of pre-existing TILs may be useful and critical for an optimal anti-Her2 mAb-based combination therapy with better therapeutic outcome.

Supplementary Figures



Supplementary Figure 1. Confirmation of *in vivo* depletion efficacy. Facs dot plots show the frequencies of CD4 and CD8 T cells in blood of WT BALB/c (A) and BALB/c-NeuT (B) at Day 9 and Day 21 which received CD4 and CD8 depleting antibodies at Day 8 and 18.

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CHAPTER 6.

MMTV-NeuT transgenic mice on C57BL/6 background: an improved mammary tumor model to study anti-neu antibody therapy

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Manuscript in preparation


Heng Sheng, come in, close the door...

Abstract

Gene amplification resulting in overexpression of Her2 is seen in up to 20-30% of breast cancers and is associated with an aggressive phenotype. Many genetic murine models are generated to study tumorigenesis driven by Her2/neu and to develop novel cancer (immuno)therapies. One of the most commonly used models, is MMTV-NeuT, a genetically engineered mouse model, with histopathological and molecular features similar to Her2 overexpressing in human primary breast cancer. However, this mouse is on BALB/c background whereas most other genetically engineered mice have been generated in, or backcrossed into C57BL/6 background, which limits the application of the BALB/c-NeuT model in preclinical studies. To overcome this problem, we generated a MMTV-NeuT mouse model on C57BL/6 background. Furthermore, we established a protocol to maintain and transplant MMTV C57BL/6-NeuT tumor cell suspensions into syngeneic C57BL/6-NeuT female mice. Here, we describe the overall characterization of the C57BL/6-NeuT mice and their mammary tumors. C57BL/6-NeuT female mice develop spontaneous mammary tumors at the age of about 250 days whereas transplanted tumor cells gave rise to a palpaple tumor within 30 days. The spontaneous and transplanted mammary C57BL/6-NeuT tumors retained the mammary tumor histologic phenotype and immune infiltration properties similar to the tumors of BALB/c-NeuT mice, as confirmed by flow cytometry studies. These results indicate that the C57BL/6-NeuT mouse strain represents an alternative model for the BALB/c-NeuT strain that can be combined with a large variety of geneticlly modified C57BL/6 mouse models in preclinical studies.

Introduction

The overexpression of ErbB2/Her2/neu, which occurs in 20-30% of breast cancer cases, correlates with poor prognosis (1). Genetically engineered mouse models of Her2 breast cancer includes MMTV NeuT transgenic mice overexpresses activated rat-neu under the control of the mammary gland specific MMTV promoter(2, 3), NeuT-knock-in-mice at the Neu locus (4), doxycycline-inducible NeuT (5) and MMTV-driven overexpression of human Her2 (6) transgenic models have contributed substantially to our understanding of the pathophysiology of Her2-driven carcinogenesis and enabled the testing of therapeutic agents targeting Her2 *in vivo*.

Several anti-Her2 mAb that specifically inhibit the *in vitro* growth of Neu expressing tumor cell lines have been developed. One of them, 4D5, was humanized and the resulting antibody was termed trastuzumab (7). Despite its successful clinical application for more than a decade, the mechanisms of action of trastuzumab is not fully understood. Inhibition of Her2-mediated cell signalling (8, 9) is a well-recognized mode of action of trastuzumab. Even though studies (10) suggested that FcγR could contribute to anti-neu mAb therapy, the role of FcγR expressing immune cells (e.g. macrophages, neutrophils, monocytes, DCs) has not been established *in vivo*. Main reason is that most cell type specific FcγR deficient mice have been generated or backcrossed into the C57BL/6 background, which restricts detailed preclinical evaluation of the mechanisms of action of anti-neu mAb in the BALB/c-NeuT model. To overcome this drawback and to provide alternative and suitable mouse models for future studies, we established a C57BL/6 MMTV-NeuT mouse strain and characterized the spontaneous and transplantable mammary tumors that arose in this mouse strain.

Materials and Methods

Generation of MMTV-NeuT mice on C57BL/6 background by speed congenic approach

To generate MMTV-NeuT mice on C57BL/6 background from BALB/c-NeuT, a speed congenics marker-assisted selection protocol (MASP) was followed (11). Approximately 20 male NeuT carriers were generated in a C57BL/6 back cross 2 (BC2) (N3) (Figure S3). These mice were analyzed using ~ 80 micro satellite markers dispersed over the genome, with an average distance of 20 cM.). The marker set consisted of MIT, Nsd and NKI (custom made) microsatellite primer sets (supplemental table 1). The PCR products were assayed on 5% agarose gels (SSLP, Simple Sequence Length Polymorphism). The two male mice with the lowest number of BALB/c genotyped markers were selected for further breeding. The BC4 (N5) was typed by a second set of ~ 80 markers at 20 cM distance (supplemental table 2) which are intermediate of the first set of markers, hence the total screening is one marker per 10cM. Again, the two male mice with the lowest number of BALB/c genotyped markers were selected for further breeding. Further generations were screened only for those specific regions containing contaminating BALB/c donor segments. In the BC5 (N6) mice full congenic offspring was identified. These mice maintained a proximal region of Chromosome 7 of BALB/c origin indicating that the NeuT transgene is integrated in that region of the genome.

Mice

BALB/c and C57BL/6 female mice were purchased from Charles River (L'Arbresle, France) and housed in the animal facility of the Leiden University Medical Center (LUMC). MMTV-NeuT mice (kindly provided by Prof. K. de Visser. NKI, Amsterdam (12). The NeuT mice either on BALB/c or C57BL/6 mice were bred in house and maintained by mating MMTV-NeuT males with wild-type BALB/c or C57BL6 females. NeuT transgenic animals were routinely checked for their genotype by PCR. Transgene-positive female animals were monitored weekly by palpation for mammary tumor development. Once palpable tumors were present, tumor size was measured twice per week, using a calliper. All mice were housed in individually-ventilated-cage (IVC) systems under specific pathogen-free conditions. The health status of the animals was monitored over time. Animals tested negative for all agents listed in the FELASA (Federation of European Laboratory Animal Science Associations) guidelines for SPF mouse colonies (13). All mouse studies were approved by the animal ethics committee of the LUMC. Experiments were performed in accordance with the Dutch Act on Animal Experimentation and EU Directive 2010/63/EU ('On the protection of animals used for scientific purposes').

***In vivo* tumor challenge**

TUBO cells are a cloned line established *in vitro* from a spontaneous BALB/c-NeuT mouse mammary carcinoma. This cell line (kindly provided by Pier-Luigi Lollini, Universtity of Bologna)) was cultured in Iscove's modified Dulbecco's medium (IMDM) (Lonza) supplemented with 16% Fetal Calf Serum (Greiner), 25µM 2-mercaptoethanol and 100 IU/ml penicillin/streptomycin (Gibco). Cell lines were mycoplasma and MAP-tested before the start of experiments. TUBO Tumor cells ($5x10^5$) in 200 µl of PBS were injected subcutaneously (s.c.) in the right flank of 8-12-week-old MMTV-BALB/c-NeuT female recipient mice.

Tumor transplantation

Mammary tumors (15 x 15 mm) were isolated from C57BL/6-NeuT female mice into 1ml of non-supplemented IMDM media in 24-well plates and manually dissociated into small pieces with scalpels and single-cell suspensions were made using 70-µm cell strainers (BD Biosciences). After washing twice with PBS, cells were re-suspended in PBS, C57BL/6-NeuT female mice were injected subcutaneously with approximately $10x10^6$ cells in 200ul of PBS. Tumor size was measured with a calliper as the product of two perpendicular diameters. Palpable established tumors were grown to a size of 50 – 100mm^2 before they were routinely passaged to the next group of C57BL6-NeuT female mice

Flow cytometry

Tumors (5x5mm in diameter) were harvested into 1ml of non-supplemented IMDM medium in 24-well plates and manually dissociated into small pieces with scalpels, incubated with 2.5 mg/mL Liberase TL (Roche) for 20 minutes at 37ºC and single-cell suspensions were made using 70-µm cell strainers (BD Biosciences). Fc-receptors were blocked with 10% normal mouse serum and anti-mouse CD16/CD32 antibody (2.4G2). Cell surface staining was performed using the following antibodies: CD3ε (clone 145-2c11), CD11b (clone M1/70),

F4/80 (clone BM8), CD45.2 (clone 104), Ly6G (clone 1A8), Ly6C (clone HK1.4). Dead cells were excluded based on 7-AAD staining (Invitrogen).

Statistical analyses

Data were analysed using Prism 7.0 (GraphPad Software). Statistical significance was calculated using the Mann-Whitney U test. Statistical significance was defined as $p < 0.05$. Tumor latency data were analysed with the Kaplan-Meier method and the log-rank (Mantel-Cox) test.

Results

MMTV-C57BL/6-NeuT mice develop multifocal spontaneous mammary tumors

MMTV-neuT C57BL/6 mice were generated by backcrossing MMTV-NeuT BALB/c mice into C57BL/6 background using a marker-assisted speed congenic approach. The breeding scheme is depicted in Fig. S3A. Compared to BALB/c-NeuT with a median of tumor onset of 100 days, the tumor onset was significantly delayed in C57BL6-NeuT mice with a median of 250 days (Fig 1A). As shown in Fig. 1B, due to the rapid tumor growth and progression most of the BALB/c-NeuT females mice had to be euthanized around 35 days after initial tumor detection (end-stage cumulative tumor burden ≥ 15mm in diameter). In contrast, it took more than 50 days for the spontaneous tumors in C57BL/6-NeuT mice to reach the end-stage size. In addition, compared to female BALB/c-NeuT mice, C57BL/6-NeuT female mice carried significantly lower number of spontaneously developing tumors as evaluated at the day the cumulative tumor burden reached 15 x 15mm (Fig 1C; Fig S4). Our studies indicate that NeuT mice in C57BL/6 background are less susceptible to the spontaneous development of MMTV driven rat-neu positive tumors.

