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Mechanisms of melanoma-targeting antibody therapy in mice

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door

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Mechanisms of melanoma-targeting antibody therapy in mice

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CHAPTER 1



General introduction

Chapter 1

Introduction

Tumors do not only consist of malignant cells that have been transformed but also include a complex cellular network of recruited immune cells and other non-malignant cells for example fibroblasts and endothelial cells. The microenvironment of the tumor contains both immune cells that attack the tumor and immune cells that foster the tumor (1). Intercellular communication between immune cells and tumor cells are driven by a network of cytokines, chemokines and the structure of the tumor tissue (2-4)). The present development of tumor therapy focuses on this communication between the tumor and the immune system in the tumor microenvironment.

The immune system can be divided into an adaptive and innate immune system. T cells and B cells are the major components of the adaptive immune system. T cells are predominantly involved in cell-mediated immunity and are defined by having a unique T cell receptor (TCR). Gene rearrangements followed by a selection process result in a large repertoire of T cells, each expressing unique TCRs, together capable of recognizing a large variety of peptides presented by MHC on the surface of cells. Due to their education process, T cells can discriminate between antigens that are 'self' or 'non-self'. T cells can be classified into two main subclasses, CD4 and CD8 T cells. CD4 T cells or T helper (Th) cells recognize peptides on MHC class II molecules on the surface of B cells, macrophages, or dendritic cells. They direct immune responses through the release of different categories of cytokines. Th cells are crucial for antibody development (e.g. class switching, affinity maturation) and the activation of CD8 T cells as well as the induction of activation of phagocytes such as macrophages. An important subgroup of CD4 T cells is regulatory T cells which suppress immune responses through the release of inhibiting cytokines. CD8 T cells, or cytotoxic T cells, mediate their response by recognition of peptides presented by MHC class I molecules expressed by most cells of the body. A cytotoxic T cell that recognizes a mutated tumor-specific or a 'non-self' viral peptide presented in MHC class I with its TCR will respond by secreting enzymes like perforin and granzymes that lyse the target cell. CD8 T cells predominantly protect against viral infection and the outgrowth of cancer cells (Reviewed in (5))

B cells and their large repertoire of antigen-specific antibodies are central elements of humoral immunity as part of the adaptive immune system which protects against almost unlimited variety of pathogens. B cells express a B cell receptor (BCR). The secreted soluble form of the BCR, consisting of two heavy and two light chains, is called an antibody. The unique binding specificities of the BCRs are created, analogous to these of TCRs, by rearranging V_H , D_H , and J_H , segments of the heavy chain gene together with rearranging V_L - J_L of the light chain gene segments generates a B-cell antibody repertoire capable of recognizing more than 5×10^{13} different antigens. Within the germinal centers of lymph nodes and spleen, mature B cells increase the affinity for their antigen through a process called affinity maturation by randomly creating mutations in their BCR DNA segments (somatic hypermutation) and switch the class of their antibodies to become antibody-producing plasma cells. This process of DNA mutation is unique for B cells and specific for the antigen binding domain region of the rearranged antibody-encoding gene. Those B cells are selected in which hypermutation results in increased affinity for foreign antigens (affinity maturation). Antibodies can class switch from IgM to four different other classes: IgA, IgG, IgD, and IgE, as reviewed in (5). There are four distinct IgG subclasses that each differ in their downstream effector pathways (see below) which have a strong impact on the outcome of an antibody response. Affinity maturation and class switching are involved for T-cell dependent antigens, like bacterial proteins and take place in germinal centers of lymph nodes and spleen. CD4 T helper cells are needed for this germinal center reaction and are called Follicular helper T cells. In addition, there are also antibodies specific for T-cell independent antigens, like repetitive structures such as polysaccharides (Reviewed in (6)). Follicular helper T cells are antigen-experienced CD4 T cells within lymph nodes and spleen and are identified by their expression of CXCR5 chemokine receptor that drives homing into these germinal centers. They play a critical role in the selection and survival of B cells that later differentiate into plasma cells which secrete high-affinity antibodies (Reviewed in (6)).

Immunoglobulin binding receptors (FcR)

Cellular receptors for the constant Fc region of antibodies, the so-called Fc receptors (FcR), regulate and execute antibody-mediated responses (7). They link the antigen specificity of the adaptive immune response with the non-specific effector functions of cells of the innate immune system such as neutrophils, monocytes, and macrophages.

FcRs are widely expressed on the cells of the immune system. Different FcRs recognize different classes of antibodies (IgG, IgA, IgM, and IgE). Four different Fc receptors that are able to recognize IgG have been identified in rodents (FcγRI, FcγRIIB, FcγRIII and FcγRIV) (7). IgG binding α-chains of the three activating FcγRI, FcγRIII and FcγRIV are associated with a shared FcR γ chain, the signal transduction subunit that is also required for their cell surface expression. Deletion of the γ chain gene leads to the loss of antibody-mediated effector cell responses (8-12). The three activating FcγRs are counterbalanced by the inhibiting receptor FcγRIIB. These four FcγRs are expressed in different combinations on a variety of immune cells, mainly myeloid effector cells. NK cells only express FcγRIII, neutrophils express FcγRIII and FcγRIV while monocytes, DCs, and macrophages can express all four receptors (Figure 1). The four receptors have different affinity for the murine IgG subclasses IgG1, IgG2a, and IgG2b (Figure 2) (13). FcγRI is the only high-affinity receptor, binding monomeric IgG2a. Glycosylation of antibodies in the Fc region affects their affinity for the different FcγR, for example, fucose-free antibodies show higher binding to FcγRIV than antibodies bearing fucose (14). To investigate the role of Fc receptors in different antibody mediated functions *in vivo* a variety of FcγR knockout mice have been generated. In the past, the most commonly used mouse to study activating FcγR was a mouse deficient for the shared FcR γ chain (11). However, the FcR γ-chain is a promiscuous signal transduction subunit associated with at least nine other receptor complexes (15). Therefore, the phenotype of the FcR γ chain knockout mouse is not exclusively determined by the absence of activating FcγR. More recently, mice selectively lacking FcγRI (16), FcγRII (17,18), FcγRIII (19,20) and FcγRIV (21,22) or FcγRIII/IV (21,23), or FcγRI/III/IV (23) or all four FcγRs (19) have been generated and characterized.

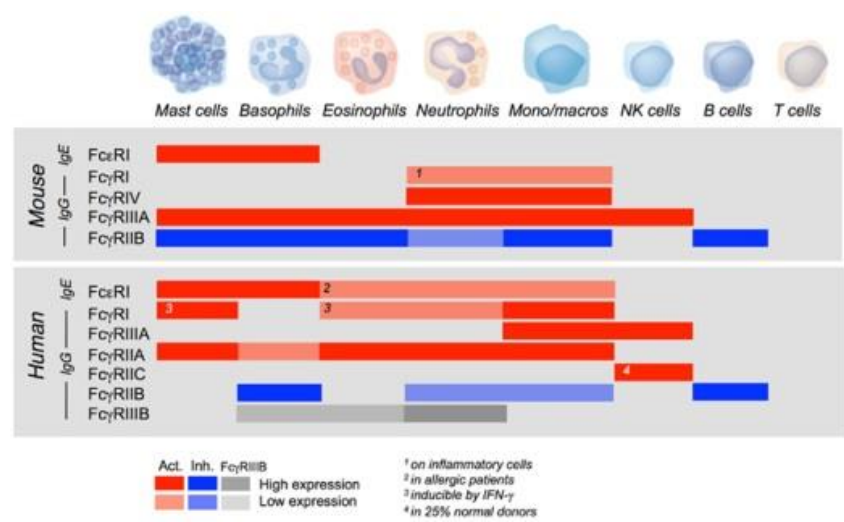


Figure 1: Expression of different mouse and human FcεR and FcγRI on different immune cells. Adopted from (24).

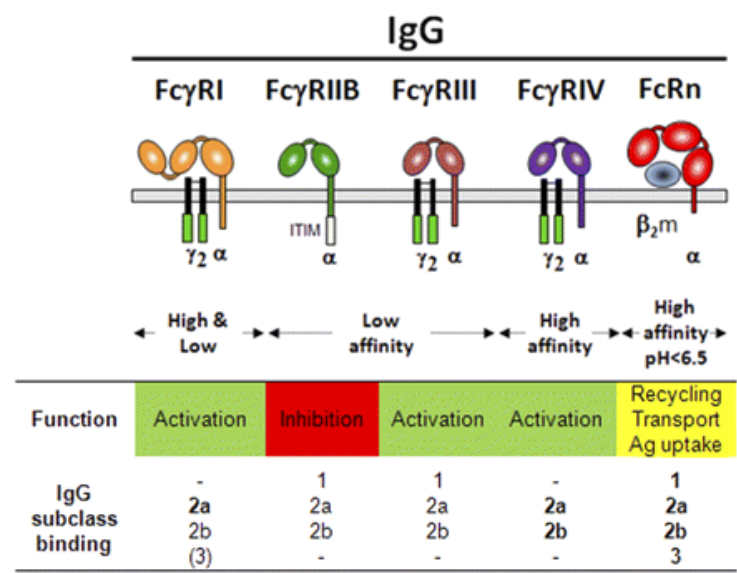


Figure 2: Affinity of mouse FcγR to different subclasses of IgG. Adopted from (25).

Differences in repertoires of FcγRs in mouse and man

Human FcγRs and mouse FcγRs have somewhat different classifications. The overall concept of activating FcγRs (FcγRI and FcγRIIA in human) associated with a dimer of the common γ-chain with an immune receptor tyrosine-based activation motif (ITAM) counterbalanced by a single chain inhibiting receptor (FcγRIIB) with an immune receptor tyrosine-based inhibiting motif (ITIM) in its cytoplasmic tail and one high-affinity FcγRI binding monomeric IgG is conserved between mice and primates. However, primates have acquired three additional FcγR genes by gene duplication: FcγRIIA, FcγRIIIB, and FcγRIIC. FcγRIIA and FcγRIIC are single chain transmembrane molecules with an ITAM motif in their cytoplasmic tail whereas FcγRIIIB is a glycosylphosphatidylinositol (GPI) anchor linked membrane receptor with no intracellular tail unable to signal however signaling via the induction of the translocation to the plasma membrane of nonreceptor tyrosine TEC kinases has been reported (26). In 90% of the human population, FcγRIIC is a pseudogene. Based on their expression patterns mouse FcγRIII and human FcγRIIA and mouse FcγRIV and human FcγRIII are considered as functional counterparts (Reviewed in (13)). In conclusion, the extrapolation of the results of the functional analysis of FcγR in mice to human must be approached with caution because of several striking differences between mouse and human FcγR.

