Mechanisms of melanoma-targeting antibody therapy in mice

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CHAPTER 6

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General Discussion

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The application of antibodies directed towards antigens on tumor cells to treat cancer, the so-called tumor-targeting antibodies, is increasing in the clinic. Currently, there are 18 tumor-targeting antibodies approved by the FDA for tumor therapy. Eight of those antibodies recognize four targets on solid tumors (including neu) while ten are specific for seven targets on leukemias/lymphomas (including CD20) (1). Most of FDA approved tumor-targeting antibodies used in cancer therapies are of the human IgG1 subclass. Human IgG1 is the IgG subclass with the highest affinity for FcγR and is the most effective inducer of FcγR-mediated antibody effector functions (2-4). Engineering antibodies to alter their binding to FcγR has shown to be one of the ways to improve their therapeutic efficacy. Bispecific antibody formats that target a tumor antigen and with the other arm the CD3 on T cells are other approved antibody modalities used already in the clinic to treat certain cancers (5). In this thesis the mode of action of different tumor-targeting antibody types (bispecific, polyclonal or monoclonal) and how their therapeutic efficacy can be improved has been studied, focusing on the role of FcγR in mouse models.

Mice deficient for all FcγR have strongly impaired downstream antibody effector functions while maintaining normal adaptive immunity

The role of FcγR receptors has been widely studied over the last 3 decades mainly by using mouse models, particularly genetically engineered models. However, there were some flaws in these studies, since most applied mice deficient for the common FcR γ chain (FcRγ$^{-/-}$ mice). Although for a long time these mice were considered to exclusively lack activating FcγRs, in recent years an increasing number of receptor molecules was found to be also associated with the common FcR γ chain, including the c-type lectin receptors MINCLE and Dectin II (7). MINCLE and Dectin II were shown to be important for initiating and suppressing cancer development. In addition, Dectin II also appeared to mediate phagocytosis of tumor cells by Kupffer cells (6-8). By using MINCLE$^{-/-}$ mice it has been shown that pancreatic tumors grow slower in the absence of MINCLE (9). Others showed that pancreatic tumors grow slower in FcRγ$^{-/-}$ mice and attributed that to the absence of FcγR suggesting a role for spontaneously developing tumor-specific antibodies in this tumor model (10). This demonstrates the need for KO mouse models lacking the functional genes encoding the

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alpha chains of the different FcγR while maintaining the expression of the common FcR γ chain.

In addition, it was shown that FcγRII and -III deficient mice that were generated by gene targeting in the 129 mouse strain-derived ES cells and backcrossed more than seven generations into C57BL/6 background, become more sensitive to the spontaneous development of autoimmunity (11) due to the presence of remaining 129 derived sequences (SLE16) flanking the FcγR KO alleles (12). Therefore, we developed a new mouse model deficient for the IgG-Fc binding alpha chains of all four FcγR on a pure C57BL/6 background (FcγRI/II/III/IV$^{-/-}$ mice) and studied the immune system of these mice *in vivo*. In chapter 2 we demonstrate that, although downstream antibody effector mechanisms are strongly impaired, FcγRI/II/III/IV$^{-/-}$ mice develop normal B and T cell responses and do not develop anti-nuclear autoantibodies (ANA) with age, indicating that previously used mouse models contained flaws. It might be very interesting to also study the growth of pancreatic tumors in our FcγRI/II/III/IV$^{-/-}$ mice in order to determine whether FcγRs are anyhow involved in this process. All this data suggests that that mouse FcγRs could be redundant.

Furthermore, we used the new FcγRI/II/III/IV$^{-/-}$ and cell type-specific FcγR KO mice to define the role of innate immune cells and their three activating FcγR in the therapeutic effect of tumor-targeting antibodies.

The FcγR-dependent downstream effector pathways in tumor-targeting antibody therapy

In chapter 3 and 4 we demonstrate that FcγR dependent downstream antibody effector pathways are indispensable for the therapeutic effect of an MCMV-TRP2 vaccine or imiquimod/IL2/TA99 combination therapy confirming the important role of FcγRs in tumor-targeting antibody therapy as suggested by a series of preclinical studies using the same mouse model of transplantable B16F10 melanoma under a variety of experimental conditions. Interestingly, different laboratories reported contradictory results regarding the involvement of individual activating FcγR in the B16F10 model using different panels of FcγR$^{-/-}$ mice and FcγR-blocking antibodies. It has been shown that the population of myeloid cells infiltrating the B16F10 melanoma varies depending on the location of the tumor (13). When seeded in the lung (14, 15), a prominent role for FcγRI was found in some studies (15) while in others only FcγRIV (16) or FcγRI in combination with FcγRIII (14) were pivotal. When seeded in the liver, a combination of FcγRI and FcγRIV was found to be important (16) and in subcutaneous tumors, only a role for FcγRIV was evident (17). Glycosylation of antibodies, which strongly influences their binding affinity to FcγR (17), varies between antibodies produced by hybridomas (18) and human cells (HEK293) transfected with a recombinant IgG gene (19, 20). Although we used a therapeutic setting, treated a subcutaneous established tumor with TA99 antibody from hybridoma cultures and combined this with the innate and adaptive immunity stimulating TLR7/8 agonist imiquimod, the outcome with respect to FcγR involvement was fairly similar to what has been published with single TA99 treatment in the prophylactic setting. Macrophages and FcγRI were absolutely required with a minor role for FcγRIV and perhaps FcγRIII. This holds true also for polyclonal IgG anti-B16F10 antibodies generated by vaccination with a Tpr2 expressing MCMV vector.