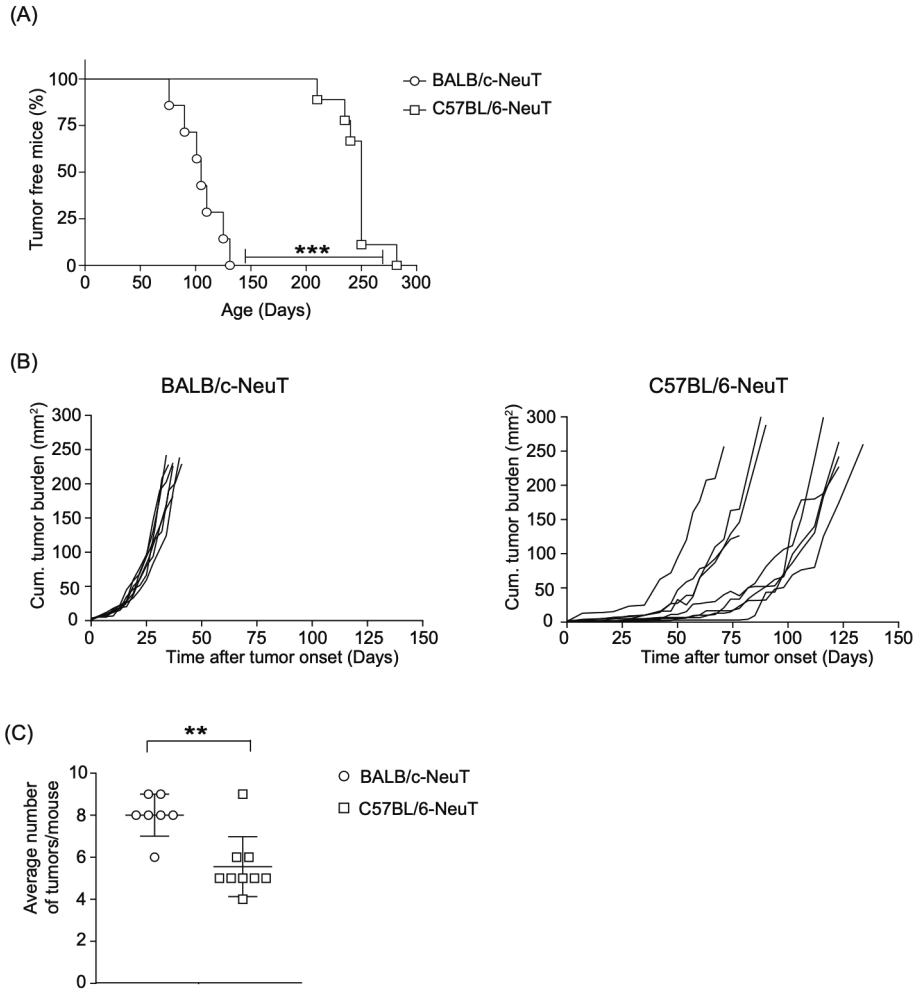

Figure 1. Differences in the tumorigenesis of MMTV BALB/c-NeuT and C57BL/6 mouse models. (A) Comparison of the kinetics of tumor latency between NeuT on BALB/c and C57BL/6 background. Mice were considered tumor-free until a palpable tumor mass of 2x2 mm was detected. Logrank test was used to determine the statistical significance of the tumor latency. (B) Cumulative tumor burden (mm^2) followed over time in individual BALB/c-NeuT and C57BL/6-NeuT. (C) Average number of primary mammary tumors per BALB/c-NeuT and C57BL/6-NeuT mouse calculated when mice reached end-stage cumulative tumor burden (15x15mm) (n=7-9/group). Statistical significance was evaluated by Mann-Whitney test (** $P < 0.01$; *** $P < 0.001$).

Transplantable C57BL/6-NeuT tumor model

Given the long tumor latency of the C57BL/6-NeuT tumors, it was difficult to follow tumor growth for preclinical studies. In addition, we attempted but failed to establish cell lines from mammary tumors that arose in the C57BL/6-NeuT, mainly due to the slow grow rate. It took two weeks to reach 80% confluency in a T175 flask (Data not shown). To overcome this, we established a protocol in which the tumor of C57BL/6-NeuT was maintained on C57BL/6 female NeuT mice by passaging tumor cell suspension directly from mouse to mouse once the

tumor burden reached 10x10mm. As shown in Fig. 2A, *ex vivo* tumor growth rate was improved from first to third passage, reaching a similar growth rate as the TUBO tumor in BALB/c-NeuT mice. In contrast, the growth of established C57BL/6-NeuT tumors was inconsistent in WT C57BL/6 mice and most of them underwent complete spontaneous regression without therapeutic intervention. Our observations suggest that tumor of C57BL/6-NeuT is more immunogenic in WT C57BL/6 than in C57BL/6-NeuT recipient mice due to the presence of the rat-neu which is a self-antigen in NeuT mice, but non-self in C57BL/6 mice. This is in keeping with our results with TUBO transplantation in the BALB/c background. Also, WT BALB/c mice showed much poorer tumor take compared to BALB/c-NeuT mice (14).

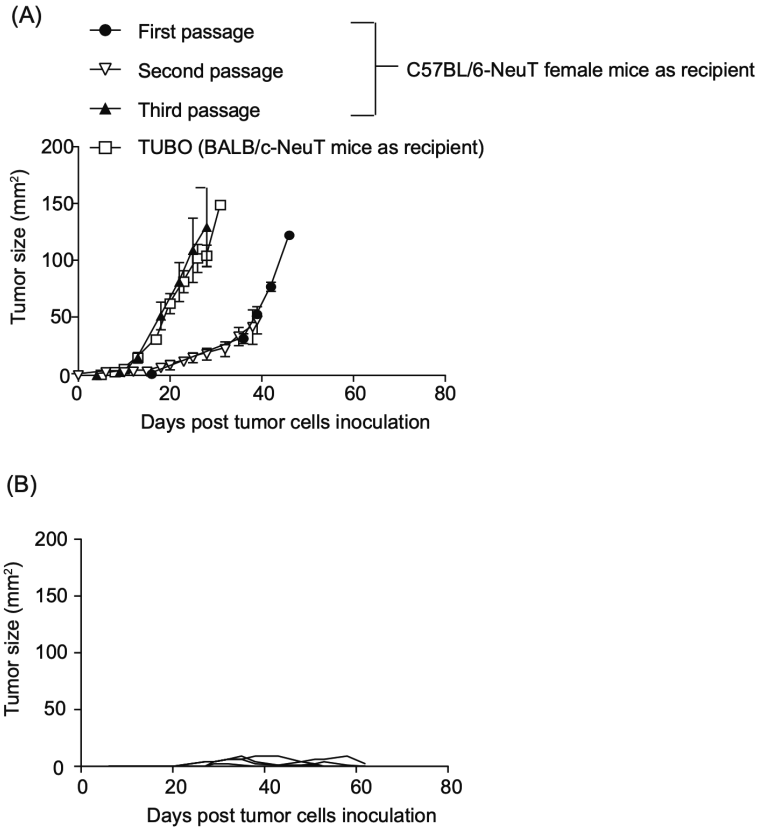

Figure 2. ***In vivo*** **outgrowth of transplantable tumor derived from C57BL/6-NeuT mouse mammary carcinomas.** (A) Comparison of the subcutaneous growth rate of BALB/c-NeuT-derived TUBO tumor in BALB/c-NeuT mice and the *ex vivo* transplanted tumor cell suspension derived from the spontaneous tumors of C57BL/6-NeuT mice (3 C57BL/6-NeuT mice per group in each passage; 5 BALB/c-NeuT mice were used for TUBO challenge). Data are represented as mean of tumor size mm^2 ± SEM. (B) Subcutanous growth rate of C57BL/6-NeuT derived tumor in WT C57BL/6 mice.

Immune infiltration profile of the subcutaneous C57BL/6-NeuT tumor

Mammary tumorigenesis in BALB/c-NeuT mice is accompanied by influx of immune cells not seen in normal mammary glands (15). We therefore examined and compared the immune infiltration profile of the transplantable tumors from C57BL/6-NeuT and BALB/c-NeuT mice. TUBO tumors transplanted on BALB/c-NeuT recipient mice and C57BL/6 NeuT tumors of seventh passage in C57BL/6-NeuT recipient mice were harvested and subjected to flow cytometric analysis. As shown in Fig.3A, there was no significant difference in $CD45^+$ leukocyte infiltrate in tumors of BALB/c-NeuT and C57BL/6-NeuT. We also checked the leukocyte subpopulations, i.e. $F480^+$ macrophages (Fig. 3B), $Ly6G^+$ granulocytes (Fig. 3C). However, no significant differences were observed in the magnitude of the two immune subsets between tumors of BALB/c-NeuT and C57BL/6-NeuT mice. In addition, both tumors showed no significant difference in $CD3^+$ T cell infiltration (Fig. 3D). Together, these data indicate that the genetic background of the NeuT mice does not influence the overall composition of the infiltrating immune cells in the tumor microenvironment.

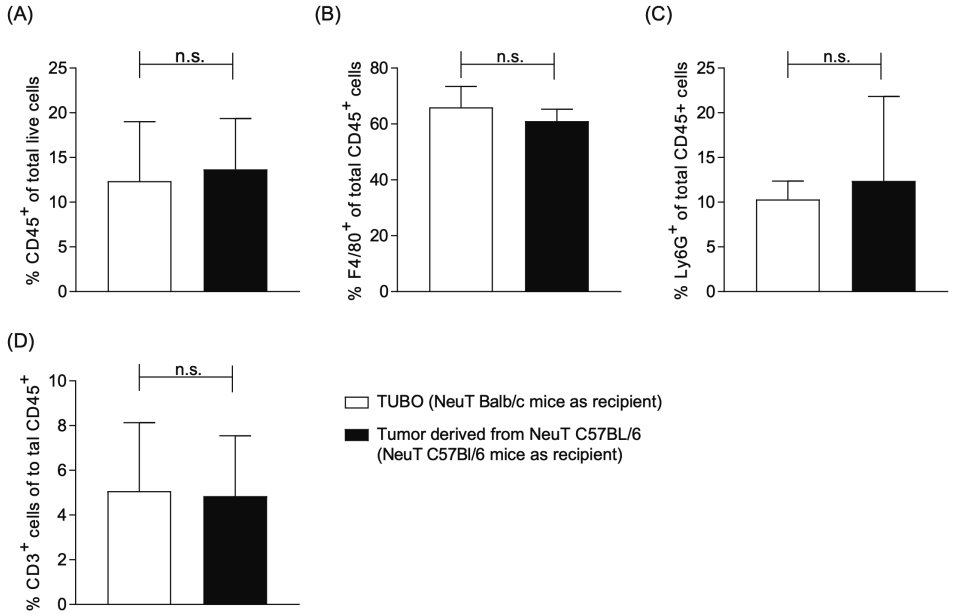

Figure 3. Similar immune infiltration profile of subcutaneous BALB/c-NeuT derived (TUBO) and C57BL/6-NeuT derived tumors. Flow cytometric analysis of (A) $CD45^+$ leukocytes, (B) $F4/80^+$ macrophages, (C) $Ly6G^+$ granulocytes and (D) $CD3^+$ T cells in established TUBO and C57BL/6-NeuT subcutaneous tumors (n = 5-8/group). Data are represented as mean ± SEM. Statistical significance was determined by Mann-Whitney test (n.s. not significant).