Immunotherapy with tumor-targeting antibodies

Tumor-targeting antibody-based therapy against cancer was initially developed to directly target and eliminate malignant cells. Combination of Rituximab (anti-CD20) with chemotherapy resulted in increased survival of patients with various B cell lymphomas (27,28). CD20 is an antigen expressed by all B cells. Its function is not fully understood. The disadvantage of the anti-CD20 therapy is that all B cells are depleted resulting in the absence of a B cell responses during infection. Antibody-based therapy against solid tumors started with the approval of trastuzumab (anti-Her2) antibody to treat breast cancer as it improved disease-free survival, although it failed to improve overall patient survival (29), indicating that patients experienced a longer cancer-free period. Many therapeutic tumor targeting antibodies function in cancer treatment by affecting the signaling transduction of its target antigen. Binding of antibodies to either CD20 or Her2/neu can lead to apoptosis or cell cycle arrest of the cancer cells (30,31). This was

initially thought to be the main function of tumor targeting antibodies whereas now FcR or complement-mediated effector functions are known to be also important for their therapeutic efficacy (Reviewed in (32)). These effector functions include antibody dependent cell-mediated cytotoxicity (ADCC) (9), antibody dependent cell-mediated phagocytosis (ADCP) (33,34), trogocytosis (35), complement dependent cell-mediated phagocytosis (CDCP) (36), and complement-dependent cell-mediated cytotoxicity (CDCC) (37). Trogocytosis, ADCC, and ADCP depend all on FcRs. ADCC is initiated when FcR expressing cells recognize a target cell that has been opsonized with antibodies. ADCC is mediated by NK cells, although there are examples that other FcR-expressing cells such as monocytes, macrophages, eosinophils, neutrophils and basophils can also mediate this (38,39). Binding of the Fc tail of antibodies to FcγRs on effector cells results in crosslinking of the FcγRs leading to release of cytokines (such as IFNγ) and cytotoxic molecules like perforin and granzymes (40). ADCP is initiated when FcR-bearing cells like macrophages, monocytes, and neutrophils recognize the Fc domains of antibodies opsonizing a target cell (33). The opsonized cells are internalized by the phagocyte and degraded. Recent observations suggest that ADCP is the most important effector mechanism of antibodies (33,41). ADCP might constitute a link to adaptive immune responses. Antibody-mediated cross-presentation of tumor-derived peptides has been observed to induce protective immunity by cytotoxic CD8 T cells (42). Trogocytosis can be described as the process wherein cell surface molecules from tumor cells end up in the receiving cells which subsequently express these surface molecules on their own cell surface. Trogocytosis has now been shown to be mediated by FcRs on neutrophils and monocytes. This could have a detrimental effect as it decreases the expression of the targeted antigen on the tumor cell (reviewed by (43)).

Almost all antibodies used in the clinic are monoclonal antibodies (mAb). However, it has been reported that polyclonal antibodies recognizing multiple epitopes on EGFR were able to induce complement-mediated tumor cell killing whereas a monoclonal antibody was not (44). Polyclonal antibodies recognizing multiple epitopes could thus induce better complement-dependent lysis. A mixture of antibodies recognizing many different epitopes has been shown to induce increased complement activation when compared to monoclonal antibodies which recognize only one epitope (45). Xu et al showed that vaccination with an MCMV vector encoding the tumor-specific TRP2 protein could

protect against outgrowth of tumor cells of B16F10 mouse melanoma in an antibody-dependent manner (46).

Tumor targeting antibodies in mouse tumor models

The B16 transplantable cell line is one of the most commonly used tumor cell lines in mice as a model for melanoma. The cell line was derived from a spontaneously developed tumor in C57BL/6J mice. B16F10 is one of numerous subclones of B16 (Reviewed in (47)). Most studies with tumor targeting antibodies in the B16F10 tumor model are based on the use of mAb clone TA99, a hybridoma producing IgG2c mAb specific for Trp1 (gp75), an antigen expressed on the surface of B16F10 cells. Several publications demonstrated the efficacy of the TA99 antibody in a prophylactic setting, starting the mAb treatment on the same day as the injection of the tumor cells. Importantly, usage of TA99 as monotherapy is ineffective when used against previously established solid B16 tumors and similar observations have been reported with tumor-targeting antibodies in clinical settings (29). In several preclinical studies, it has been investigated whether tumor-targeting antibody therapy can be improved by combining it with a variety of immune stimuli. Combination of TA99 and Fc-IL2 with peptide vaccination and anti-PD-L1 or IFN α alone or poly I:C alone resulted in increased survival of B16F10 tumor-bearing mice (42,48,49). The therapeutic effect of these combinations depends on various immune effector cells such as neutrophils, macrophages, NK cells and CD8 T cells (42,48,49).

A few combination therapies have been tested to improve anti-Her2/neu therapy against breast cancer which was reported to require FcR (50). Synergism of anti-PD1 and anti-CD137 with anti-Her2 improved therapeutic efficacy (51). This treatment was dependent on IFN type I and II confirming studies using TA99 antibody in combination with IFN type I inducing compounds, such as poly I:C or directly administered interferon (48).

Engineered antibodies

One of the simplest ways to improve the efficacy of a tumor-targeting antibody is to use an IgG subclass which has the highest affinity for the activating Fc γ R and the lowest affinity for the inhibiting Fc γ R. In mice that is the IgG2a subclass while in humans it is

the IgG1 subclass (25,52). Other ways to improve the therapeutic efficacy of tumor-targeting antibodies is to engineer them to enhance their tumor-killing capability by increasing their interaction with complement. It has been shown that particular amino acid substitutions could increase the efficacy of ADCC 1000-fold and of ADCP 10-fold (34). Other amino acid changes could augment antibody mediated CDC to kill tumor cells (53).

Other methods to engineer mAbs are to create bispecific modalities that can recognize two different antigens on the same antibody. They can target immune cells with one arm of the antibody and tumor cells with the other arm. The development of Quadroma technology made it possible to somatically link antibodies and create bispecific antibodies (54). The first clinically approved bispecific antibody was the mouse-rat hybrid catumaxomab (55) which binds CD3 on T cells and Epcam on tumor cells. Cross-linking of CD3 activates T cells and leads to cytolytic response toward target cells (55). By its Fc region, Catumaxomab could as well recruit FcγR-bearing effector cells such as NK cells and macrophages into the tumor. In 2009 Catumaxomab was approved by EMA to treat malignant ascites in patients that have carcinomas positive for Epcam (55). Since Catumaxomab was engineered from mouse and rat antibodies, it induced unwanted neutralizing responses in patients, resulting in its clearance from the body, thereby limiting its application (56). This bispecific antibody was withdrawn from the market. Newer bispecific formats are based on small single chains lacking Fc domains. These are called BiTE (bispecific T cell engager) and are supposed to function in the same way as Catumaxomab except that they do not engage FcγR. The best known is BiTe is Blinatumomab which targets CD3 and CD19, a surface marker on most B cells. This therapeutic compound has been approved by the FDA and EMA for treating refractory B cell Acute Lymphocytic Leukemia (57). Efforts have been made to improve bispecific formats and many are being studied in clinical and preclinical settings, like DARTS (58), Duobody format (59), Crossmab format (60) and single chain triple body format (61-63). IgG4 antibodies naturally exchange Fab arms and this knowledge was exploited to optimize *in vitro* production of bispecific antibody formats (59). Several engineering strategies are available to prevent FcγR binding, for example, the LALA mutation (64) in the Fc tail of the antibody.

Outline of this thesis

In **chapter 2** of this thesis, we first describe the characterization of a newly generated mouse model that lacks all four FcγRs. FcγR have little or no role in the ontogeny and maintenance of the immune system and their role in regulating adaptive immunity appears redundant. In **chapter 3**, we intended to characterize the role of FcγRs in protection against B16F10 melanoma outgrowth by MCMV-TRP2 prophylactic vaccination. Polyclonal TRP2-specific antibodies induced by the vaccine prevented outgrowth of B16F10. This anti-tumor effect depended on FcγRs expressed by macrophages. In **chapter 4**, we aimed to improve the therapeutic efficacy of tumor-targeting antibodies by combination therapy. We found that a therapeutic combination of TA99 antibody, imiquimod (a Toll-like receptor 7/8 activator), and IL2 results in the delayed outgrowth of established B16F10 melanoma. This depended on FcγRs, macrophages and CD8 T cells. In addition, we showed that the numbers of intratumoral FcγR-expressing macrophages were increased in animals treated with this combination therapy. In **chapter 5**, we intended to analyze how the bispecific CD3xTA99, which combines the specificity for CD3 with that for the mouse melanoma Trp1 surface antigen, functions in the treatment mechanism. We showed that the delayed tumor outgrowth after administration depended on CD4 or CD8 T cells and promoted the migration of both macrophages and T cells into the tumor.

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CHAPTER 2

2

A Restricted Role for Fc γ R in the Regulation of Adaptive Immunity.

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A Restricted Role for FcγR in the Regulation of Adaptive Immunity

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By their interaction with IgG immune complexes, FcγR and complement link innate and adaptive immunity, showing functional redundancy. In complement-deficient mice, IgG downstream effector functions are often impaired, as well as adaptive immunity. Based on a variety of model systems using FcγR-knockout mice, it has been concluded that FcγRs are also key regulators of innate and adaptive immunity; however, several of the model systems underpinning these conclusions suffer from flawed experimental design. To address this issue, we generated a novel mouse model deficient for all FcγRs (FcγRI/II/III/IV^{-/-} mice). These mice displayed normal development and lymphoid and myeloid ontogeny. Although IgG effector pathways were impaired, adaptive immune responses to a variety of challenges, including bacterial infection and IgG immune complexes, were not. Like FcγRIIb-deficient mice, FcγRI/II/III/IV^{-/-} mice developed higher Ab titers but no autoantibodies. These observations indicate a redundant role for activating FcγRs in the modulation of the adaptive immune response *in vivo*. We conclude that FcγRs are downstream IgG effector molecules with a restricted role in the ontogeny and maintenance of the immune system, as well as the regulation of adaptive immunity.

Adequately defining the *in vivo* role of the receptors for IgG, FcγRs, is severely hampered, not only by the complexity of the FcγR gene family itself but also because of their functional redundancy with the complement system. FcγRs and complement link innate and adaptive immunity on two levels: they mediate the activation of downstream effector pathways of innate immune cells by Ag-specific IgG, and they are involved in the IgG immune complex (IC)-mediated regulation of adaptive immunity.