In summary, nine experimental setups of TA99 treated B16F10 models (as depicted in Table 1) can be divided into the following groups, based on the involvement of FcγR and effector cell types: Five with a prominent role for FcγRI, five with a role for FcγRIV and six with a prominent role for Macrophages. There were three exceptions: (1) an exclusive role of FcγRIV when antibodies were used produced by HEK293 cells, (2) no role for FcγRIV when another detection method for tumor outgrowth was used and (3) an exclusive role of neutrophils when a neutrophil-specific syk KO and intravenous injection of TA99 was used. These conflicting results suggest that some experimental conditions have a strong impact on the outcome of tumor therapy experiments with respect to the role of FcγR.

It was surprising that vaccination with MCMV-TRP2 results in an antibody response that protects against outgrowth of the B16F10 tumor because, in contrast to Trp-1 which is recognized by the TA99 monoclonal antibody, Trp-2 is not expressed at the cell surface. However, a previous study showed that a DNA vaccine of a TRP2-encoding sequence induced a cross-reactive antibody response against TRP1 because these proteins are quite homologous (21). Therefore, it is highly likely that the MCMV-TRP2 vaccine also induces antibodies against TRP1 which makes a direct comparison of the efficacy of MCMV-TRP2 vaccination and TA99 treatment possible. While TA99 is of one subclass of IgG (IgG2c) targeting one epitope of the TRP1 protein, the MCMV-TRP2 vaccine induces polyclonal antibody responses consisting of a variety of subclasses of Ig with a variety of avidity and binding to multiple epitopes. Previous studies have shown that polyclonal antibodies and a combination of antibodies specific for a larger variety of epitopes of the same target induce better complement-mediated killing of tumor cells compared to monoclonal antibodies (22-26), making vaccination an attractive alternative for injection of monoclonal antibodies. In the absence of all four FcγR, the protection against B16F10 outgrowth was completely abolished, suggesting that complement is redundant or plays a negligible role in this model.

Whereas macrophages seem to be crucial, NK cells were not involved in the *in vivo* antibody-dependent killing mechanism of B16F10 tumors (13, 16, 27). In most *in vitro* studies using tumor-targeting humanized therapeutic antibodies it has been demonstrated that human NK cells, expressing FcγRIIA, are effective in antibody-dependent killing of tumor cells (28). The discrepancy between mouse and human can be explained by species differences as it has been reported that mouse NK cells, expressing very low levels of FcγRIII are poor elicitors of ADCC/ADCP compared to rat and human NK cells. In contrast, human and mouse macrophages show the similar antibody-dependent killing of tumor cells *in vitro* (29). This might explain why in mice FcγRI, exclusively expressed on mononuclear cells, including macrophages, plays a dominant role in IgG tumor-targeting antibody therapy.

Induction of tumor-specific T cells in tumor-targeting antibody therapy

The induction of ADCC and ADCP is most likely not sufficient to completely clear the body from cancer cells. It is generally assumed that the induction of a long-lasting tumor-specific cytotoxic T cell immunity is required. TA99 antibody monotherapy has no effect on an established B16F10 tumor and the therapeutic effect in a prophylactic setting is T cell independent (30). Previous studies have shown that the therapeutic efficacy of TA99 tumor targeting antibodies can be substantially increased by combination with checkpoint blocking antibodies (anti-PD-L1), TLR-4 ligands and peptide vaccination, and IL2 with a long half-life (Fc-IL2). The therapeutic effect was absent in $CD8^+$ T cell-depleted mice (31-33). In chapter 4 it is shown that the therapeutic efficacy of the combination of TA99, IL2, and imiquimod is also dependent on $CD8^+$ T cells. The mentioned combination therapies have in common that T cells are activated alongside the direct targeting of the tumor with TA99 antibody. The underlying mechanism of T cell priming in these improved antibody therapies is currently unknown (31-33). Though one study showed that the therapy was abrogated in a mouse with impaired cross-presentation capability (31). Moreover, It is known that imiquimod treatment results in increased IFNα secretion (34) that activates cross-presentation (35). The therapeutic efficacy of TA99 is strongly improved when combined with IL2 and IFNα (32). This is likely the scenario how an effective $CD8^+$ T cell response is induced in the combination therapy of TA99, IL2 and imiquimod as schematically depicted in Figure 1, which combines results presented in this thesis and those from literature. Other studies using tumor-targeting antibodies in combination with immune stimuli such as anti-GD2/IL2 immunocytokine and anti-CD40/CpG combination therapy against $GD2^+$ tumor cells also showed dependency on $CD8^+$ T cells (36). Together, these results hint to the importance of the activation of CD8$^+$ cytotoxic T cells for the improvement of tumor-targeting antibody therapy.