Discussion

MMTV-BALB/c-NeuT mice, carrying an activated rat neu oncogene driven by the mammary gland specific MMTV promoter in the H-2^d BALB/c background, have greatly contributed to our understanding of the development and progression of mammary tumors driven by Her2 and have emerged as valuable tools for preclinical research. Furthermore, this model is immunocompetent, enabling the evaluation of immunotherapies targeting Her2. Most genetically engineered mice have been generated in, or backcrossed into the C57BL/6 background, which limits the application of the BALB/c-NeuT model in preclinical studies. Here, to enable future preclinical studies, we established a MMTV-C57BL/6-NeuT mouse model that can be crossed with any available C57BL/6 genetically modified strain while maintaining the full C57BL/6 background. Our work demonstrated that the onset of spontaneous mammary tumors in C57BL/6-NeuT mice is significantly longer than in BALB/c-NeuT. Similar observations have been reported for (MMTV FVB x C57BL/6 Neu) F1 female mice. These F1 mice exhibited longer tumor latency (18 months) than MMTV-FVB Neu mice (7 – 12 months) (16) and developed tumors only after they experienced two to four pregnancies (17). Interestingly, when F1 animals were backcrossed onto the FVB strain, the resulting offspring developed tumors with a latency similar to that of the parental FVB Neu transgenic strain. Of note, MMTV-FVB Neu or FVB x C57BL/6 Neu mice on mixed background express the wild-type rat-neu and they have longer tumor latencies (7-18 months dependent on the genetic background) as compared to BALB/c-NeuT mice (tumor latency of 3 months) carrying the activated rat-neu transgene. The constitutive activated rat-neu contains a missense mutation at residue 664 resulting in the substitution of a valine for a glutamine residue (18). Despite of the overexpression of activated rat-neu, the development of spontaneous tumors was significantly delayed when BALB/c-NeuT mice (tumor latency of 3 months) were backcrossed into C57BL/6 background (tumor latency of 8 months). This suggests that the C57BL/6 background has a profound impact on the mammary tumor progression driven by overexpressed wild-type or activated rat-neu. Similarly, there are significant differences in the tumor latency of MMTV-Ras mice dependent on the strain backgrounds. The tumor onset in MMTV-Ras FVB/N mice is 5-6 months and 9-10 months in FVB/N x C57BL/6 x BALB/c mixed genetic background (19). Furthermore, no spontaneous tumors were found in human ErbB2 transgenic mice in C57BL/6 background (20). One hypothesis might be that the increased tumor latency observed in C57BL/6 background is due to a lower activity of the MMTV promoter resulting in lower levels of rat-neu compared with FVB or BALB/c mice. However, it has been shown that the levels of rat-neu protein were similar in mammary glands of MMTV Neu FVB or MMTV Neu FVB x C57BL/6 F1 animals (16), suggesting that the genetic backgrounds does not affect the expression level of rat-neu. Another explanation is that C57BL/6 mice contain tumor suppressing genes or intrinsic immune components that influence the mammary tumorigenesis induced by the activated rat-neu. Nevertheless, it is possible that additional passages of rat-neu expressing tumor cells in recipient C57BL/6-NeuT mice will result in the selection of more aggressive tumor cells that can be established as a cell line *in vitro*. Most importantly, the transplantable C57BL/6 MMTV-NeuT tumor model shows a growth rate similar to that of the BALB/c-NeuT TUBO cell line, making it a suitable model

for *in vivo* studies. More aggressive tumor cells can also be achieved by introducing other oncogenes. Li et al. (21) demonstrated that when mutated p53 oncogene (p53-172H; 172Arg-to-His p53 mutation) is introduced in FVB neu mice, tumor latency in the bi-transgenic mice is shortened to 5 months compared to 7.8 months in FVB neu mice. Therefore, it would be interesting to know if shorter tumor latency can be achieved in mutated p53 expressing C57BL/6-NeuT.

Although the rat-neu protein shows less than 6% amino acid difference with the mouse homologue (22), it is a foreign antigen in WT mice. However, rat-neu is a self-antigen in NeuT transgenic mice. This might be the explanation for the almost complete spontaneous rejection of the TUBO cell line transplanted on non-syngeneic WT BALB/c mice (14) and the C57BL6-NeuT cell line transplanted on non-syngeneic WT C57BL/6 mice. The attenuated growth of the rat-neu$^+$ tumors in WT background suggests that preclinical studies using transplanted tumors derived from genetically engineered mice must be carried out in fully syngeneic donor mice to fully recapitulate the tumorigenesis of the spontaneous tumors. Ignoring these conditions might be the explanation for the reported T cell dependency of anti-neu antibody therapy in WT BALB/c mice transplanted with rat-neu$^+$ tumors (10). Cytokines, secreted by either cancer cells or tumor associated immune cells, are part of the tumor microenvironment (TME) and can regulate the immune status of the TME (23). Although our work suggests that there is no difference in the composition of the immune cell populations in the TME between BALB/c neuT and C57BL/6 neuT mice, we cannot completely rule out the possibility that the cytokine profiles in the tumors in both mouse strains are different having a different impact on tumor latency. Moreover, tumor-promoting non-immune cells such as endothelial cells and fibroblasts in the TME might differ between both tumor models.

In patients, the efficacy of Trastuzumab correlates positively with the presence of allelic variants of FcγRIII with higher affinity for IgG (24, 25) suggesting a contribution of FcγR to the therapeutic efficacy of Trastuzumab. Although a preclinical study has suggested that FcγRs are essential for anti-neu mAb therapy in TUBO bearing BALB/c mice (10), detailed analysis of the contribution of the different FcγR and FcγR expressing effector cells was hampered because most FcγRKO mouse models are in C57BL/6 background. This can be solved now by breeding the C57BL/6-NeuT mice with the available various cell type specific FcγRKO mouse strains to test the role of FcγR expressing effector cells in the response to anti-neu mAb treatment of rat-neu$^+$ mammary tumors and ultimately evaluate new strategies for enhancing the efficacy of this tumor specific antibody therapy as we demonstrated in the B16F10 melanoma mouse model with the tumor specific anti-Trp1 antibody (26). Especially the role of FcγR and FcγR expressing antigen-presenting cells in the 'vaccine effect' of tumor targeting antibodies, believed to have the potency to induce a long lasting adaptive anti-tumor immune response, can be studied in this setting.

Supplementary figures

chrom 1		chrom 2		chrom 3		chrom 4	
D1Nki103	3.3	D2Nki101	3.1	D3Mit117	5.8	D4Mit149	3.6
D1Mit211	25.9	D2Mit11	71.7	D3Mit46	30	D4Mit108	38
D1Mit478	52.5	D2Mit94	81	D3Mit241	67	D4Mit178	74
D1Mit215	79	D2Mit42	104.4	D3Mit158	109.8	D4Mit31	106.6
D1Mit54	120.2	D2Mit340	149.8	D3Mit17	143	D4Mit127	148.7
D1Mit105	159	D2Mit148	177	D3Nki108	160		
D1Mit407	196						

chrom 5		chrom 6		chrom 7		chrom 8	
D5Mit145	6.5	D6Mit138	4.2	D7Mit77	27.6	D8Nki114	3.1
D5Mit13	36.3	D6Mit274	48.6	D7Mit120	54	D8Mit65	42.1
D5Mit356	72.3	D6Mit209	76.4	D7Mit353	99.5	D8Mit80	90.6
D5Mit139	123.6	D6Mit104	111.8	D7Mit101	125.6	D8Mit13	126.32
D16Mit197	151	D6Mit15	147.1	D7Nki119	152.4		

chrom 9		chrom 10		chrom 11		chrom 12	
D9Mit227	41.7	D10Mit123	9.9	D11Mit71	6.8	D12Nki101	3.8
D9Mit32	67.1	D10Mit194	46.6	D11Mit51	37.8	D12Mit147	36.4
D9Mit115	102.3	D10Mit134	104	D11Mit320	70.7	D12Mit34	70.9
D9Mit18	120.1	D10Nki103	127.6	D11Mit42	114	D12Nds2	116.6

chrom 13		chrom 14		chrom 15		chrom 16	
D13Mit17	20.7	D14Mit109	12.6	D15Mit53	13.3	D16Mit182	5.1
D13Mit283	61.5	D14Mit28	58.9	D15Mit121	58.3	D16Mit211	35.6
D13Mit53	109	D14Mit92	82.3	D15Mit2	91.6	D16Mit86	86.9
		D14Mit107	122.7				

chrom 17		chrom 18		chrom 19	
D17Nki107	3.4	D18Nki105	8	D19Mit68	3.6
D17Mit50	44.4	D18Mit120	36.2	D19Mit63	35.7
D17Mit72	78.2	D18Mit154	76.1	D19Mit71	59.7

Figure S1. The first marker set for the genotyping of the generation BC2.

chrom 1		**chrom 2**		**chrom 3**		**chrom 4**	
D1Mit294	11.7	D2Nki112	27.8	D3Mit131	17.6	D4Mit1	17.9
D1Mit318	33.7	D2Mit436	80.9	D3Mit6	48.9	D4Mit288	56.8
D1Nki108	60.7	D2Mit45	93.2	D3Mit40	87.3	D4Mit145	94.4
D1Mit308	110.6	D2Mit132	115.9	D3Mit319	128.3	D4Mit12	124.4
D1Nki111	142.4	D2Nds3	130.4	D3Mit18	147.7		
D1Mit403	175.6	D2Mit265	174.4				
chrom 5		**chrom 6**		**chrom 7**		**chrom 8**	
D5Nki103	23.7	D6Mit159	29.7	D7Mit191	18.9	D8Mit4	30.8
D5Mit255	55.0	D6Mit183	53.3	D7Mit176	63.2	D8Mit25	64.6
D5Mit10	104.2	D6Mit284	92.6	D7Mit30	81.4	D8Nki108	77.5
D5Mit31	138.6	D6Mit61	128.5	D7Mit237	115.8	D8Mit271	114.3
				D7Nki127	139.1		
chrom 9		**chrom 10**		**chrom 11**		**chrom 12**	
D9Nki106	3.1	D10Mit214	25.6	D11Mit80	20.6	D12Mit291	17.5
D9Mit4	52.1	D10Mit42	82.7	D11Mit274	56.7	D12Mit88	55.7
D9Mit8	76.5	D10Mit266	115	D11Mit39	88.4	D12Mit5	80.9
D9Mit37	110.8						
chrom 13		**chrom 14**		**chrom 15**		**chrom 16**	
D13Mit165	44.2	D14Nki112	9.4	D15Mit8	33.5	D16Mit34	15.9
D13Mi233	36	D14Mit133	29.5	D15Mit69	70.6	D16Mit185.2	60.5
D13Mit233	83.6	D14Mit34	62.5			D16Mit65	72.8
D13Mit78	118.8	D14Mit194	93.8				
chrom 17		**chrom 18**		**chrom 19**			
D17Nki110	25.4	D18Mit172	23.6	D19Mit23	20.4		
D17Mit139	51.7	D18Mit74	52.5	D19Mit19	39.4		
D17Nki114	94.7	D18Mit9	68.5	D19Mit36	53.9		

Figure S2. The second marker set for the genotyping of the generation of BC4.