Four FcγRs have been identified in the mouse. The IgG binding α-chains of activating FcγRI, FcγRIII, and FcγRIV are associated with the FcR γ-chain, a signal transduction subunit that is also required for cell surface expression (1). The activating FcγRs are

counterbalanced by the inhibiting receptor FcγRIIb. The four FcγRs are expressed in different combinations on a variety of immune cells, primarily myeloid effector cells.

The *in vivo* role of FcγRs has been extensively studied by analyzing the phenotype of mice deficient for one or combinations of two or three FcγRs or the FcR γ-chain. By establishing a variety of disease models, such as arthritis, hemolytic anemia, anaphylaxis, and lupus-like disease in these knockout (KO) mice, we and others have shown that FcγRs play an important role in the downstream Ab effector pathways that drive pathogenesis in these diseases (2). However, by using Abs with a mutation in their Fc domain, which destroy FcγR binding without affecting interactions with complement, it has recently been shown that several

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; ADCP, Ab-dependent cell-mediated phagocytosis; ANA, anti-nuclear Ab; BM-DC, bone marrow-derived DC; CAIA, collagen Ab-induced arthritis; DC, dendritic cell; DNP-HSA, 2,4-dinitrophenylated human serum albumin; ES, embryonic stem; IC, immune complex; KO, knockout; LUMC, Leiden University Medical Center; WT, wild-type.

IgG downstream effector functions also can be mediated by complement (3).

Mice deficient in the early pathway components C1q, C3, and C4 and the complement receptors Cr1/Cr2 have impaired humoral responses to T cell-dependent and T cell-independent Ag (4–6), indicating that the complement system plays an important role in priming and regulating the adaptive immune response (7). Moreover, C1q-deficient mice spontaneously develop lupus-like disease. A series of observations suggests that FcγRs also play a role in priming and regulating adaptive immunity and the maintenance of immune tolerance. Ag-specific IgG1, IgG2a, and IgG2b enhance Ab and CD4⁺ T cell responses to soluble protein Ag via activating FcγRs, probably by increasing Ag presentation by dendritic cells (DCs) to Th cells (8). With Ag-specific IgG3, an IgG subclass not interacting with FcγR, this process is complement dependent (9). In FcR γ-chain-KO mice, immunized with the model Ag keyhole limpet hemocyanin, the delayed-type hypersensitivity response after challenge is significantly decreased compared with that in wild-type (WT) mice. Moreover, the secondary responses of CD4⁺ T cells to Ag and Ab formation were also reduced in these mice (10). These data suggest that activating FcγRs on APCs facilitate Ag presentation, resulting in efficient priming of Th cell responses *in vivo* in an IC-dependent manner that is required for a full-blown Ab response. We (11–13) and other investigators (14) have shown that soluble IgG ICs enhance cross-presentation by DCs, resulting in strong proliferation of Ag-specific CTLs. It is generally believed that FcγRs play an important role in this process (15).

Combined, these observations suggest an important role for activating FcRs in modulating the adaptive immune response. In addition, cross-linking the BCR with FcγRIIb by IgG ICs results in downregulation of Ab production. FcγRIIb-deficient mice develop higher Ab titers compared with WT mice (16). Moreover, it has been shown that FcγRIIb-deficient mice spontaneously develop lupus-like disease when backcrossed onto the C57BL/6 background (17).

In conclusion, many *in vivo* observations in WT and FcγR-KO mice suggest a pleiotropic role for FcγR in the immune system; however, many of these studies are flawed. Several studies were performed in FcR γ-chain-deficient mice. The FcR γ-chain is a promiscuous signal transduction subunit that is associated with at least nine other receptor complexes (18). Most FcγR-KO mice have been generated by gene targeting in 129-derived embryonic stem (ES) cells, followed by backcrossing onto the C57BL/6 background. We have shown that, after backcrossing, the remaining 129-derived sequences flanking the FcγRIIb-KO allele, including the hypomorphic autoimmune susceptibility *SLAM* locus (19), cause the autoimmune phenotype of the FcγRIIb-KO mouse on a mixed 129/C57BL/6 background, whereas the FcγRIIb deficiency only enhances the lupus-like disease (20). In many *in vivo* cross-presentation studies, bone marrow-derived DCs (BM-DCs) loaded *ex vivo* with IgG IC of the model Ag chicken OVA induced strong proliferation of adoptively transferred OVA Ag-specific T cells (11, 12, 14). However, the endogenous anti-OVA cytotoxic T cell response was very low (21). Moreover, *in vivo* cross-presentation of IgG IC-derived Ag was impaired in C1q-deficient mice (22).

To address these issues, we generated a novel C57BL/6 mouse model deficient for all four FcγRs, but expressing the FcR γ-chain, and analyzed its phenotype. Although, as expected, we could confirm that a variety of IgG downstream effector pathways were impaired, the overall characteristics of the immune system of these mice and WT control mice were very similar. Their B and T cell responses were not impaired. Like FcγRIIb-deficient mice,

FcγRI/II/III/IV^{−/−} mice developed higher Ab titers, but no auto-antibodies, with age. We conclude that, in contrast to complement, FcγRs have little or no role in the ontogeny and maintenance of the immune system. Their role in priming and regulation of the adaptive immune response appears to be redundant.

Materials and Methods

Mice

Mice were housed, and all experiments were performed, in the specific pathogen-free animal facilities of the laboratory animal facility (PDC) of Leiden University Medical Center (LUMC) or the University of Cambridge (*Salmonella* infection). 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 (23).

All mouse studies were approved by the animal ethics committee of 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*). C57BL/6J mice were purchased from Charles River. All FcγR-KO mice were generated in the transgenic mouse facility of LUMC (Fig. 1, Supplemental Fig. 1). The EIIaCre deleter strain (on C57BL/6J background, *n* = 20) was a kind gift of Dr. H. Westphal. The Flp deleter strain C57BL/6-Tg(CAG-flpe)36Itto/IttoRbrcc was purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were routinely checked for their genotype by PCR.

Cells and cell lines

B3Z is an OVA 257–264 (SIINFEKL)-specific H-2Kb-restricted costimulatory independent T cell hybridoma cell line. Thioglycollate-elicited (*i.p.*) macrophages from WT C57BL/6 and FcγRI/II/III/IV^{−/−} mice were isolated by abdominal lavage with 5 ml of PBS, 48 h after *i.p.* injection of 1.5 ml of 4% thioglycollate medium (Becton Dickinson, Mountain View, CA).

Collagen Ab-induced arthritis

Mice were injected *i.v.* with 4 mg of a mixture of four mouse anti-mouse collagen type II IgG mAbs (equimolar mix of M2139 [IgG2b] + CIIC1 [IgG2a] + CIIC2 [IgG2b] + UL1 [IgG2b]) on day 0, as well as with 100 µg of LPS from *Escherichia coli* 055:B5 (L2880; Sigma-Aldrich, St. Louis, MO) in 100 µl of PBS *i.p.* on day 3. On day 10, 4 mg of a mixture of four mouse anti-mouse collagen type II IgG mAbs was injected *i.p.* to boost the response. From day 7 onward, the development of arthritis was monitored daily in a blinded manner using a caliper to measure footpad swelling (24).

Anaphylaxis

Mice were sensitized by *i.v.* injection of 400 µg of pyrogen-free mouse anti-TNP IgG2a in saline and challenged 4 h later by *i.v.* injection of 1 mg of pyrogen-free 2,4-dinitrophenylated human serum albumin (DNP-HSA; A6661; Sigma-Aldrich) in saline (25). For the monitoring of blood pressure, mice were anesthetized by *i.p.* injection of ketamine (75 mg/kg), DEXDOMITOR (0.2 mg/kg), and atropine (0.5 mg/kg) in saline. After induction of anesthesia, the femoral artery and femoral vein were catheterized. The artery catheter was connected to the blood pressure monitor, and blood pressure was allowed to stabilize for ≥5 min. Subsequently, mice were injected with DNP-HSA *i.v.* via the femoral vein catheter. Blood pressure was monitored for ≥30 min after OVA injection, using a physiological pressure transducer (AD Instruments, Colorado Springs, CO). The signal was acquired and digitized in PowerLab, sampled at 200 Hz, and analyzed offline using LabChart (both from AD Instruments).

Ab-dependent cellular phagocytosis

WT C57BL/6 and FcγRI/II/III/IV^{−/−} mice were injected *i.p.* with 25 µg of rat IgG2b 2.43 Ab (produced *in-house*) to deplete CD8⁺ T cells. One day before and 3 d after the depleting-Ab injection, CD8⁺ T cell numbers were analyzed in blood using flow cytometry and quantified as a percentage of total CD3⁺ cells. As determined with surface plasmon resonance, rat IgG2b Ab has a binding preference for activating mouse FcγR (activating/inhibitory FcγR binding = 40) (26).

In vitro uptake and cross-presentation of IC-derived Ag

In vitro uptake and cross-presentation of OVA IC-derived Ag by DCs were studied by preforming complexes of OVA and rabbit polyclonal anti-OVA Ab (Cappel) at a 1:30 ratio and incubating 5 µg of these complexes with 25,000 BM-DCs in normal DC-conditioned medium, as described (27).