Bispecific antibodies that activate T-cells

Usage of CD3-targeting bispecific antibodies for the treatment of cancer is an upcoming field. Blinatumomab is a CD19-directed T cell engager and has already been approved by the FDA for the treatment of refractory B-cell precursor acute lymphoblastic leukemia (ALL) patients (5). In chapter 5, it is shown that treatment of TRP1-expressing B16F10 melanoma with a CD3-targeting bispecific antibody that binds to TRP1 on the tumor cells and activates T cells induced clear delay of tumor outgrowth. This therapeutic effect was operational in the absence of either CD4 T cells or CD8 T cells, but not anymore when both subsets were depleted. Previous reports showed that CD3-bispecific antibodies induce recruitment and activation of T cells and results in expression of known activation markers like CD25 and CD69 and secretion of cytokines like IFNγ, TNF-α, IL-2, IL-4, IL-6, and IL-10 (37-40). In addition, it has been demonstrated that bispecific antibodies, binding CD3 on T cells combined with binding to different tumor target molecules on different tumor types, can induce perforin/granzyme-dependent cytotoxicity against tumor cells by both CD4$^+$ and CD8$^+$ T cells. However, CD4$^+$ T cells are less capable to elicit cytotoxicity (41-46). In chapter 5, our studies clearly show a higher killing of KPC3-TRP1 tumor cells compared to B16F10 tumor cells. This difference is probably caused by the difference in expression level of TRP1 which is higher on KPC3-TRP1 cells than on B16F10 cells. Interestingly, the in vivo tumor control of B16F10 was more convincing than that of KPC3-TRP1, suggesting that other mechanisms than direct target cell killing were important *in vivo* or that TRP1 levels might be increased by tumor cells *in vivo*.

CD3-targeting bispecific antibodies have been in development for the treatment of cancer for a long time, but it is only recently that one compound has been approved by the regulatory bodies FDA and EMA (European Medicine Agency) for clinical use. The therapeutic effect of around 60 different bispecific antibodies is currently tested in preclinical models and in clinical trials. Blinatumomab has predominantly been used for hematological cancer and approved by FDA and catumaxomab by EMA against carcinoma ascites. Both of these tumors are easily accessible and penetrable by T cells and antibodies (reviewed in (47)). One of the challenges in this field is how to exploit CD3-bispecifics against tumors that have low T cell influx and have a low penetrance of antibodies. In Chapter 5 it is shown that the bispecific CD3xTA99 works in both subcutaneous melanoma and pancreas carcinoma models. Both tumors have a relatively low influx of T cells and future investigation might focus on means to enhance this, in addition to protocols that are capable to induce tumor-specific T cell memory.

Concluding remarks

Most studies described in this thesis investigate different immune mechanisms revolving tumor-targeting antibodies leading to eradication of tumors. To fully understand antibody-mediated effector functions in Chapter 2 mice are characterized that lack all FcγR, showing a strong role of FcγR in downstream antibody effector pathways, especially when expressed by macrophages. We demonstrate that control of tumor outgrowth by tumor-specific polyclonal antibodies induced by MCMV-TRP2 vaccination in a prophylactic setting (Chapter 3) and by combining the TA99 monoclonal antibody with imiquimod and IL2 in a therapeutic setting (Chapter 4) depends on FcγRI-expressing macrophages. Combining the tumor-targeting TA99 antibody with the immune stimulatory molecules imiquimod and IL2 resulted in a strong, $CD8^+$ T cell-dependent, therapeutic effect corroborating recent results from other groups that used different combinations of immune-stimulating compounds. However, our studies also eluded to the superior anti-tumor effects of CD3-bispecific antibodies. Together, this strongly advocates for examination of extended combinations of immune stimulatory molecules and tumor-targeting antibodies, which induces acute anti-tumor immune reactivity and, simultaneously, promotes long-lasting T cell responses to prevent recurrences by immune memory.

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Table 1: Comparison of different papers investigating the role of Fc receptors in the treatment of B16F10 melanoma with tumor targeting antibodies (13-17, 27, 48-51).