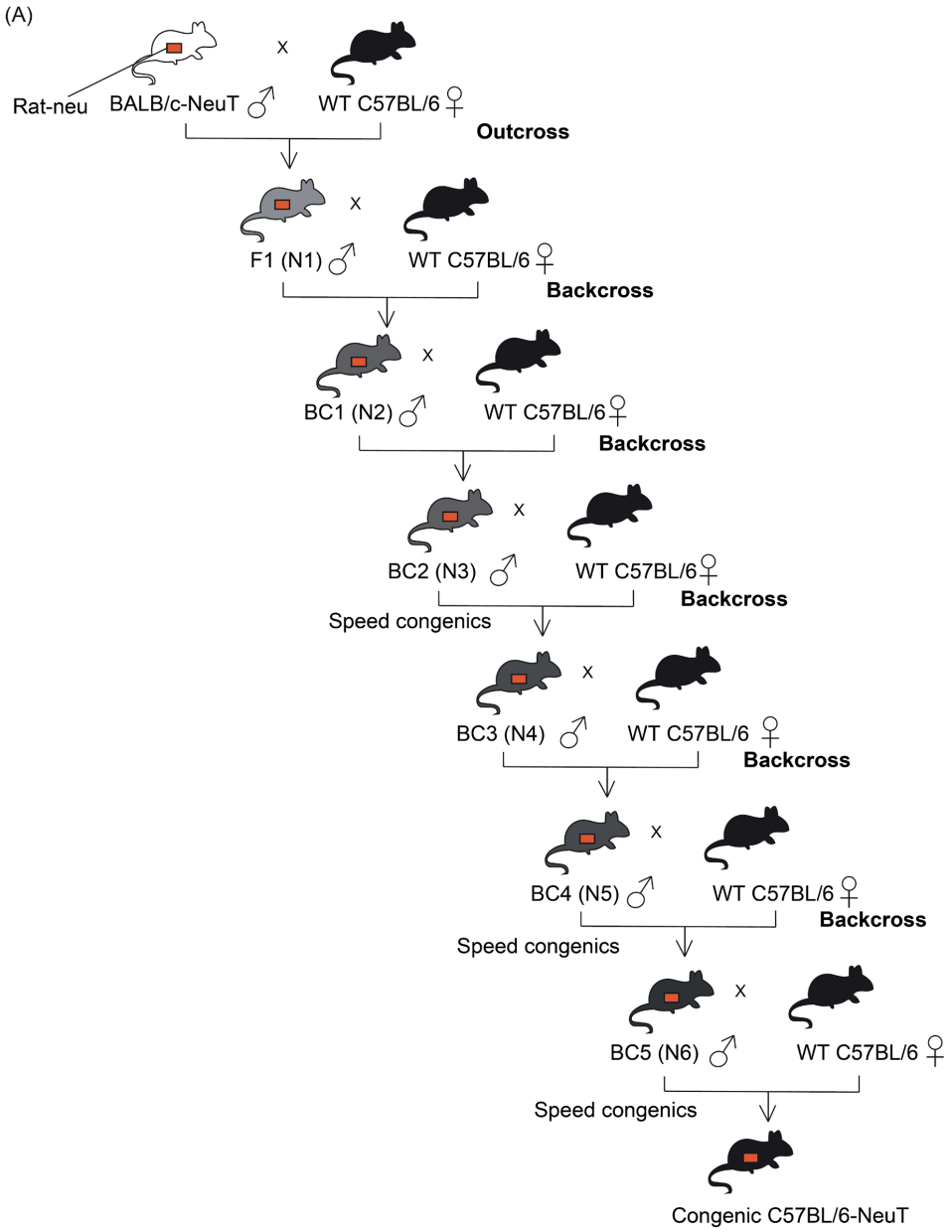

Figure S3. Generation of the C57BL/6-NeuT strain by means of marker assisted breeding. (A) Diagram illustrating the breeding approach to obtain congenic C57BL/6-NeuT mouse strain, based on 6 generations of backcrossing. WT C57BL/6 female mice were used to breed with rat-neu^{+} (BALB/c-NeuT) male mice. Microsatellite analysis was performed with two generations of mice (generation BC2 and BC4). C57BL/6-NeuT female mice from generation BC5 or onwards were used for studies. F1: first filial generation; N: number of backcross generations; BC: Backcross.

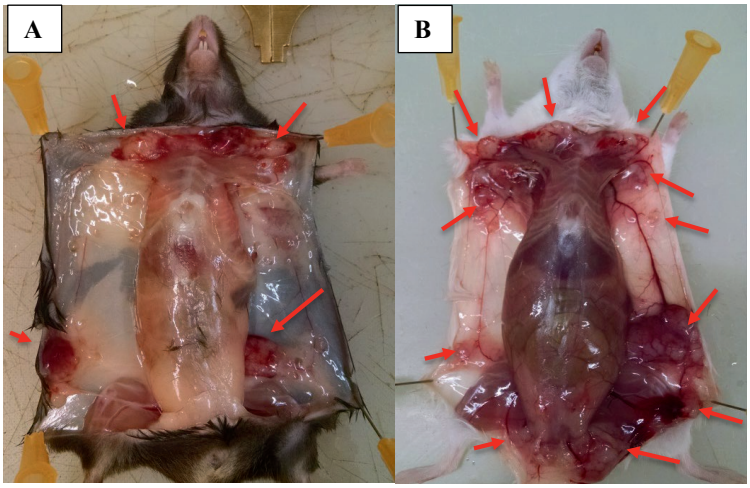

Figure S4. MMTV-C57BL/6-NeuT mice develop spontaneous mammary tumors. (A) Gross observation of a female C57BL/6-NeuT mouse and (B) BALB/c-NeuT mouse with multiple spontaneous mammary tumors (as pointed with black arrows).

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CHAPTER 7.

General discussion

General Discussion

This thesis displays a variety of approaches, analysed in preclinical mouse models, which potentially improves the effectiveness of antibody therapy in cancer. These studies underscore that the therapeutic outcome of a particular antibody-based immunotherapy can vary depending on the chosen tumor model and experimental design. Altogether this demonstrates that there is a strong need for adequate experimental design of therapy efficacy studies in mice which more accurately answers clinically relevant questions. Using well-defined experimental conditions in multiple mouse models allows a more careful interpretation of the results which increases their translational value.

Role of FcγR mediated activation of downstream effector mechanisms in antibody-based immunotherapy

I. Anti-PD-L1 antibody

As summarized in Table 1, it has been shown by many laboratories that, at least in pre-clinical mouse models, interaction with FcγR can either reinforce or hamper the therapeutic effect of immunomodulatory antibodies. For example, activating FcγR are essential for the optimal therapeutic efficacy of anti-CTLA-4 mAb (1). The mode of action of this antibody involves not only the blocking of inhibitory signalling of CTLA-4 on both effector and regulatory T cells, but also the ADCC of Tregs in the TME mediated by FcγR on macrophages (2). On the other hand, FcγR binding can significantly impair the anti-tumor activities of anti-PD-1 mAb therapy. Based on these studies, it will be important to understand how FcγR interactions may impact the therapeutic efficacy of immunomodulatory antibodies for cancer. In **Chapter 2**, it is shown that in CT26 tumor-bearing BALB/c mice the therapeutic efficacy of anti-PD-L1 mIgG2a (functionally resembling human IgG1), the IgG subclass with the highest binding affinity for mouse activating FcγRI, III and IV, is higher compared to other IgG isotypes. This was associated with a reduction of a high PD-L1 expressing myeloid cell subset in the CT26 tumor microenvironment. Furthermore, higher therapeutic efficacy of anti-PD-L1 mIgG2a was also observed in PD-L1$^{-/-}$ CT26 tumors, suggesting that the enhanced therapeutic effect of anti-PD-L1 mIgG2a can be attributed to the modulation of PD-L1^{+} immune cells in the TME. This might have implications for PD-L1 blockade therapy in human. There are four anti-PD-L1 antibodies used in the clinic. Avelumab is an intact IgG1 whereas atezolizumab (mutant hIgG1), durvalumab (mutant hIgG1) and BMS-936559 (hIgG4) are either mutated or of a human IgG subclass with low affinity for activating FcγR. Avelumab, has been approved for the treatment of metastatic Merkel cell carcinoma and metastatic urothelial carcinoma (3). Additionally, avelumab triggers more effectively NK cell-mediated ADCC than atezolizumab *in vitro*. However, little information of the Fc-mediated effect of avelumab on the TME is available. Yet, a positive correlation between a high PD-L1 expression in tumor-associated immune cells and favourable outcome of Avelumab treatment has been described in patients with metastatic breast cancer (4). This data may support the possibility that Avelumab modulates PD-L1^{+} immune cells in the TME. Future investigation of the presence of FcγR expressing effector cells in different human tumor types, different PD-L1-

expressing myeloid cell subsets, and the efficacy of the different IgG isotypes of anti-PD-L1 antibody should provide useful information regarding the role of FcγR in the therapeutic efficacy of PD-L1 checkpoint inhibitors.

In the MC38 tumor model, the therapeutic efficacy of anti-PD-L1 mIgG2a was not superior to the anti-PD-L1 mAb of the other IgG subclasses. The results in **Chapter 2** indicate that the additive therapeutic activity of anti-PD-L1 mIgG2a is dependent on the genetic background. There are three IgG2 subclasses in mouse inbred strains, namely IgG2a, 2b and 2c. Mouse IgG2b (binding preferentially to mFcγIIb, III and IV) is encoded by the Igh-3 gene and can be found in all mouse strains. On the other hand, mIgG2a and 2c are allelic variants encoded by the Igh-1a and Igh-1b, gene respectively, with 15% difference in amino acid sequence (5, 6). The BALB/c mouse strain expresses IgG2a while C57BL/6 mice expresses IgG2c (5). IgG2a and IgG2c are generally considered to be equivalent (6). It has been demonstrated that anti-trinitrophenyl (TNP) IgG2a produced similar passive systemic anaphylaxis in BALB/c and C57BL/6 mice injected with TNP-BSA intravenously, indicating that the sequence variations between IgG2a and IgG2c do not affect anaphylaxis induction (7). However, in terms of induction of other Fc-mediated effector functions (e.g. ADCC, ADCP, complement activation), it remains unclear whether there is any difference between IgG2a and IgG2c. Most importantly, IgG2a might be immunogenic in C57BL/6 mice and this could increase the clearance rate of injected IgG2a resulting in the reduction of antitumor activity over time (1). In a preliminary study, we found that anti-PD-L1 mIgG2a cleared faster in C57BL/6 than in BALB/c mice (unpublished results). Interestingly, this increased clearance was not observed in C57BL/6 FcγRI/II/III/IV$^{-/-}$ mice, suggesting that FcγR may be responsible for the clearance of the IgG2a. Therefore, it is likely that the absence of the FcγR-dependent additional therapeutic effect of IgG2a in C57BL/6 mice is due to anti-IgG2a antibody responses. It would be interesting to investigate if anti-PD-L1 mIgG2c could bypass anti-drug antibody responses and elicit stronger antitumor efficacy than anti-PD-L1 mIgG2a in the C57BL/6 MC38 tumor model.