Rabbit IgG binds to all mouse FcγRs (27, 28). Alexa Fluor 488-labeled OVA was used for uptake and was measured using flow cytometry, with or without quenching of extracellularly bound fluorescent OVA by trypan blue (Sigma-Aldrich). For cross-presentation, 25,000 BM-DCs were incubated with 50,000 B3Z cells. B3Z cells recognize the minimal SIINFEKL OVA-CTL epitope in MHC class I. Recognition leads to

upregulation of the transcription factor NFAT, which activates a *LacZ* reporter gene by binding to its IL-2 promoter (29). After overnight incubation with BM-DCs, B3Z cells were incubated with a lysis buffer containing CPRG substrate for β-gal (PBS + 1% 9 mg/ml CPRG + 0.9% 1 M MgCl₂ + 0.125% Nonidet P-40 + 0.71% 14.3 M 2-ME) at 37°C until the color reaction had progressed sufficiently for readout in a plate reader measuring OD

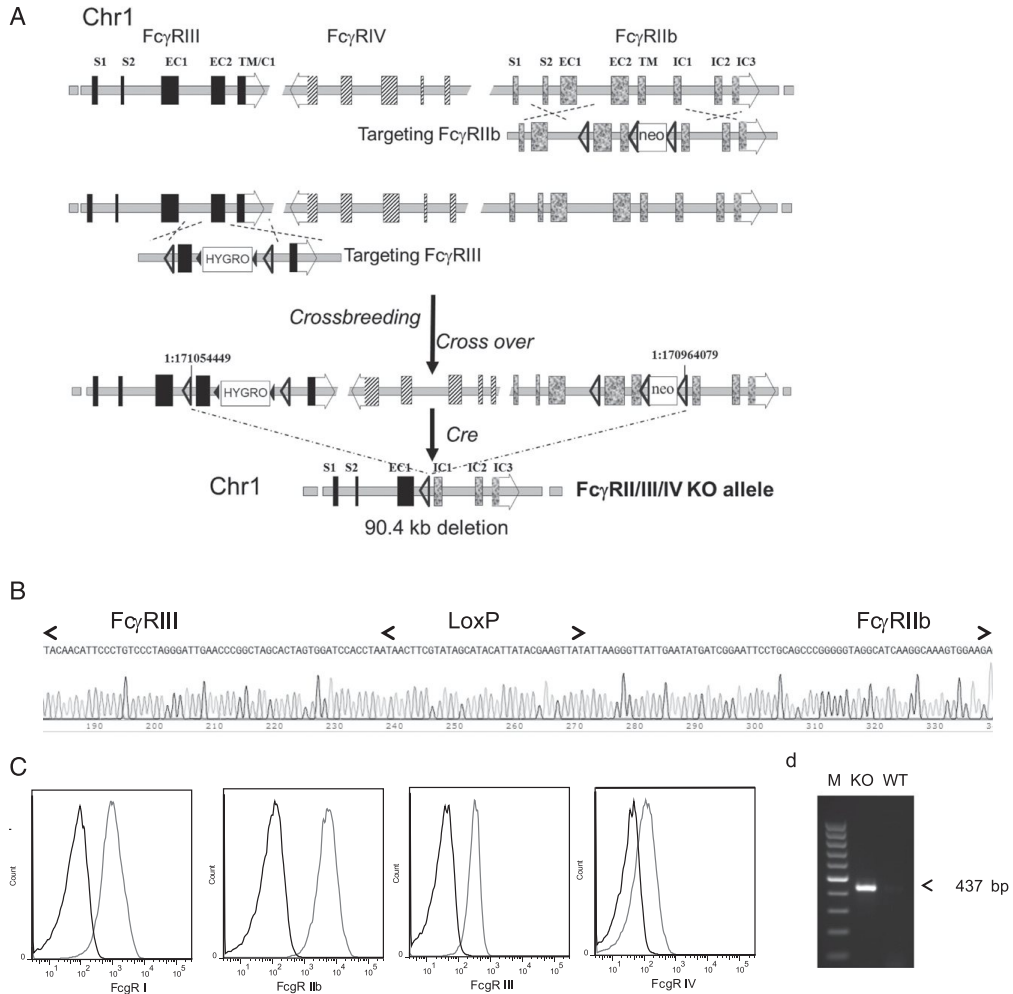


FIGURE 1. Generation of the FcγRII/III/IV^{-/-} mouse strain. FcγRIIb^{fl/fl} mice (14) were crossed with FcγRIII^{fl/fl} mice (Supplemental Fig. 1). Offspring were selected for cross-over between both floxed alleles. By crossing FcγRIIb^{fl/fl}/FcγRIII^{fl/fl} mice with the EIIaCre deleter strain, a 90.4-kb fragment between the two most distant LoxP sites, containing the main part of the FcγRIIb and FcγRIII genes and the complete FcγRIV gene, was removed, resulting in an FcγRII/III/IV-KO allele. The presence of the deletion was confirmed by PCR and DNA sequencing. FcγRII/III/IV^{-/-} mice were crossed with our previously generated FcγRI^{-/-} mice (16), and FcγRII/III/IV^{-/-} offspring were selected. The absence of all four FcγR was confirmed by FACS analysis. **(A)** From top to bottom are depicted the genomic structure of the WT FcγRIIb/FcγRIV/FcγRIII gene cluster on chromosome 1 (Chr1), the gene-targeting strategy for the generation of the floxed FcγRIIb and FcγRIII genes, and the genomic structure after the cross-over between the two floxed genes and, subsequently, after Cre-mediated recombination. The locus is shown in reverse orientation in relation to the chromosomal nucleotide numbering. The exact location of the borders of the deletion (NC_000067.6:g.171054449_170964079del according to HGVS nomenclature) on Chr1 are depicted based on the mouse reference genome build GRM38.p3 (C57BL/6J) provided by the Genome Reference Consortium. **(B)** Core sequence flanking the remaining LoxP site within the 437-bp PCR fragment. **(C)** Flow cytometry of thioglycollate-elicited peritoneal cells from FcγRII/III/IV^{-/-} mice (black lines) and WT C57BL/6 mice (gray lines) stained with fluorescently labeled Abs specific for F4/80 and CD11b and Abs specific for the different FcγRs, as indicated. **(D)** Agarose gel electrophoresis of the unique PCR fragment bridging the 90.4-kb deletion. By using an FcγRIII-specific "Geno Fw" 5'-GAGGGCATCC-GATTTCATTA-3' primer, an FcγRIIb-specific "Null B Rev" 5'-GCTTCATTGACCTGCCTAC-3' primer, and genomic DNA from an FcγRII/III/IV^{-/-} mouse as a template, a unique 437-bp fragment with the remaining LoxP site was synthesized. M, 100-bp ladder.

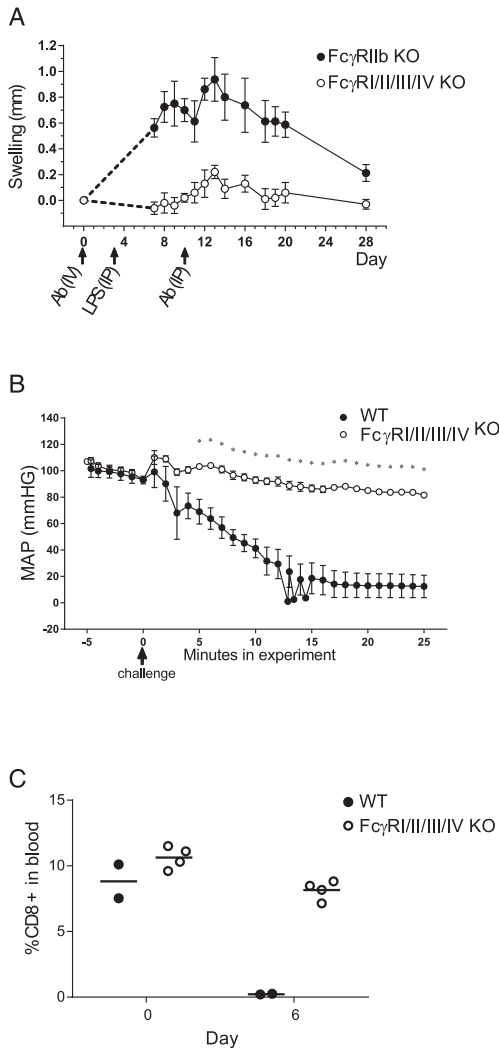


FIGURE 2. IgG downstream effector functions are impaired in Fc γ RII/III/IV $^{-/-}$ mice. **(A)** CAIA. Footpad swelling was measured, in mice of each phenotype, using a caliper. The average of combined left and right footpad swelling of the forepaws was plotted and expressed as the mean (+ SEM) increase in footpad thickness. The area under the curve was calculated per mouse from day 7 until day 28 ($n = 5$ mice per group). One representative experiment of two performed is shown. The response of Fc γ RII/III/IV $^{-/-}$ mice was significantly lower compared with the response of Fc γ RIIb $^{-/-}$ mice. $p = 0.0159$, Mann-Whitney U test. **(B)** Passive systemic anaphylaxis. Time course of blood pressure, shown as mean arterial pressure (MAP) + SEM, in Fc γ RII/III/IV $^{-/-}$ and WT C57BL/6 mice passively sensitized by i.v. injection of mouse anti-TNP IgG2a and challenged 4 h later with DNP-HSA ($n = 6$ mice per group). Each time point was analyzed by a separate t test, and the curves are significantly different ($p < 0.01$) from 5 min onward, as indicated by an asterisk (*). **(C)** ADCP. Mice were injected with CD8-depleting Ab (2.43). The number of CD8 $^{+}$ T cells in blood was determined before and after Ab injection by flow cytometry and is depicted as the percentage of CD8 $^{+}$ /CD3 $^{+}$ cells in the total lymphocyte (Figure legend continues)

at 590 nm. A peptide with the minimal OVA epitope SIINFEKL (100 ng/ml in PBS) that directly binds to MHC class I was used as a positive control, and unstimulated D1 cells [a DC line (27)] were used as negative controls.

In vivo cross-presentation of IC-derived Ag

CD8 $^{+}$ T cells were isolated from spleen and lymph nodes from OT-I/CD45.1 mice with a BD Mouse CD8 T Lymphocyte Enrichment Set and labeled with CFSE. Three million OT-I T cells were injected i.v. in recipient mice. One day later, 200 μ g of rabbit polyclonal anti-OVA Ab (Cappel) or nonspecific rabbit serum (negative control) was injected i.v., followed 30 min later by 5 μ g of OVA (Worthington Biochemicals) or peptide (synthesized in-house) with the minimal SIINFEKL OVA epitope (positive control). Three days later, mice were sacrificed, spleens were isolated, and proliferation of CFSE-labeled OT-I T lymphocytes was analyzed in single-cell suspensions by flow cytometry gated on CD8 $^{+}$ and CD45.1 $^{+}$ cells.

Quantitation of IC clearance

Age- and weight-matched naive mice received an i.v. injection of 100 μ g of rabbit IgG anti-OVA (Cappel), followed 15 min later by an i.v. injection of 5 μ g of Alexa Fluor 488/647-labeled OVA (Life Technologies). At the indicated time points, blood was drawn, and serum was collected. Five microliters of serum was mixed with sample buffer, heated at 95°C for 5 min, and loaded on SDS/PAGE gels. Fluorescent OVA was quantified directly from the SDS/PAGE gels using a Typhoon 9410 Variable Mode Imager (GE Healthcare Bio-Sciences) and ImageQuant TL 8.1 software (GE Healthcare Life Sciences).

At different time points after injection, mice were sacrificed and a single lobe of liver was isolated and imaged by IVIS Spectrum (PerkinElmer) using excitation at 605 nm and measuring emission at 680 nm with an exposure time of 2 s.