Paper	Model	Start and Ab	KO used	Cell depletion	Detection	Which cell and FcγR involved	Difference
(48)	Lung metastasis	TA99 day 0 IgG1, IgG2b and IgG2c defucosylation	FcγRI KO FcγRIII KO FcγRIV blocking (9E9)		Size (Caliper)	IgG2c and Defucosylated IgG2b it is FcγRIV	Own TA99 production “Metastatic”
(15)	Lung metastasis	TA99 day 0 (+ MPL)	FcγRI KO FcγIII KO FcR γ chain KO		Numbers/size	FcγRI is central	Effector cells involved: Alveolar Mϕ? “Metastatic”
(16)	Liver metastasis	TA99 day 0	FcγRI KO FcγRIII KO FcγRIV blocking (9E9) FcγRI/IIII KO	Kupfer cell depletion (clodronate)	Numbers	FcγRI and FcγRIV and Kupfer cells	Effector cells involved: Kupffer cells “Metastatic”
(17)	Skin s.c	TA99 day 0	FcγRI KO FcγRIII KO FcγRIV KO FcγRI/III KO		Size (Caliper)	Only FcγRIV	Own TA99 production “Metastatic”
(14)	Lung metastasis	TA99 day 0 (+ CTX)	FcγRI KO FcγRIII KO FcγRIV KO FcR γchain KO FcγRI/II/III KO FcγRII/III/IV KO FcγRIII and IV blocking MoAb		Bioluminescence load	FcγRI and FcγRIII but NOT FcγRIV	Effector cells involved: Alveolar Mϕ? Other detection method “Metastatic”
(49)	Skin s.c	TA99 day 0/2	hMRP8Cre X Syk$^{fl/fl}$ (neutrophil spec.)	Clodronate; anti-GR-1 NK cell depl. + Gadolinium	Bioluminescence load	Neither Mϕ nor NK cells, but neutrophils	Other detection Method IV injection TA99 “Metastatic”
(27)	Liver metastasis	TA99 day 0			Intravital imaging	Kupfer cells (Mϕ)	Kupfer cells (Mϕ) “Metastatic”
(50)	Skin s.c	MCMV-TRP2 vaccination	FcγRI/II/III/IV KO FcγRI KO; FcgRIII KO FcγRIV KO FγRIII/IV KO	Clodronate, anti-GR-1, NK cell depletion	Size (Caliper)	Central role FcγRI and Mϕ minor role of FcγRIV No role for neutrophils	Polyclonal anti-TRP2 Prophylactic
(51)	Skin s.c	TA99 Day 5 + IL2 + Aldara	FcγRI/III/IV KO FcγRI KO FcγRIII/IV KO NK spec. FcγRIII KO Neutrophil spec. FcγRIII/IV KO	Clodronate, anti-GR-1, NK cell depletion	Size (Caliper)	FcγRI on Mϕ, minor role FcγRIII and/or FcgRIV on Mϕ FcγR independent small contribution NK cells No role (FcγR on) neutrophils T cell dependent	Effect of Aldara and IL-2 “Therapeutic”

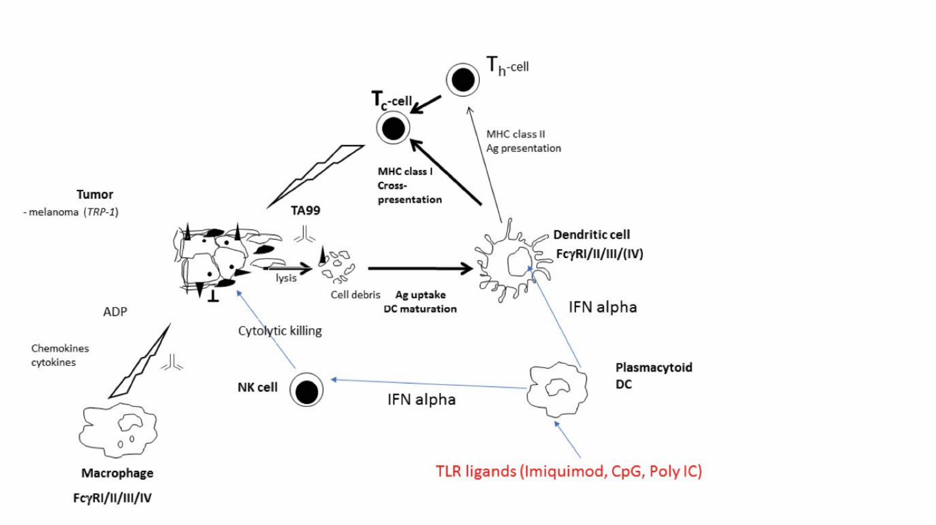

Figure 1: Schematic overview of the mode of action of the combination therapy of imiquimod and TA99 and how the mechanism could be when using previous studies to fill in the gap (31-35, 51).