Table 1. Summary of the *in vivo* studies investigating the role of FcγR in the immunostimulatory antibody therapy

Antibody	Direct biological effect	Requirement for Fc receptors	Consequences of Fc interactions
Anti-death receptor 4 or 5 (DR4 or DR5)	Apoptosis induction by binding of mAb	Yes	Interactions with either activatory and/or inhibitory FcγR provide a crosslinking scaffold that promotes DR4/5-mediated tumor cell apoptosis(8) Enhancing FcγRIIb engagement increases apoptotic and antitumor potency of anti-DR5 antibody(9)
Agonistic anti-GITR	Promotes $CD8^+$ T cell functions	Yes	Interactions with activating FcγR to deplete $GITR^+$ Treg cells in mice(10)
Agonistic anti-CD40	Activation of a variety of immune cells	Yes	FcγRs provide a crosslinking scaffold and improve agonistic activity of anti-CD40 mAb(8) (12, 13)
Anti-41BB	Promotes $CD8^+$ T cell functions	Yes	Anti-41BB IgG2a depletes Treg, . Hinge-engineered anti-4-1BB mIgG2a/h2B harness both mechanisms of action resulting in enhanced antitumor therapy(14)
Anti-TIGIT	Blocking interactons between TIGIT and CD155; eliminating immune suppression	Yes	Interaction with FcγR on APCs enhances antigen-specific T cell responses and antitumor activity (15)
Anti-CTLA4	Blocking interaction between CTLA4 and CD80/86; eliminating immune suppression	Yes	Requires activating FcγR to deplete $CTLA\text{-}4^+$ Treg cells in mice(1, 10, 16, 17) Fc-FcγR co-engagement on APCs by anti-CTLA-4 antibodies improves T cell signaling and function(15)
Anti-PD-1	Blocking interaction with PD-L1/2; eliminating immune suppression	No	Engagement of FcγR reduces the antitumor activity of anti-PD-1 by eliminating CD8 T cells(18) Anti-PD-1 are captured from T cell surface by macrophages via FcγR(19) Engagement of FcγR induces $Fc\gamma RI^+$ macrophages to phagocytose $PD\text{-}1^+$ T cells (20)
Anti-PD-L1	Blocking interaction with PD-1 eliminating immune suppression	Depending on the genetic background	Engagement of activating FcγR enhances the antitumor activity of Anti-PD-L1 (clone 14D8) and correlates with modulation of intratumoral myeloid subset in MC38 tumor model(18) Engagement of activating FcγR enhances the antitumor activity of Anti-PD-L1 in CT26 but not MC38 tumor model. The additional therapeutic efficacy correlates with the reduction of $PD\text{-}L1^+$ myeloid cells in the CT26 tumor (21)

II. Anti-Trp1 (TA99) antibody

There is indirect evidence that also the therapeutic efficacy of tumor targeting antibodies such as anti-HER2 and anti-EGFR depends partially on their interaction with FcγR (22, 23). To study what FcγR and what FcγR expressing effector cells might play a role, in **Chapter 4**, the anti-trp-1 mAb therapy in B16F10 mouse melanoma model has been explored. Because trp-1 is not a signalling molecule, the therapeutic effect of anti-Trp-1(TA99) is exclusively dependent on its ability to activate downstream effector mechanisms to kill the tumor, making it an optimal model to study these mechanisms without the confounding effect of other mechanisms such as interference with cell signalling. TA99 is effective at preventing melanoma formation in prophylactic setting, however, it has no effect in established tumors (therapeutic setting). Apart from optimisation of the mAb:FcγR interaction as shown in **Chapter 2**, maximising antibody Fc-mediated effector functions of macrophages has been proposed as another mechanism by which therapeutic mAb function can be improved (24, 25). In **Chapter 4**, the combined TA99/Imiquimod/IL2 treatment resulted in effective B16F10 tumor control that was dependent predominantly on FcγRI, macrophages, $CD8^+$ T cells and NK cells. On the other hand, FcγRIII on NK cells and FcγRIV on neutrophils were not involved in TA99 dependent killing mechanisms of the B16F10 tumor. This was confirmed by using NK cell-specific FcγRIII knockout and neutrophil-specific FcγRIV knockout mice. Instead, the production of IFNγ by NK cells is apparent upon imiquimod therapy. Since IFNγ induces the expression of FcγRI and downregulates FcγRIIb expression (26), we hypothesized that IFNγ producing NK cells are required as immunoregulatory cells for the stimulation of macrophage activity. This finding might explain why FcγRI in mice, exclusively expressed on mononuclear cells, including macrophages, plays a dominant role in TA99 combination therapy. FcγRI is the only FcγR class capable of binding monomeric IgG with high affinity and can mediate ADCC/ADCP and facilitate antigen presentation (27). However, it is currently unclear how macrophages elicit tumor cell killing after TA99 combination therapy of established B16F10 tumors. In prophylactic setting, macrophages are critical for the elimination of tumor cells via ADCP following TA99 mAb therapy. The involvement of FcγR and macrophages in TA99/Imiquimod/IL2 combination therapy in therapeutic setting are similar to what has been published with TA99 monotherapy in prophylactic setting. ADCP by macrophages is therefore a likely direct tumor killing mechanism in TA99 combination therapy. Triggering TLR7/8 by imiquimod has pleiotropic effect on immune responses. In this study, imiquimod sustained the retention or infiltration of CD86 and FcγR expressing inflammatory macrophages which is associated with the optimal therapeutic efficacy of TA99 combination therapy. Increasing the Activating:Inhibitory [A:I] FcγR ratios in macrophages with an immunomodulatory agent (STING agonist) has been shown to induce additional therapeutic activity of anti-CD20 mAb in mouse tumor models (24). Similarly, cyclophosphamide treatment significantly upregulates activatory FcγR and downregulates FcγRIIb on the bone marrow macrophages, resulting in an improvement of the therapeutic efficacy of anti-EGFR and anti-Her2 mAb in a humanized mouse model for metastatic breast cancer (25). These studies demonstrated that high [A: I] FcγR binding ratio is critical for antitumor activities of therapeutic antibodies. It would therefore be interesting to investigate whether imiquimod can directly modulate the expression of FcγR on inflammatory macrophages as a mechanism to augment TA99-mediated tumor cell killing *in vivo*. From our results and literature data, it can be concluded that FcγR can contribute

to the antitumor activity of a range of therapeutic mAbs. Therefore, a better understanding of Fc-FcγR interactions may provide rational for the design of more efficacious antibody therapy.

Involvement of antitumor T cell immunity is beneficial for antibody therapy

The induction of ADCC and/or ADCP by tumor-specific antibodies is usually not sufficient to eradicate the tumor completely. It has now become increasingly apparent that in addition the induction of antitumor T-cell responses is required for complete tumor eradication. In **Chapter 4**, the therapeutic efficacy of combined melanoma therapy of TA99 antibody, imiquimod and IL-2 is also dependent on $CD8^+$ T cells as the therapeutic efficacy was lost in the absence of $CD8^+$ T cells. However, the underlying mechanism of T cell priming in this combination therapy requires further investigations. Though one study demonstrated that the strong antitumor efficacy of the combination of IL-2 with extended half-life, TA99, anti-PD-1 mAb and vaccination was lost in $Batf^{-/-}$ mice with impaired cross-presentation capability, indicating that cross-presentation by DCs is essential in TA99 combination therapy (28). In addition, imiquimod treatment can result in the secretion of IFN-α (29), which can activate and enhance cross-presentation by DCs (30, 31). Another study demonstrated the important role of cross-presentation by DCs for the therapeutic efficacy of the combination therapy of TA99, IFN-α and IL-2 with extended half-life (31). Therefore, treatment with TA99, imiquimod and IL-2 is likely to promote also cross-presentation of tumor antigen to educate $CD8^+$ T cells to further eradicate tumor cells.

Not only the induction of antitumor T-cell responses is important for optimal tumor targeting antibody therapy, but also tumor infiltrating T cells may have a predictive value for the therapeutic efficacy as shown with anti-neu mAb therapy. In **Chapter 5**, it is clearly demonstrated that in a mouse mammary tumor large numbers of already present T cells are associated with stronger efficacy of anti-HER2 mAb therapy. There is an increasing number of observations in the clinic showing that the presence of high levels of tumor infiltrating lymphocytes (TILs) or TIL-associated genes in the TME are associated with a better response to anti-HER2 therapy in $HER2^+$ breast cancer (32-35). This suggest that TILs play a significant role in anti-HER2 therapy and opens up important questions for patient stratification. The results presented in in **Chapter 5** support these findings suggesting that the number of T cells already present in the tumor before treatment is predictive whether an effective anti-tumor response will be induced by the anti-HER2 mAb.

There are multiple lines of evidence that T cells are essential in anti-PD-L1 mAb therapy (36, 37). However, induction of durable and robust antitumor T cell responses by anti-PD-L1 mAb therapy only occurs in a subset of patients. In various tumors, signalling by transforming growth factor-β (TGF-β) fosters tumor growth by promoting angiogenesis, fibroblast activation, epithelial-to-mesenchymal (EMT) transition, metastasis, and immunosuppression (38). Most importantly, signalling by TGF-β has been found to promote resistance to anti-PD-L1 mAb (atezolizumab) therapy in cancer patients (39). **In Chapter 3**, it is shown, in the MC38 tumor model, that combined treatment with an anti-PD-L1 mAb and an TGF-β inhibitor (LY364947) results in improved overall survival compared to the monotherapies. This correlated with an increase of $CD8^+$ T cells in the TME. Other studies using anti-PD-L1 mAb in combination with either Garlunisertib (TGF-β inhibitor, LY2157299)[25] or TGF-β blocking

antibodies [24] showed improved T cell penetration into the centre of mouse solid tumors. However, the underlying mechanisms through which TGF-β inhibition improves the penetration of T cell into the tumor are currently unknown. The studies by Mariathasan (39) and Tauriello et al[25] suggest that TGF-β signalling in stromal fibroblasts is the major mechanism that contributes to the exclusion of T cells from mouse solid tumors. Moreover, it has been shown that cancer-associated fibroblasts (CAFs) contribute to the development of a physical barrier that interferes with T-cell infiltration (40). Altering tumor stroma by inhibiting TGF-β signalling in cancer-associated fibroblasts is likely the underlying mechanism of the improved infiltration of $CD8^+$ T cells in MC38 tumors in the combination therapy of TGF-β inhibitor and anti-PD-L1 mAb therapy.