Flow cytometry

Single-cell suspensions were made from spleen, thymus, lymph nodes, bone marrow, and from lavage of the peritoneal cavity 24 h after injection of 1.5 ml of thioglycollate. For analysis of myeloid cells from spleen, organs were incubated for 30 min with Liberase (Sigma-Aldrich), according to the manufacturer's protocol. Cells were blocked with 10% normal mouse serum. 7-Amino-actinomycin D (7-AAD; Life Technologies) was used to exclude dead cells.

Abs for the following surface markers were used in this study: CD11c (clone HL3), CD8b (clone 53-5.8), CD19 (clone 1D3), CD90.1 (clone H1S51), and Fc γ RI (clone X54-5/7.1) (all from Becton Dickinson); CD3e (145-2c11), B220 (RA3-6B2), and CD45.2 (clone 104) (all from eBioscience); CD4 (clone RM4-4), F4-80 (clone BM8), Ly6C (HK1.4), and Ly6G (clone 1A8) (all from BioLegend); Fc γ RIIb (clone Ly17.2, produced in-house); Fc γ RIII (clone 275003; R&D Systems); and Fc γ RIV (clone 012; Sino Biological).

Serum levels of IgG subclasses

Serum was collected from 10-mo-old naive Fc γ RII/III/IV $^{-/-}$ and WT C57BL/6 mice. ELISA was performed with goat anti-mouse IgG (Becton Dickinson) and goat anti-mouse IgG, IgG1, IgG2a, or IgG2b HRP (SouthernBiotech, Birmingham, AL) and TMB substrate (Dako). The reaction was stopped with 1 M H₂SO₄, and absorption was measured at 450 nm.

For Ag-specific Ab titers in serum, mice were immunized s.c. with 50 μ g of TNP-BSA in 100 μ l of CFA (1:1 emulsion with PBS) at day 0 and were boosted at days 14 and 28 with 25 μ g of TNP-BSA in 100 μ l of IFA (1:1 emulsion with PBS). Ab titers in sera collected at day 36 were assessed with ELISA. Streptavidin-coated 96-well plates were incubated with 1 nmol/ml biotin-BSA and blocked with 5% nonfat milk. Secondary Ab was goat anti-mouse HRP. Substrate ABTS (code number S1599; Dako) was added, and absorption was measured at 415 nm. Serum levels of IgG class autoantibodies were determined using ELISA plates coated with 5 μ g/ml dsDNA, 5 μ g/ml histone (both from Sigma-Aldrich), or 4 μ g/ml chromatin, as previously described (30). Serum levels of binding activities against dsDNA, histone, and chromatin were expressed in units by reference to a standard curve obtained by serial dilution of a standard serum pool from (NZB $\times$ NZW) F_1 mice aged >8 mo, containing 1000 U activities per milliliter. Serum levels of IgG Abs were measured using HRP-conjugated anti-mouse IgG secondary Abs (SouthernBiotech) and were detected at OD 450 nm using TMB Substrate Reagent (BD).

population. Data shown are from one of two experiments with similar results ($n = 4$ Fc γ RII/III/IV $^{-/-}$ and $n = 2$ WT mice per group). $p = 0.15$ at day 0, $p = 0.0001$ at day 6 versus WT C57BL/6 mice, t test.

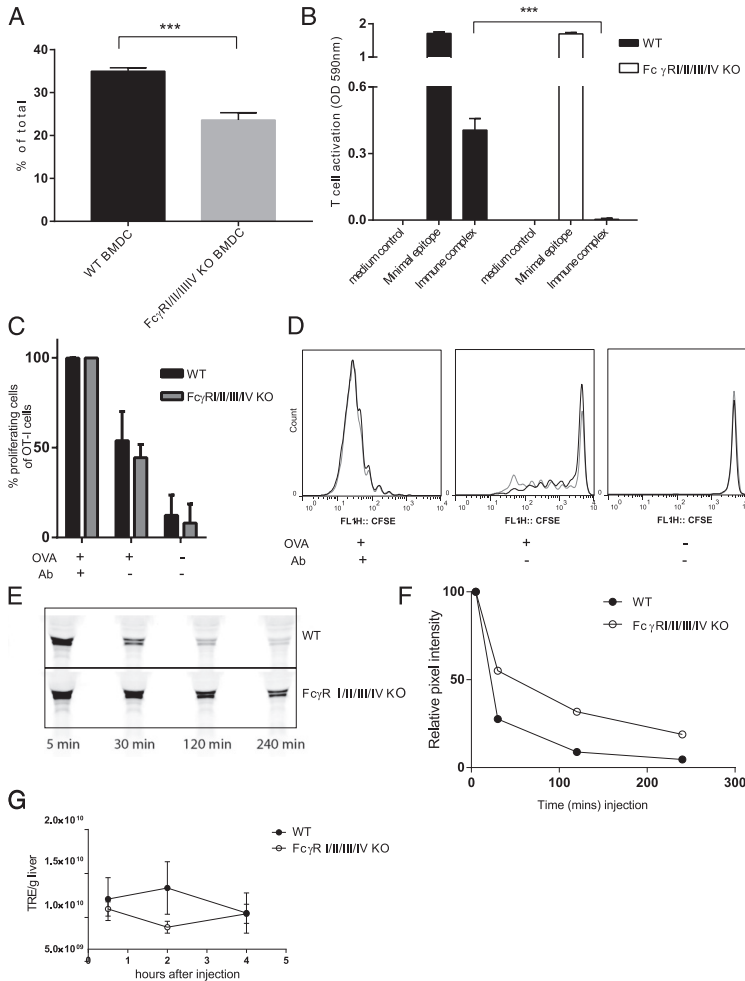


FIGURE 3. FcγR involvement in the cross-presentation of IgG IC-derived Ag. **(A)** Uptake of IgG IC-derived Alexa Fluor 488-labeled Ag by BM-DCs from WT and FcγRII/III/IV^{-/-} mice, as measured by flow cytometry (data are mean + SD of $n = 3$ samples). Extracellular binding was quenched by the addition of trypan blue. Depicted is the percentage of Alexa Fluor 488⁺ cells of the total cell count. One representative experiment of two experiments performed is shown. **(B)** BM-DCs from FcγRII/III/IV^{-/-} and WT C57BL/6 mice were incubated with OVA-IgG IC and subsequently cocultured with T cell hybridoma B3Z, which recognized an OVA-CTL epitope in MHC class I. Recognition leads to activation of the *LacZ* reporter gene, which was measured with a β-galactosidase assay, and analyzed as absorption of light at OD 590 nm. Minimal SIINFEKL OVA epitope was included as an MHC class I-loaded positive control in both DC types. Data are mean + SEM ($n = 4$ samples per group). **(C and D)** WT C57BL/6 and FcγRII/III/IV^{-/-} mice were injected with CFSE-labeled OT-I T cells and subsequently injected with OVA, with or without anti-OVA IgG. In vivo cross-presentation was determined by analyzing CFSE dilution of OT-I cells using flow cytometry. Depicted are the percentage of proliferating OT-I cells (CFSE fluorescence is diluted at least once) of total OT-I gated cells as mean of group + SD (**C**) and representative CFSE plots (**D**). Data shown are from one of two experiments with similar results ($n = 5$ mice per group). $p = 0.63$ for OVA+Ab, $p = 0.15$ for OVA alone, $p = 0.73$ for naive mice versus WT C57BL/6 mice, t test. Western blot analysis of the presence of Alexa Fluor 488-labeled OVA in the serum of mice at different time points after i.v. injection of the OVA anti-OVA IgG IC (**E**) and quantification of fluorescent OVA in Western blot samples (**F**). Data are representative samples from three mice per experiment. Three experiments with similar results were performed. **(G)** At different time points after injection, mice were sacrificed, and a single lobe of liver was isolated and imaged. Signal quantification of Alexa Fluor 488-labeled IgG ICs was performed. The fluorescent signal is shown as the total radiant efficiency (TRE), expressed in photons per second ($\mu\text{W}/\text{cm}^2$). The TRE/g liver in the time is shown. Data are from one of two experiments with similar results ($n = 3$ mice per group). *** $p < 0.001$, t test.

Infection with *Salmonella* live vaccine

Mice were infected with *Salmonella typhimurium* SL3261, an attenuated *aroA* strain (31). Live bacteria for parenteral immunization were prepared from a 16-h static culture of *S. typhimurium* SL3261 in LB Broth, diluted 1/100 in PBS, and administered by i.v. injection into the tail vein ($\sim 10^6$ CFU per mouse). The actual inoculum dose was determined by plating dilutions on LB Agar.

Salmonella induces Th1 T cell responses. IFN- γ and IL-2 production correlate well with Th1 responses to *Salmonella*. T cell-stimulation assays and cytokine measurement postinfection with *Salmonella* live vaccine were performed as follows: CD4⁺ T cells were positively enriched from spleens using magnetic bead-conjugated Abs (Miltenyi Biotec), according to the manufacturer's instructions. Purity was assessed by flow cytometry on a FACSCalibur (Becton Dickinson). CD4⁺ T cells were stimulated with *Salmonella* Ag or anti-CD3 ϵ (clone 145-2C11) and anti-CD28 (clone 37.51; both from eBioscience) as positive control in the presence of mitomycin C-treated (25 mg/ml; 37°C for 30 min) splenic APCs. *Salmonella* Ag was alkali-treated *S. typhimurium* SL1344. The levels of IFN- γ and IL-2 produced at 24 and 72 h were determined using DuoSet ELISA kits (R&D Systems), according to the manufacturer's instructions.

Postinfection with *Salmonella* live vaccine, anti-LPS Abs were detected by ELISA as follows: *S. typhimurium* LPS (Sigma-Aldrich) was dissolved in water containing sodium deoxycholate (0.5% w/v). Microtiter plates (Greiner Bio-One) were coated overnight at 37°C with 5 μ g/ml LPS in carbonate buffer. Serum sample serial dilutions in PBS-Tween + 1% BSA were applied in duplicate and incubated. Plates were washed, and total Ab was detected with HRP-conjugated goat anti-mouse Ab (SouthernBiotech); detection was with SIGMAFAST OPD substrate (Sigma-Aldrich), with absorbance read using a FLUOstar Omega (BMG Labtech).

Complement analysis

Mice were euthanized using CO₂, and plasma samples were collected via heart puncture and put directly on ice. Plasma was collected with syringes pretreated with EDTA and tubes with a final EDTA concentration of 10 mM. Blood was kept on ice for 30–120 min and centrifuged twice at 3000–5000 $\times g$ for 10 min at 4°C. Samples were pooled, aliquoted into single-use batches, and stored at -80°C .