In **Chapter 3**, the combined effect of anti-PD-L1 mAb and LY364947 was not observed in poorly immunogenic KPC1 tumor model. Compared to immunogenic MC38 tumor model, KPC tumors lack expression of neoepitopes (41). However, when the poorly immunogenic KPC tumor expresses ovalbumin, inoculation of this tumor cell line led to T-cell dependent tumor rejection in C57BL/6 mice (41). This suggests that the limited effect of checkpoint inhibitor in KPC tumor model may due to the low antigenic diversity and generally low amount of tumor targeting T cells (41, 42). Similar to KPC tumor model, human pancreatic cancers contain low mutation burden (TMB) and is not responsive to immune checkpoint inhibitors. The low immunogenic and aggressive pancreatic cancers is therefore a challenge for the successful application of cancer immunotherapies (43). For this, we have tried several combinations with agonistic anti-CD40 and mesothelin peptide vaccination (to elicit T cell responses against endogenous mesothelin antigen expressed in KPC) or CXCR2 inhibition (to block CXCR2 signaling which promotes pancreatic tumorigenesis), which all failed to improve meaningful long-term survival of the KPC tumor bearing mice (unpublished data). Other published studies have reported that the infiltration and accumulation of T cells in KPC tumors is restricted by the immunosuppressive TME. It has been shown that the KPC tumor produces high levels of the granulocyte-macrophages colony-stimulating factor (GM-CSF), which is associated with an increase of immunosuppressive $CD11b^+$ myeloid cells in the TME. Interestingly, genetic ablation or neutralisation of GM-CSF leads to decreased myeloid cell infiltration, promoting CD8 T cell-driven antitumor immunity (44, 45). Another study suggests that $Ly6C^{low}$ $F4/80^+$ tumor-associated macrophages regulate infiltration of T cells into the tumor. Depletion of macrophages using clodronate restores the therapeutic efficacy of gemcitabine/agonistic anti-CD40 mAb combination therapy which is dependent on $CD8^+$ T cells. These studies indicate that the KPC tumor may retain antigenicity that are recognized by T cells and understanding the suppressive pathways by which KPC tumor evades immune recognition is required to effectively design therapeutic regimens. Similarly, in **Chapter 3**, we found that neutralizing immunosuppressive TGF-β signalling alone was more effective at controlling KPC1 tumor outgrowth than anti-PD-L1 mAb therapy. TGF-β inhibitor treatment unexpectedly decreased the relative amount of intratumoral $CD4^+$ T cells, suggesting a potential tumor-promoting role of such $CD4^+$ T cells. Recent data in mice suggest that IL-17 production by $CD4^+$ T cells is required for the initiation and progression of pancreatic cancer (46, 47). Moreover, KPC tumor growth was slower in the CD4KO mice, and they had longer survival than the CD8KO mice. Consistent with this finding, weekly depletion of $CD4^+$ T cells resulted in a significant delay in the formation of pancreatic intraepithelial neoplasia (PanIN),

thus suggesting a pro-tumorigenesis role for $CD4^+$ T cells (47). As $Foxp3^+$ $CD4^+$ T cells were not affected by TGF-β inhibitor in **Chapter 3**, it remains to be investigated which $CD4^+$ T cell subsets in the KPC tumor were reduced upon the inhibition of TGF-β signalling. Together, this suggests that $CD4^+$ T cells might be important regulators of the KPC tumors in hampering an antitumoral response and this may be considered when designing a strategy for enhancing the therapeutic efficacy of combining TGF-β inhibitor and anti-PD-L1 mAb against pancreatic cancer.

Using the correct FcγR KO mouse model in antibody therapy research

Clynes et al.(22) were the first to show a critical role for FcγR-mediated effector functions by demonstrating loss of efficacy of anti-HER2 /neu and anti-CD20 antibody in FcR common γ-chain-deficient mice lacking all activating FcγR. However, this study does not exclusively address the role of activating FcγR as the FcR γ-chain is a promiscuous signal transduction subunit that is associated with a number of other receptor molecules, including the C-type lectin receptors Mincle and Dectin-II (48) which can manifest distinct antitumor functions in innate antitumor responses. For example, Dectin-II mediates phagocytosis of tumor cells by Kuffer cells (macrophages) suppressing cancer development (49). Therefore, the decreased efficacy of anti-her2 and anti-CD20 mAb in $FcR\gamma^{-/-}$ mice compared to WT animals, might be explained by the absence of Dectin-2 mediated anti-tumor activity in these KO mice. For this reason, mice deficient for FcγRs (lacking the functional genes encoding the ligand binding alpha chains of different FcγR while maintaining the expression of the common FcRγ chain)(50) are essential to investigate the exclusive role of FcγR (50) (**Chapter 2, 4**). Single, double, triple or quadruple FcγR knockout mouse strains (50-52) and cell type specific FcγR knockout mice (i.e. NK cell/ neutrophil specific $Fc\gamma RIII/IV^{-/-}$ mice [53], **Chapter 4**) have been generated and used extensively to further delineate the role of the individual activating FcγRs in the therapeutic efficacy of IgG mAbs in mice. By using the B16F10 mouse melanoma model and various FcγR (conditional) knockout mouse strains in **Chapter 4,** it is shown that activating FcγR expressing macrophages but not other FcγR expressing effector cells (NK cells and neutrophils) are likely to contribute to the TA99 combination therapy. In studies of others specific $Fc\gamma R^{-/-}$ mice were also instrumental in determining an essential role of activating FcγR in anti-CTLA4 therapy by facilitating ADCC of immunosuppressive Tregs in the TME (17). Taken together, preclinical studies using different $Fc\gamma R^{-/-}$ mice highlight a potential role of these mouse models for the development of rational synergistic therapies that aim to boost the Fc-dependent anti-tumor functions of therapeutic antibodies **(Chapter 4)**.

In **Chapter 5,** it is shown that anti-neu mAb therapy can efficiently delay tumor outgrowth in BALB/c-NeuT mice, however, the role of activating FcγR and FcγR expressing immune cells has not been studied in this model, *in vivo*. A previous report showed that FcγRIV expression on the RAW264.7 murine macrophage cell line was required for anti-her2 mAb-mediated cancer cell killing *in vitro* (54). However, RAW264.7 cells are established from an ascites of a tumor induced in a BALB/c mouse (55) and may not accurately represent monocytes/macrophages *in vivo* subsets that are responsible for the antitumor activities of anti-Her2 mAb. Furthermore, the role of monocytes/macrophages in antibody therapy might be different in different tissue location. For this, Lehmann et al. showed that TAMs derived from

Ly6C^{hi} monocytes were critical for TA99 therapy of B16F10 subcutaneous tumor, while the destruction of B16F10 tumors in the lung required the presence of alveolar macrophages (56). It has been shown in FcR γ-chain-deficient mice that the antitumor activity of anti-neu mAb requires the presence of the FcR γ-chain (57). As mentioned earlier, mice deficient for the FcγR ligand binding alpha chains but not for the FcR γ-chain are more suitable for the analysis of the role of activating FcγRs. However, the analysis of the role of the individual FcγR and the FcγR expressing effector cells in anti-neu mAb therapy is hampered by the genetic background of the BALB/c-NeuTmodel because most FcγR and cell type specific FcγRKO mice are on the C57BL/6 background. In **Chapter 6**, a novel C57BL/6-NeuT mouse model was generated that can be crossed with the different available C57BL/6 FcγR (conditional) knock-out mice in order to study the role of the different FcγR and FcγR expressing myeloid effector cells in anti-neu mAb therapy by analogy with what was demonstrated with TA99 antibody therapy in the B16F10 melanoma model (**Chapter 4**).

Understanding the preclinical tumor models for cancer immunotherapy research

Studies in this thesis were conducted using five commonly used syngeneic tumor models, which cover a broad range of tumor-immunogenicities. Both MC38 and CT26 tumors demonstrate high response rates to immunotherapy (**Chapter 2**) and are commonly referred to as "hot tumors" or T-cell-inflamed tumors. In contrast, immunotherapy is less effective to "cold tumors" or non-T-cell-inflamed tumors such as KPC (**Chapter 3**), B16F10 (58), and TUBO (59). It is clear that KPC, B16F10 and TUBO tumors contain fewer infiltrating T cells than CT26 or MC38 tumors. The low tumor T cell infiltrate can be explained by the absence of sufficiently immunogenic tumor antigens for T cell recognition (60), making the poor immunogenic tumor less likely to respond to immunotherapy. The MC38 and CT26 tumor cell lines are derived from chemically induced colon adenocarcinoma in C57BL/6 and BALB/c mice (61, 62) respectively, and these tumor cell lines harbour a good number of potential neoantigens that can be recognised by T cells. This is in contrast to the murine tumor cell lines derived from genetically engineered mice (TUBO, KPC). Genetic tumor models have a very low number of strong driver mutations (i.e. Kras, p53, HER2) sufficient to induce the development of de novo tumors. Unlike carcinogen-induced tumor models, genetically engineered tumor models are not exposed to mutagenic or carcinogenic substances and therefore display limited antigenicity that may be recognized by the immune system. The B16F10 is a spontaneously developed tumor with relatively low immunogenicity as discussed later.

The immunologic diversity of these five different tumor models broadly represents the diversity of human tumors and therefore represent a useful toolbox to evaluate different therapeutic strategies. In **Chapter 2** the immunologically "hot" CT26 and MC38 tumor models have been used to evaluate cancer immunotherapy based on anti-PD-L1 immune checkpoint blockade antibodies. In **Chapter 3 and 4**, the immunologically relatively "cold" B16F10 and KPC tumor models have been used to evaluate the capacity of combination therapies to modulate the TME leading to improved immune activation and immune cell infiltration into these tumors. It is noteworthy that the B16F10 model that was used in **Chapter 4** lacks mutations in BRAF, seen in at least 60% of the human melanomas (63, 64). Therefore, the

B16F10 tumor cell line is not a suitable model for assessing therapies based on (e.g. BRAF inhibitors) targeting BRAF. Nevertheless, the relatively "cold" B16F10 tumor cell line expresses several melanoma-associated antigens such as Trp-2/gp100 that may serve as a target for autologous T cells. As in addition several neoantigens have been identified from the B16F10 melanoma cell line (65), one cannot rule out the possibility that the observed antitumor $CD8^+$ T cells responses in the study in **Chapter 4** are neoantigen specific. Similarly, KPC pancreatic tumor model is similar in many aspects to the human pancreatic cancer. For example KPC tumors mirror similar levels of fibrosis and immunobiology as is seen in human pancreatic tumors(66). As a result, KPC model may be used to understand by which tumors regulate T cell exclusion and how immunosuppressive TME can be modulated to invoke tumor-specific T cell responses **(Chapter 3)**. Taken together, B16F10 and KPC can be leveraged to guide the development of immunotherapy-based combination therapy for poorly immunogenic human tumors.

Apart from the tumor immunogenicity of each tumor model, extrinsic factors such as experimental conditions can also have a huge impact on the results of the mechanistic/efficacy studies of antibody therapy. For example, opposing results have been reported regarding the involvement of the individual activating FcγR in TA99 therapy in different studies. In the B16F10 lung metastasis model, TA99 treatment was found to require FcγRI (52) or FcγRIV (67) or the combination of FcγRI and FcγRIII (68). Furthermore, FcγRI and FcγRIV (69) were simultaneously required for TA99 treatment of liver metastases. In the subcutaneous model, only a role for FγRIV (51) was evident. These contradictory results can probably be attributed to differences in experimental settings such as location of the tumor (lungs vs subcutaneous vs liver), treatment schedule (therapeutic vs prophylactic) and production process of the TA99 mAb (HEK cells vs hybridoma). Likewise, using rat-neu^+ TUBO tumor bearing WT BALB/c mice might have the risk of leading to an overestimation of the contribution of the adaptive immune response in anti-neu therapy (**Chapter 5**, summarized in Table 2). Using TUBO bearing non-isogenic recipient WT BALB/c mice, the results presented in **Chapter 5** are in agreement with the studies of Park et al (57), Stagg et al (70) and Mortenson et al (71) demonstrating that effective anti-neu mAb therapy requires $CD8^+$ T cells. Park et al. observed that also in isogenic recipient mice tolerant for rat-neu that $CD8^+$ T cells are involved in anti-neu mAb therapy (57). However, instead of using BALB/c-NeuT mice, Neu-Tg F1 (FVB/N-Tg/MMTV-neu x BALB/c) mice were used as the recipient for TUBO cells. Of note, unlike BALB/c-NeuT mice, this transgenic mouse carries the wild type form of the rat-neu gene (72). The data generated in Neu-Tg F1 recipient mice raised the question if in these mice the TUBO tumor cell line is really isogenic. Firstly, the point mutation in the activated rat-neu might result in the generation of a neo-epitope which can potentially increase the immunogenicity of TUBO cells in neu-Tg F1 mice. Secondly, BALB/c-NeuT transgenic mice were originally generated in C57BL/6 x DBA x CD1 mixed background (73) and subsequently backcrossed into BALB/c for about 12 generations (74, 75). Such a mouse is not fully BALB/c but congenic for the C57BL/6 x DBA x CD1 derived flanking region of the NeuT transgene containing still hundreds of genes of C57BL/6 x DBA x CD1 origin similar to what has been demonstrated for KO mice generated by gene targeting in 129 deived ES cells and subsequently backcrossed into C57BL/6 (76, 77).The TUBO and some other rat-neu^+ tumor cell lines (H2N100, H2N113, and H2N67) (70, 75) were derived from tumors spontaneously developed in BALB/c-NeuT