Measurement of functional pathway activities in plasma of mice was performed, as described (32). In brief, complement activation was induced by incubation of serial dilutions in ELISA plates (Nunc MaxiSorp plates; Thermo Fisher Scientific) coated with human IgM, mannan, and LPS to induce the classical pathway, lectin pathway, and alternative pathway, respectively. Activation of complement was quantified at the level of C3 deposition, using an Ab directed against mouse C3b/C3c/iC3b, or at the level of C9 deposition, using a rabbit anti-mouse C9 polyclonal Ab. Complement activity in the experimental samples was calculated using CD1 serum as a standard (100 AU/ml).

Complement factors in plasma were quantified using specific sandwich ELISAs. C3 was quantified in the form of C3b/C3c/iC3b, as previously described. C1q was quantified using rabbit anti-mouse C1q polyclonal Ab (33). Mouse properdin was measured using coating with an anti-mouse properdin mAb and detection with rabbit anti-mouse properdin polyclonal Ab-DIG, whereas C6 and C9 were quantified using rabbit polyclonal anti-mouse C6 and rabbit polyclonal anti-mouse C9 (34).

Histology

Complete necropsy was performed following standard procedures. Tissues were fixed in 4% neutral buffered formalin, embedded in paraffin, sectioned at 5 μ m, stained with H&E, and evaluated by light microscopy. Histopathological analysis was performed by a veterinary pathologist certified by the European Board of Veterinary Specialisation. All main organs were

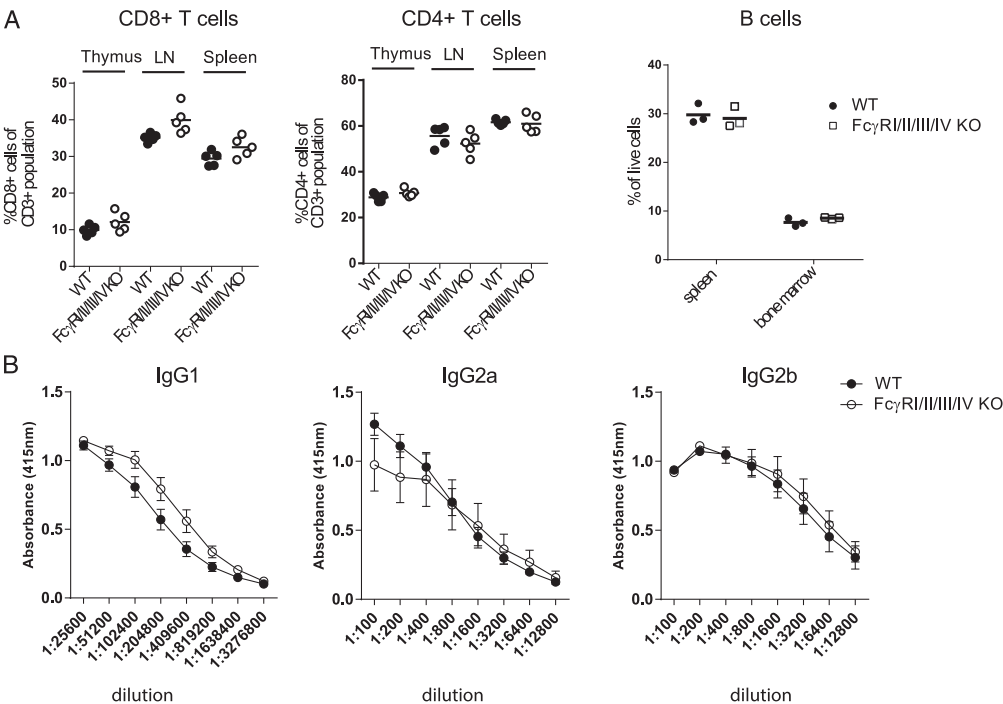


FIGURE 4. Adaptive immune system is normal in Fc γ RII/III/IV^{-/-} mice. **(A)** Lymphoid organs were harvested from 2-mo-old Fc γ RII/III/IV^{-/-} and WT mice. Single-cell suspensions were labeled with fluorescent Abs and analyzed using flow cytometry. Each circle represents an individual mouse. Data are from one of two experiments with similar results. **(B)** Mice were immunized three times with TNP-BSA. Serum samples were taken 8 d after the first boost and 7 and 12 d after the second boost. Titers of anti-BSA Abs were determined with ELISA. Data from day 12 are shown. Other time points showed similar results. Data are mean + SEM ($n = 8$ mice per group).

analyzed. Light microscopy photographs were taken with a DP26 Olympus camera.

Metabolic parameters

Twelve-week-old mice were weighed, and lean and fat mass was assessed by magnetic resonance imaging-based body composition analysis (Echo MRI; Echo Medical Systems, Houston, TX). Blood was drawn from the tail vein of overnight-fasted mice into capillary tubes coated with Paraoxon (Sigma-Aldrich). After centrifugation, plasma was collected, and triglyceride, total cholesterol, free fatty acid, glucose, and insulin levels were determined using commercially available kits ([1488872 and 236691] Roche Molecular Biochemicals, Indianapolis, IN; [NEFA-C] Wako Chemicals, Neuss, Germany; [ab83390] Abcam, Cambridge, U.K.; InstruChemie, Delfzijl, the Netherlands; and Crystal Chem, Elk Grove Village, IL, respectively). Indirect calorimetry measurements were performed using metabolic cages (LabMaster System; TSE Systems, Bad Homburg, Germany), as previously described (35).

FcγR expression during embryonic development

Total RNA was extracted using QIAzol (5346994; QIAGEN). One microgram of total RNA was used for reverse transcription with the ReverAid H Minus First Strand cDNA Synthesis Kit (K1632; Thermo Fisher Scientific). Quantitative RT-PCR was performed in triplicate on a C1000 Touch Thermal Cycler with SYBR Green (170-8887; both from Bio-Rad). Data were normalized to β-actin. The following primers were used: β-actin forward: 5'-GGCTGTATCCCTCCATCG-3'; β-actin reverse: 5'-CCAGTTGGTAACAATGCCATGT-3'; FcγRI forward: 5'-AAGTGGCTGGTCCCGAGTC-3'; FcγRI reverse: 5'-CTGCAGCCTGTGATTTC-3'; FcγRIIb forward: 5'-AATTGTGGCTGCTGTCAGT-3'; FcγRIIb reverse: 5'-GTTCTCTGGGAGAGCTGGA-3'; FcγRIII forward: 5'-TGGGGACTACTACTGCAAGG-3'; FcγRIII reverse: 5'-AGAAATAAAGGCCGTGTC-3'; FcγRIV forward: 5'-TGGAATGTACAGGTGCCAGA-3'; and FcγRIV reverse: 5'-TTCCGTACAGGCTGTTTTC-3'.

Results

Generation of the FcγRI/II/III/IV quadruple-KO mouse model

To overcome the drawbacks of the existing FcγR-KO mouse models, we generated a novel mouse model on the C57BL/6 background that is deficient for all four FcγR ligand-binding chains while maintaining the promiscuous Fcγ signal transduction subunit. To this end, we crossed a newly generated mouse model with a 90.4-kb deletion on chromosome 1 deficient for the *FcγRI/II/III/IV*^{-/-} gene cluster (Fig. 1) with our previously generated mouse model with deletion of the FcγRI gene (25) located on chromosome 3 (Fig. 1, Supplemental Fig. 1). FcγRI/II/III/IV quadruple-KO (FcγRI/II/III/IV^{-/-}) offspring developed normally and showed normal breeding characteristics. The phenotype of the FcγRI/II/III/IV^{-/-} mouse was analyzed in a series of in vitro and in vivo assays.

IgG downstream effector functions are impaired in FcγRI/II/III/IV^{-/-} mice

To confirm that the novel FcγRI/II/III/IV^{-/-} mouse model had impaired known FcγR-dependent IgG downstream effector functions, we studied IgG collagen Ab-induced arthritis (CAIA), IgG-induced passive systemic anaphylaxis, and IgG-induced Ab-dependent cell-mediated phagocytosis (ADCP) in these mice. As expected, FcγRI/II/III/IV^{-/-} mice were almost completely resistant to CAIA initiated by i.v. injection of a mixture of four anti-collagen IgG Abs (Fig. 2A). CAIA cannot be induced easily in WT C57BL/6 mice, whereas FcγRIIb^{-/-} mice are more sensitive (36). Therefore, we compared FcγRI/II/III/IV^{-/-} mice, which lack FcγRIIb, with FcγRIIb^{-/-} mice. In contrast to FcγRIIb^{-/-} mice, FcγRI/II/III/IV^{-/-} mice showed little footpad

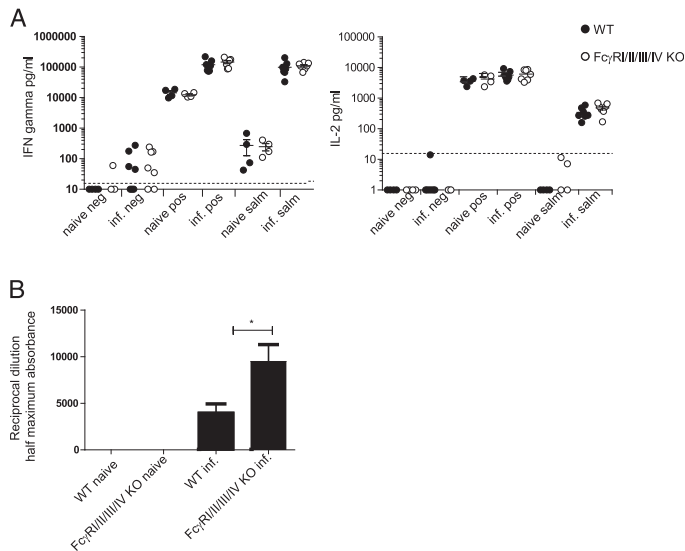


FIGURE 5. T and B cell responses to *Salmonella* infection were similar in FcγRI/II/III/IV^{-/-} and WT control mice. **(A)** CD4⁺ T cells were positively enriched from splenocytes of groups of seven WT C57BL/6 and seven FcγRI/II/III/IV^{-/-} mice infected 10 wk earlier with *S. typhimurium* SL3261. Groups of four WT C57BL/6 and four FcγRI/II/III/IV^{-/-} naive mice were also included in the experiment. The cells from individual mice were exposed to *Salmonella* Ag (salm), anti-CD3 and anti-CD28 (pos) as a positive control, or medium (neg) as a negative control. IFN-γ (left panel) and IL-2 (right panel) were measured in the supernatants by ELISA after 72 and 24 h, respectively. Data of one representative experiment of two performed are shown. Statistical analysis using ANOVA did not show significant differences between WT C57BL/6 and FcγRI/II/III/IV^{-/-} mice. **(B)** Anti-*S. typhimurium* LPS Abs were measured by ELISA in the sera of groups of five WT C57BL/6 and five FcγRI/II/III/IV^{-/-} mice, infected as in (A). Groups of four naive mice were included as controls. Ab titers are expressed as the reciprocal of the dilutions, giving a reading equal to half of the maximal absorbance. Data of one representative experiment of two performed are shown. **p* < 0.05, *t* test.

swelling, which confirms our previous results with K/BXN serum-induced arthritis (37).

Fc γ RII/III/IV^{-/-} mice were also resistant to passive systemic anaphylaxis, whereas WT C57BL/6 mice were not. This was induced by challenging mice, which were sensitized by i.v. injection of IgG2a anti-TNP, with DNP-HSA (Fig. 2B). The in vivo phagocytosis of CD8⁺ T cells by ADCP after i.p. injection of rat IgG2b anti-CD8 Ab was completely abrogated in Fc γ RII/III/IV^{-/-} mice (Fig. 2C). Together, these results confirm that, in our Fc γ RII/III/IV^{-/-} mouse model, IgG downstream effector functions are strongly impaired in a variety of in vivo experimental IgG-induced inflammation models.

In vivo cross-presentation of soluble IgG IC-derived Ag is normal in Fc γ RII/III/IV^{-/-} mice

It has been demonstrated, with a variety of in vitro and in vivo experiments, that Fc γ Rs on APCs facilitate the presentation of soluble IC-derived Ag to cytotoxic T cells: the process of cross-presentation (12, 14, 15). In accordance with these observations, the uptake of fluorescently labeled OVA-IgG ICs by DCs from Fc γ RII/III/IV^{-/-} mice was strongly impaired in vitro compared with the uptake by DCs from WT C57BL/6 mice (Fig. 3A). Moreover, as shown in Fig. 3B, the in vitro presentation of SIINFEKL peptide processed from OVA ICs to B3Z hybridoma

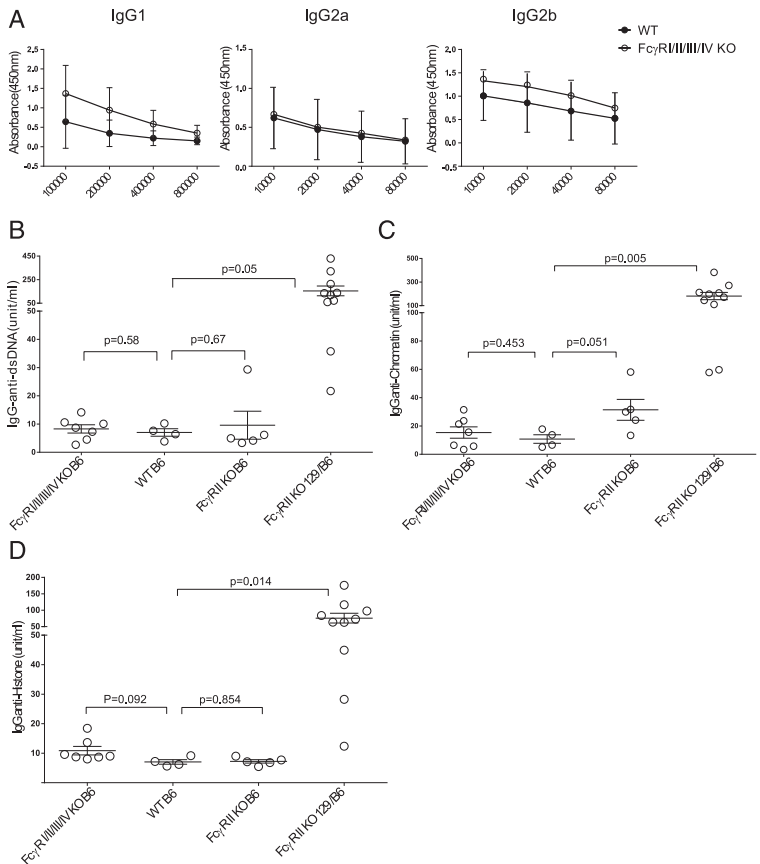
cells by Fc γ RII/III/IV^{-/-} BM-DCs was strongly inhibited. SIINFEKL synthetic peptide was used as control, and it was presented by BM-DCs from Fc γ RII/III/IV^{-/-} and WT C57BL/6 control mice with similar efficiency, indicating that MHC class I expression was comparable between genotypes. These data show that Fc γ Rs are required for the in vitro IC uptake and subsequent MHC class I-restricted presentation of IC-derived peptides.

Surprisingly, proliferation of adoptively transferred OT-1 CD8⁺ T cells was indistinguishable between Fc γ RII/III/IV^{-/-} and WT C57BL/6 control mice. No difference in CFSE division was observed after activation by ICs, which were formed in situ by i.v. administration of OVA and, subsequently, anti-OVA Abs (Fig. 3C, 3D). Compared with WT C57BL/6 mice, Fc γ RII/III/IV^{-/-} mice showed a delay in the clearance of IgG ICs from the circulation (Fig. 3E, 3F), whereas the uptake of IgG ICs by the liver appeared to be decreased (Fig. 3G). These observations imply that, in vivo, Fc γ Rs are dispensable for cross-presentation of IgG IC-derived Ag, but they are involved in the clearance of IgG ICs from the circulation.

Adaptive immunity is not impaired in Fc γ RII/III/IV^{-/-} mice

To further characterize the adaptive immune system in Fc γ RII/III/IV^{-/-} mice, the B and T lymphocyte compartments were analyzed by flow cytometry using a panel of fluorescently labeled Abs specific

FIGURE 6. IgG titers in older Fc γ RII/III/IV^{-/-} and WT control mice. (A) IgG1, IgG2a, and IgG2b titers were determined in sera of Fc γ RII/III/IV^{-/-} and WT C57BL/6 mice by ELISA (*n* = 3 mice per group). One representative experiment of two performed is shown. IgG anti-dsDNA Ab titers (B), IgG anti-chromatin titers (C), and IgG anti-histone titers (D) were determined by ELISA using anti-mouse γ -chain-specific secondary Abs. Each circle represents a mouse. Data are mean $\pm$ SEM with respective *p* values.



for B and T lymphocyte surface markers. No differences in the percentages of CD8⁺ and CD4⁺ T cells in the thymus, lymph nodes, or spleen were found between WT C57BL/6 and FcγRI/II/III/IV^{-/-} mice (Fig. 4A). Also, CD19⁺ B220⁺ B cell numbers in the spleen and bone marrow were comparable between these mice.

After vaccination with BSA-TNP in CFA, no gross differences were observed in BSA Ag-specific Ab titers between WT C57BL/6 and FcγRI/II/III/IV^{-/-} mice, with the exception of a small increase in IgG1 in FcγRI/II/III/IV^{-/-} mice (Fig. 4B), which can be explained by the absence of FcγRIIb on B cells in FcγRI/II/III/IV^{-/-} mice. These results are in agreement with those of a previous experiment using a milder immunization protocol (long synthetic peptide in saline with CpG) in our FcγRI/II/III/IV^{-/-} mice (38), indicating that FcγRs are dispensable for T cell-dependent B cell responses against a protein Ag.

To further test the functionality of the adaptive immune response in FcγRI/II/III/IV^{-/-} mice, the ability of these mice to respond to a bacterial infection was analyzed. We inoculated FcγRI/II/III/IV^{-/-} and WT C57BL/6 mice with a live vaccine consisting of the non-virulent SL3261 attenuated *aroA* *S. typhimurium* strain and analyzed the T and B cell responses (Fig. 5). We did not observe significant differences in the induction of T cell responses between the groups. B cell responses were not hampered in FcγRI/II/III/IV^{-/-} mice. The higher *Salmonella*-specific Ab responses detected in these mice are in keeping with the absence of FcγRIIb on their B cells (Fig. 5B).

Normal Ab levels and no autoantibody formation in aging FcγRI/II/III/IV^{-/-} mice

Serum IgG titers in 10-mo-old naive mice were comparable between FcγRI/II/III/IV^{-/-} and WT C57BL/6 mice (Fig. 6A). FcγRIIb^{-/-} mice on a mixed 129/C57BL/6 background develop high anti-nuclear Ab (ANA) titers with age (17), whereas FcγRIIb^{-/-} mice on a pure C57BL/6 background develop few ANAs (20). The FcγRI/II/III/IV deletion (on chromosome 1) was generated in C57BL/6-derived ES cells, whereas FcγRI^{-/-} mice were generated by gene targeting of the FcγRI gene (on chromosome 3) in 129-derived ES cells (25) and subsequent backcrossing onto the C57BL/6 background ($n > 12$). We compared ANA titers in the serum of old FcγRI/II/III/IV^{-/-} mice with those in old FcγRIIb^{-/-} mice on a mixed 129/C57BL/6 background ($n \geq 8$) and FcγRIIb^{-/-} mice on a pure C57BL/6 background. Only FcγRIIb^{-/-} mice generated by gene targeting in 129-derived ES cells and backcrossed onto the C57BL/6 background developed high ANA titers (Fig. 6B–D), confirming that 129-derived FcγRIIb-flanking sequences (*SLE16* on chromosome 1) (39) determine the development of ANAs. The absence of FcγRIIb does not lower the threshold for the development of autoimmunity in FcγRI/II/III/IV^{-/-} mice.