mice. Therefore, TUBO contains a substantial NeuT transgene flanking genomic region containing many genes of C57BL/6 x DBA x CD1 origin. The neu-Tg F1 mouse used by Park et al. as recipient strain for the TUBO tumor does not contain the NeuT flanking region of C57BL/6 x DBA x CD1 origin. Because of the many genetic differences between inbred strains, the presence of the NeuT transgene flanking region of C57BL/6 x DBA x CD1 origin in the TUBO tumor most likely increases the immunogenicity of the TUBO tumor in FVB/N-Tg/MMTV-neu x BALB/c F1 mice. Therefore the results of Park et al. showing the importance of CD8 T cells for anti-neu mAb therapy are not conclusive. As shown in **Chapter 5**, a role of $CD8^+$ T cells in anti-neu mAb therapy was ruled out by transplanting TUBO onto the fully syngeneic BALB/c-NeuT recipient mouse. Until now, only a study by Stagg et al. (70) provides evidence that $CD8^+$ T cells play a role in the therapeutic efficacy of anti-neu mAb therapy in a fully syngeneic setting. In their study, using rat-neu^+ tumor cell lines (H2N100, H2N113, and H2N67) derived from BALB/c-NeuT mice, $CD8^+$ T cell depletion significantly decreased the therapeutic efficacy of anti-neu mAb in H2N100 tumor bearing BALB/c-NeuT mice. This is not in agreement with the findings in **Chapter 5**, but it could be due to a more intense treatment schedule by Stagg et al. that may result in sustained inflammation breaking immunological tolerance of low avidity anti-rat-neu T cells. Taken together, the studies in the B16F10 and TUBO tumor models demonstrate that smarter preclinical studies are needed using more carefully designed animal tumor models during early stages of the development of therapeutic mAbs.

Table 2. Comparison of different publications investigating the adaptive immunity upon treatment of the rat-neu$^+$ mammary tumor model with anti-neu mAb

	Park et al. (57)	**Stagg et al. (70)**	**Mortenson et al. (71)**	**Sow et al. (78)**
Tumor cell line	TUBO	H2N100; H2N113	TUBO	TUBO
Recipient mice	Neu-Tg F1 (FVB/N-Tg/MMTV-neu x BALB/c); WT BALB/c	BALB/c-NeuT; WT BALB/c	WT BALB/c; Neu-Tg F1 (FVB/N-Tg/MMTV-neu x BALB/c)	BALB/c-NeuT; WT BALB/c
Treatment protocol with 100µg anti-neu mAb	Day 11 and 18	Day 8, 10, 12, 14, 16, 18, 20 and 23	Day 11 and 18	Day 10, 15 and 20
CD8$^+$ T cell depletion	Decreased therapeutic effect in TUBO tumor bearing WT BALB/c and neu Tg F1	Decreased therapeutic effect in H2N100 bearing WT BALB/c and BALB/c-NeuT	Decreased therapeutic effect in TUBO bearing WT BALB/c	N.D.
CD4$^+$ T cell depletion	N.D.	Therapeutic effect is not hampered	Decreased therapeutic effect in TUBO bearing WT BALB/c	N.D.
CD8$^+$ CD4$^+$ T cell depletion (mice and findings)	N.D.	N.D.	N.D.	therapeutic effect is not hampered
Neutralization of IFNγ	N.D.	Decreased therapeutic effect in H2N113 bearing WT BALB/c and BALB/c-NeuT	N.D.	N.D.
Conclusion	T cells are essential for therapeutic effect of anti-neu mAb	IFNγ producing CD8$^+$ T cells are essential for therapeutic effect of anti-neu mAb	CD4$^+$ T cells are essential for therapeutic effect of anti-neu mAb	Adaptive immunity does not play a role in therapeutic effect of anti-neu mAb in NeuT mice

N.D. Not determined

Concluding remarks

The durable clinical benefits obtained with antibodies targeting tumor antigens and immune checkpoints have reinvigorated the field of cancer immunotherapy. This thesis illustrates in a variety of preclinical mouse models several strategies to improve anti-tumor immunostimulatory and tumor targeting antibody therapy. In **Chapter 2**, it is demonstrated that the therapeutic efficacy of anti-PD-L1 mAb can be potentially enhanced by engaging activating FcγR. As described in **Chapter 3** this can also be achieved by combining anti-PD-L1 mAb with a TGF-β inhibitor. As for tumor targeting mAbs it is shown in **Chapter 4** that the combination of TA99 mAb, Imiquimod and IL-2 results in significant tumor control in the aggressive pre-clinical B16F10 melanoma tumor model by synergy between innate and adaptive immunity. The study in **Chapter 5** unveils that the tumor immunogenicity can affect the overall therapeutic efficacy of tumor targeting antibodies. Furthermore, the C57BL/6-NeuT mouse strain described in **Chapter 6** will be a valuable mouse model to help determine the contribution of the individual FcγR and FcγR expressing effector cell types in the therapeutic efficacy of anti-neu mAb. In addition, the findings in **Chapter 2, 3 and 5** suggest that antibody-based immunotherapies may not work for all murine tumor models. This may have important implications for the preclinical evaluation of antibody therapy. As with all animal models, mouse tumor models only mimic certain aspects of the human disease. There are several factors with regard to mAb therapy in cancer that need to be taken into account, including how well the biology, expression and distribution of mouse FcγR mimics of their human counterpart. Moreover, factors such as immune cell infiltrate (78, 79), and/or neoantigen load (80, 81) of a tumor can also affect the overall response to immunotherapy. Collectively, a greater understanding of murine tumor models and careful interpretation of the underlying therapeutic efficacy and mechanism of actions of antibody-based immunotherapy are required in order to increase the confidence in the translational of murine studies to human.

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8

CHAPTER 8.

Samenvatting in het Nederlands

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Antistoffen die binden aan eiwitten op het oppervlak van tumoren (tumor antigenen) of aan eiwitten die een belangrijke regelende functie hebben in de afweer ('immune check-points') worden met succes toegepast in de kliniek om kanker te bestrijden. Binding van de antistoffen aan tumorantigenen kan niet alleen direct de groei van de tumor remmen of de tumorcellen doden maar ook indirect een tegen de tumor gerichte afweerreactie activeren zoals uit recent onderzoek is gebleken. Antistoffen bestaan uit een variabel deel dat het antigeen bindt en een constant deel (Fc deel) dat kan binden aan Fc-receptoren (FcγR), moleculen op het oppervlak van een aantal verschillende type afweercellen. Binding aan FcR van antistoffen die met hun variabel deel aan een tumorcel zijn gebonden, activeert afweerreacties die de tumorcel kunnen doden. Antistoffen die aan immune check-points binden blokkeren de afweerremmende signalen die normaal nodig zijn om een schadelijke overreactie van de afweer te voorkomen. Dit resulteert in de (re)activatie van natuurlijke tegen de tumor gerichte afweerreacties. Omdat zowel bij preklinische als klinische toepassing de reacties op antistoftherapie sterk variëren was het algemene doel van het in dit proefschrift beschreven onderzoek om verschillende behandelingsstrategieën in een aantal preklinische muismodellen te onderzoeken om daarmee de ontwikkeling van verbeterde antistoftherapie te bevorderen.
Immuun checkpoint PD-L1 remt de spontane, tegen de tumor gerichte, reactie van de T-cellen van het afweersysteem. Daarom verhogen PD-L1 blokkerende antistoffen de tegen de tumor gerichte afweer. Het is echter niet duidelijk of de interactie van anti-PD-L1 antistoffen met FcR positief of negatief bijdraagt aan de anti-PD-L1 antistof therapie. In hoofdstuk 2 zijn vier vormen van anti-PD-L1 antistof gebruikt die uitsluitend van elkaar verschilden in de sterkte van de binding van hun Fc deel aan FcR. Alle antistoffen waren even effectief in het onderdrukken van de uitgroei van een dikke darmtumor, MC38 genaamd, in muizen. De therapeutische werkzaamheid van deze anti-PD-L1 antistoffen was noch verminderd noch verhoogd in muizen waarin alle FcR ontbraken, hetgeen suggereert dat FcγR geen rol spelen in anti-PD-L1 therapie in het MC38 tumormodel in muizen. Interessant is dat in een ander dikke-darmkankermodel, CT26, de hoogste therapeutische werkzaamheid werd waargenomen bij muizen die anti-PD-L1 ontvingen met de sterkste binding aan FcR. Hieruit kan worden geconcludeerd dat interactie van anti-PD-L1 met FcR de therapeutische werking niet negatief beïnvloedt, maar mogelijk kan bijdragen aan een verhoging van de therapeutische effectiviteit van de anti-PD-L1 antistof afhankelijk van het tumormodel.

Het is aangetoond dat verhoging van de signaalstof TGF-β geproduceerd door cellen in de micro-omgeving van de tumor het therapeutisch effect van anti-PD-L1 vermindert. In Hoofdstuk 3 laat in het MC38 model zien dat de combinatie van een TGF-β remmer en anti-PD-L1 antistof effectiever is dan TGF-β remmer of anti-PD-L1 antistof monotherapie. Dit ging gepaard met een verhoogde infiltratie van $CD8^+$ T-cellen in de tumor in vergelijking met monotherapie. TGF-β was echter even effectief als de combinatietherapie bij het onderdrukken van de uitgroei van de agressieve alvleesklier tumor KPC1. Deze resultaten suggereren dat de synergistische anti-tumoractiviteit van anti-PD-L1 en de TGF-β-remmer afhankelijk is van het gebruikte tumormodel. Dat benadrukt het belang van de evaluatie van de werkzaamheid van de verschillende therapieën in verschillende tumormodellen.

Eerder werd aangetoond dat behandeling met een antistof gericht tegen een tumor antigeen op een uitgegroeid melanoom weinig therapeutisch effect sorteert in muizen. In hoofdstuk 4 laten we zien dat de toediening van de signaalstof IL-2 en een de afweer stimulerend middel, imiquimod, aan muizen met een melanoom, het therapeutische effect van een melanoom-specifieke antistof sterk verbetert wat resulteert in een significant hogere overleving van de melanoom dragende muizen. Door muizen te gebruiken die een enkel type FcγR of FcγR op een bepaald type afweercel misten en door selectief specifieke typen afweercellen te verwijderen, konden we aantonen dat een bepaalde FcγR, FcγRI, op een bepaald type afweercel, de macrofaag, essentieel was voor de effectiviteit van de combinatie therapie. In aanwezigheid van imiquimod bleken er meer actieve macrofagen met meer FcγR op hun celoppervlak in de micro-omgeving van het melanoom aanwezig te zijn. Bovendien bleek een ander type afweercel, de killer T-cel, onontbeerlijk voor de therapeutische werking van de combinatietherapie. Deze bevindingen tonen aan dat het verhogen van de aanwezigheid in de tumor micro-omgeving van bepaalde afweercellen met veel FcγR op hun celoppervlak door een middel zoals imiquimod, dat de afweer stimuleert, kan dienen als een strategie om de effectiviteit van antistoftherapie te verbeteren en tonen tevens het belang aan van het initiëren van T-celreacties.

De belangrijke rol van T-cellen in de werkzaamheid van de tegen tumoren gerichte antistoffen wordt verder toegelicht in hoofdstuk 5. Tumoren wijken af van normaal gezond weefsel en kunnen daarom meestal herkend worden door het afweersysteem dat will zeggen dat ze immunogeen zijn. De mate van immunogeniciteit varieert echter tussen tumoren. Hoofdstuk 5 laat zien dat sterk immunogene tumoren een groter aantal infiltrerende T-cellen in hun micro-omgeving bevatten dan minder immunogene tumoren. De verhoogde aanwezigheid van T-cellen was geassocieerd met een verhoogde anti-tumoractiviteit van een therapeutische antistof die een tumor antigeen, neu, op borsttumoren herkent. Dit suggereert dat het indelen in groepen van kankerpatiënten met neu^{+} borsttumoren op basis van de reeds in de tumor aanwezige T-cellen nuttig kan zijn bij het voorspellen van de respons bij anti-neu antistof therapie. Eerdere studies suggereren dat FcγR zouden kunnen bijdragen aan anti-neu antistof-therapie. Het meest recente onderzoek naar de rol van FcγR werd echter uitgevoerd in BALB/c-muizen die deficiënt waren voor de gemeenschappelijke FcR-γ keten. Deze muizen hebben weliswaar geen functionele activerende FcR maar missen daarnaast ook andere relevante receptoren waar de gemeenschappelijke FcR γ keten ook deel van uit maakt. De rol van FcγR in anti-neu antistof therapie is daarom niet eenduidig vastgesteld. Bovendien is de bijdrage van elk type FcγR en type afweercel dat FcγR op zijn celoppervlak heeft aan anti-neu antistof therapie niet *in vivo* vastgesteld omdat de meeste FcγR knock-out muizenstammen een C57BL/6 genetische achtergrond hebben, terwijl het MMTV-NeuT borsttumor muismodel een BALB/c achtergrond heeft. Hoofdstuk 6 beschrijft het genereren en karakteriseren van een MMTV-NeuT muismodel op een C57BL/6 achtergrond. Belangrijk is dat er geen verschil was in de infiltratie van afweercellen in de tumoren in BALB/c-NeuT en C57BL/6-NeuT muizen wat suggereert dat C57BL/6-NeuT een goed alternatief tumormodel kan zijn voor preklinische studies. In toekomstige studies kan met behulp van verschillende celtype-specifieke FcγR-deficiënte C57BL/6-NeuT muizen de specifieke bijdrage van elke FcγR in de therapeutische werkzaamheid van anti-neu mAb-therapie worden vastgesteld. Dergelijke informatie kan

helpen bij het ontwerpen van nieuwe strategieën voor het verhogen van de therapeutische effectiviteit van antilichamen gericht tegen tumoren.

Summary

Antibodies that bind to proteins on the surface of tumors (tumor antigens) or to proteins that have an important regulatory function in the immune system ("immune checkpoints") are successfully used in the clinic to fight cancer. Binding of the antibodies to tumor antigens can not only directly inhibit tumor growth or kill the tumor cells but can also indirectly activate an immune response directed against the tumor, as recent research has shown. Antibodies consist of a variable region that binds to the antigen and a constant region (Fc region) that can bind to Fc receptors (FcγR), molecules on the surface of a number of different types of immune cells. Binding to FcγR of antibodies, bound to a tumor cell with their variable region, activates immune responses that can kill the tumor cell. Antibodies that bind to immune checkpoints block the immune-inhibiting signals that are normally needed to prevent a harmful over-reaction of the immune system. This results in the (re) activation of natural anti-tumor responses. As the response to antibody therapy varies widely in both preclinical and clinical application, the overall goal of the study described in this thesis was to investigate different treatment strategies in a number of pre-clinical mouse models to facilitate the development of improved antibody therapy.

The PD-L1 immune checkpoint inhibits the spontaneous response of the T cells of the immune system against the tumor. Therefore, PD-L1 blocking antibodies increase the immune response against the tumor. However, it is not clear whether the interaction of anti-PD-L1 antibodies with FcR contributes positively or negatively to the anti-PD-L1 antibody therapy. In Chapter 2 four forms of anti-PD-L1 antiserum are used that differed only in the strength of the binding of their Fc part to FcγR. All antibodies were equally effective in suppressing the growth of a colon cancer called MC38 in mice. The therapeutic efficacy of these anti-PD-L1 antibodies was neither reduced nor increased in mice lacking all FcγR, suggesting that FcR did not play a role in anti-PD-L1 therapy in the MC38 tumor model in mice. Interestingly, in another colon cancer model, CT26, the highest therapeutic efficacy was observed in mice receiving anti-PD-L1 with the strongest binding to FcγR. From this it can be concluded that interaction of anti-PD-L1 with FcγR does not negatively influence the therapeutic effect but may increase the therapeutic efficacy of the antibody depending on the tumor model.

Increased production of TGF-β by cells in the microenvironment of the tumor has been shown to reduce the therapeutic efficacy of anti-PD-L1. In Chapter 3 it is demonstrated in the MC38 model that the combination of a TGF-β inhibitor and anti-PD-L1 antibody was more effective than TGF-β or anti-PD-L1 monotherapy. This was accompanied by an increased infiltration of $CD8^+$ T cells into the tumor compared to monotherapy. However, TGF-β was as effective as the combination therapy in suppressing the growth of the aggressive pancreatic tumor KPC1. These results suggest that the cooperative anti-tumor activity of anti-PDL1 and the TGF-β inhibitor is dependent on the tumor model. This emphasizes the importance of evaluating the efficacy of different therapies in different tumor models.

It was previously shown that treatment with an antibody directed against a tumor antigen on an established melanoma has little therapeutic effect in mice. In chapter 4 we show that the

administration of the cytokine IL-2 and an immune-stimulating agent, imiquimod, to mice with an established melanoma, greatly improves the therapeutic effect of a melanoma-specific antibody, resulting in a significantly higher survival of the melanoma bearing mice. By using mice that missed a single type of FcγR or the FcγR on only a particular cell type and by selectively removing specific types of immune cells, we were able to demonstrate that one particular FcγR, FcγRI, on a particular type of immune cell, the macrophage, was essential for the effectiveness of the combination therapy. In the presence of imiquimod, more active macrophages with more FcγR were found to be present on their cell surface in the microenvironment of the melanoma. Moreover, a different type of immune cell, the killer T cell, was indispensable for the therapeutic effect of the combination therapy. These findings show that increasing the presence in the tumor microenvironment of certain immune cells with high FcR on their cell surface by an agent such as imiquimod that stimulates the immune system can serve as a strategy to improve the effectiveness of antibody therapy and also show the importance of initiating T cell responses.

The important role of T cells in the effectiveness of tumor targeting antibodies is further explained in Chapter 5. Tumors deviate from normal healthy tissue and can therefore usually be recognized by the immune system, ie they are immunogenic. However, the degree of immunogenicity varies between tumors. In chapter 5, it is shown that highly immunogenic tumors contain a greater number of infiltrating T cells in their microenvironment than fewer immunogenic tumors. The higher infiltration of T cells was associated with the increased anti-tumor activity of a therapeutic antibody that recognises the tumor antigen rat-neu on mammary tumors. This suggests that classification into groups of cancer patients with $Her2^{+}$ tumors based on the number of T cells already present in the tumor may be useful to predict the response with anti-Her2 antibody therapy. Previous studies suggest that FcγR could contribute to anti-neu antibody therapy. However, most recent research on the role of FcγR was conducted in BALB/c mice that were deficient for the common FcR-γ chain. Although these mice do not have functional activating FcγRs, they also lack other relevant receptor complexes of which the common FcR-γ chain is also part. The role of FcγR in anti-neu antibody therapy has therefore not been clearly established. In addition, the contribution of each type of FcR and type of immune cell that expresses FcγR, to anti-neu antibody therapy has not been established *in vivo* because most FcγR knockout mouse strains are on C57BL/6 background, whereas the mouse MMTV-NeuT mammary tumor model is on BALB/c background. Chapter 6 describes the generation and characterization of a MMTV-NeuT mouse on C57BL/6 background. Importantly, there was no difference in the infiltration of immune cells into the tumors in BALB/c-NeuT and C57BL/6-NeuT mice, suggesting that C57BL/6-NeuT could be an alternative tumor model for preclinical studies. In future studies, with different cell type-specific FcR-deficient C57BL/6-NeuT mice the specific contribution of each FcγR to the therapeutic efficacy of anti-neu mAb therapy can be determined. Such information would help to design new strategies for increasing the therapeutic effectiveness of tumor targeting antibodies.

Curriculum vitae

Heng Sheng Sow was born on 29th October 1986 in Kuala Lumpur, Malaysia. He received his bachelor's degree in Biotechnology in 2009 from UCSI University in Kuala Lumpur. In 2010, he started his master's degree in Cancer immunotherapy and biotechnology at the University of Nottingham, England where he started a research project on the humanization of novel anti-glycan antibody for human pancreatic cancer under the supervision of Dr Ian Spendlove. After obtaining his master's degree in 2011, he went to Australia for a 6-month internship at University of Queensland, Diamantina Institute to study the combination therapy of metronomic chemotherapy and γδ T cell vaccination in a B cell lymphoma mouse model. Heng Sheng started his PhD study in October 2019 in the department of Human Genetics (head professor Silvère van der Maarel) under the supervision of Associate professor Sjef Verbeek, Dr Marieke Fransen and Professor Ferry Ossendorp (Department of Immunohematology and Bloodtransfusion) of the Leiden University Medical Center, the Netherlands. His PhD project focused on different cancer immunotherapy approaches exploiting tumor targeting and immunostimulatory antibodies. The project was part of the EU funded Marie-Sklodowska-Curie Initial training Network (ITN) TIMCC (Tumor Infiltrating Myeloid Cell Compartment) initiated and coordinated by Sjef Verbeek. Heng Sheng also spent the last 6 months of his PhD in Prof Peter ten Dijke's lab to investigate the therapeutic efficacy of combined inhibition of TGF-β signalling and the PD-L1 immune checkpoint. From 2018 to 2020, he worked for Epsilogen therapeutics and Prof. Sophia Karagiannis at King's College London where he investigated the mechanism of action and therapeutic efficacy of tumor targeting IgE antibodies. Currently, he works at University of Southampton, under the supervision of Dr Sean Hua Lim.

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