FcγR deficiency does not affect the development and homeostasis of the myeloid cell compartment

Because the adaptive immune system was not impaired in FcγRI/II/III/IV^{-/-} mice, we focused on the innate immune system. Considering the extensive expression of FcγR on myeloid cells, we envisaged that FcγR-mediated interactions might influence the development or differentiation of myeloid cells and that the absence of these receptors in FcγRI/II/III/IV^{-/-} mice could cause alterations in the innate immune compartment. Therefore, we evaluated the relative numbers of CD11c⁺/CD8a⁺ and CD11c⁺/CD8a⁻ DCs in bone marrow and spleen (Fig. 7A) and the relative numbers of the different myeloid subsets in spleen, using a gating strategy described by Rose et al. (40) (Fig. 7B). We found no variation in the percentage of either subset of cells between

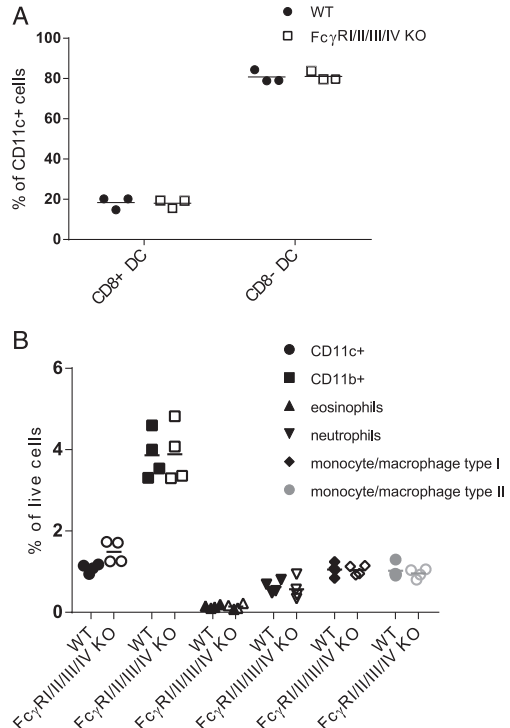


FIGURE 7. No differences in myeloid cell compartments between FcγRI/II/III/IV^{-/-} and WT C57BL/6 control mice. **(A)** Spleens of 3-mo-old FcγRI/II/III/IV^{-/-} and WT C57BL/6 mice were incubated with Liberase, and single-cell suspensions were labeled and analyzed by flow cytometry. Graphs show the percentage of CD8⁺ or CD8⁻ cells of CD11c⁺/CD19⁻/B220⁻/T-AAD⁻ cells ($n = 3$ mice per group). Data are from one of two experiments with similar results. **(B)** Spleens of 3-mo-old FcγRI/II/III/IV^{-/-} and WT C57BL/6 mice were incubated with Liberase, and single-cell suspensions were labeled and analyzed by flow cytometry. Gating strategy was according to Rose et al. (40), in short, gated on 7-AAD⁻/CD19⁻/CD3⁻ and followed by CD11c⁺ for the CD11c⁺ group, CD11b⁺ for the CD11b⁺ group, F4-80⁺/Ly6G⁺ for neutrophils, F4-80⁺/Ly6G⁻/Ly6C⁺, SSC high for eosinophils, F4-80⁺/Ly6G⁻/Ly6C⁺, SSC low for monocyte/macrophage type I, and F4-80⁺/Ly6G⁻/Ly6C⁻, SSC low for monocyte/macrophage type II ($n = 4$ mice per group). Data shown are from one of two experiments with similar results. Statistical analysis, performed using the Sidak multiple-comparison test, showed that the groups were not significantly different from each other.

FcγRI/II/III/IV^{-/-} and WT C57BL/6 mice. These results indicate that FcγR deficiency does not influence the development, differentiation, or homeostasis of the cells on which they are normally most prominently expressed.

No difference in complement, overall organ architecture, or metabolic homeostasis between FcγRI/II/III/IV^{-/-} and WT C57BL/6 control mice

Because a direct connection between complement and FcγR effector pathway activation has been reported (41), we analyzed the complement system in FcγRI/II/III/IV^{-/-} mice. We quantified complement activity in an ELISA-based system (32) upon initiation of the three pathways of complement activation. At the levels of C3 and C9 deposition, there were no differences in complement

activity between $\text{Fc}\gamma\text{RI/II/III/IV}^{-/-}$ and WT C57BL/6 mice (Fig. 8A). In line with this, circulating levels of individual components, including C1q, properdin, and activated C3 or C9, also were not different between $\text{Fc}\gamma\text{RI/II/III/IV}^{-/-}$ mice and WT C57BL/6 control mice (Fig. 8B). Circulating plasma levels of C6 were higher in $\text{Fc}\gamma\text{RI/II/III/IV}^{-/-}$ mice (Fig. 8B); however, this did not result in increased terminal pathway complement activity (Fig. 8A). Similarly, complement activity measured at the level of C3 deposition was comparable between the groups.

Furthermore, we examined histological sections of several organs, including kidney, liver, lung, and spleen, of 47-wk-old female mice. In keeping with the flow cytometry data, no abnormalities in the overall architecture of these organs were detectable in $\text{Fc}\gamma\text{RI/II/III/IV}^{-/-}$ mice compared with WT C57BL/6 control mice (Supplemental Fig. 2). There also were no differences with regard to BALT composition in representative lung sections. Glomerular and kidney pathology was also absent. Lymphoid aggregates were absent in representative liver sections from both groups.

It has been postulated that the adaptive immune system, the intestine, and microbiota govern a homeostatic metabolic function (42). B cells and pathogenic IgG promote insulin resistance in

mice fed a high-fat diet (43). Moreover, we (35) have recently shown that mice deficient for the $\text{Fc}\gamma\text{R } \gamma$ -chain are protected against diet-induced obesity and insulin resistance, suggesting a role for activating $\text{Fc}\gamma\text{Rs}$ in intestinal and systemic metabolic homeostasis. Therefore, we measured a series of metabolic parameters in $\text{Fc}\gamma\text{RI/II/III/IV}^{-/-}$ mice. Statistical analysis using unpaired t tests did not reveal significant differences between measured parameters of $\text{Fc}\gamma\text{RI/II/III/IV}^{-/-}$ and WT C57BL/6 control mice (Supplemental Table I).

Discussion

To our knowledge, the novel mouse model presented in this article is the first C57BL/6 model exclusively and completely deficient for all four $\text{Fc}\gamma\text{Rs}$. This enabled us to study the consequences of complete $\text{Fc}\gamma\text{R}$ deficiency without confounding factors. The phenotype of the $\text{Fc}\gamma\text{RI/II/III/IV}^{-/-}$ mouse demonstrates the dominant role of $\text{Fc}\gamma\text{Rs}$ in IgG downstream effector pathways, whereas complement is dispensable. These results confirm older studies with single, double, or triple $\text{Fc}\gamma\text{R-KO}$ mouse strains or $\text{Fc}\gamma^{-/-}$ mice lacking all three activating receptors. However, the overall immune system of $\text{Fc}\gamma\text{RI/II/III/IV}^{-/-}$ mice was surpris-

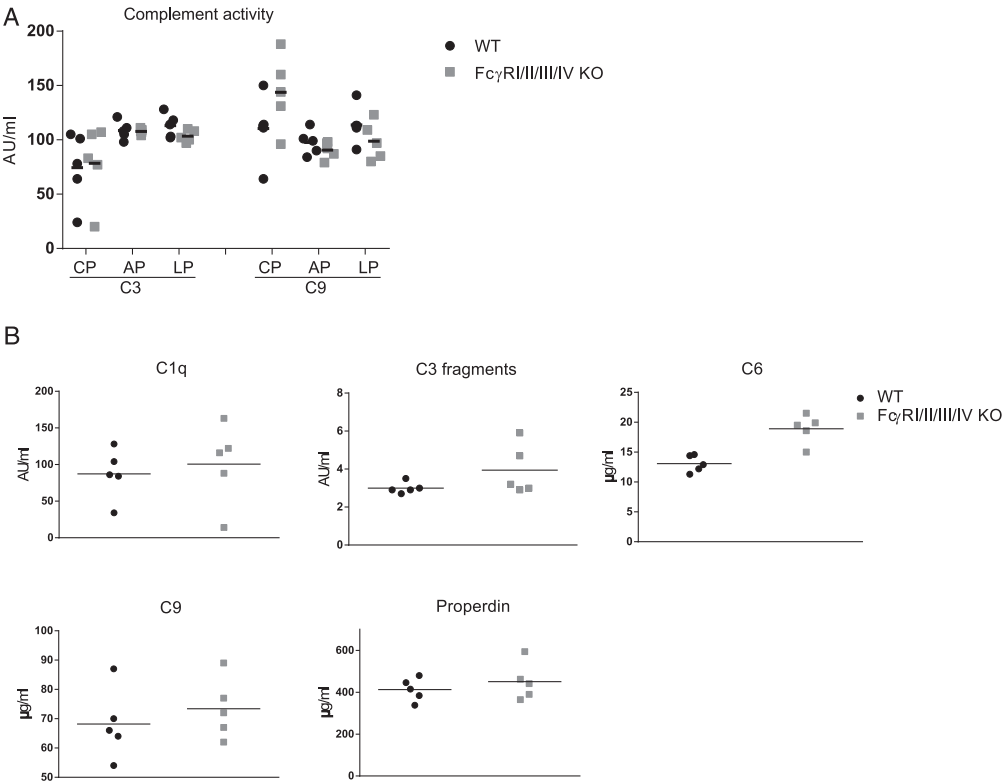


FIGURE 8. No differences in plasma concentration or activity of complement factors between $\text{Fc}\gamma\text{RI/II/III/IV}^{-/-}$ mice and WT C57BL/6 control mice. (A) Functional complement activity in plasma of WT C57BL/6 and $\text{Fc}\gamma\text{RI/II/III/IV}^{-/-}$ mice was determined for all three pathways at the level of C3 deposition and at the level of C9 deposition. Samples were tested in serial dilutions, quantified in comparison with a standard CD1 serum, and depicted as arbitrary units (AU/ml) ($n = 5$ mice per group). Statistical analysis with the Sidak multiple-comparison test revealed no significant difference between $\text{Fc}\gamma\text{RI/II/III/IV}^{-/-}$ and WT C57BL/6 mice. (B) Individual complement factors were quantified in plasma of five mice per genotype using specific ELISAs. This includes C1q as inducer of the classical pathway, properdin as stabilizer of the alternative pathway, and the central component C3 and C6 and C9 as part of the terminal pathway. Data are shown as the amount per milliliter of serum of the indicated factors in micrograms (properdin, C6, and C9) or in AU (C1q and C3 fragment). AP, alternative pathway; CP, classical pathway; LP, lectin pathway.

ingly normal. Lymphoid organs, subsets of lymphoid and myeloid cells, complement, and metabolic homeostasis were comparable to WT mice.

Mammals are exposed to their mother's Abs before birth. Also, before birth they have developed immune cells expressing a variety of FcγRs (Supplemental Fig. 3) that can directly interact with the Fc portion of these Abs. The high-affinity FcγRI binds monomeric IgG, whereas the other FcγRs are low-affinity receptors that bind ICs. After birth, animals develop their own Abs in response to threats from the outside world. The lack of aberration in the phenotype of the FcγRI/II/III/IV^{-/-} mouse suggests that the absence of all FcγRs has little impact on the ontogeny and functionality of the immune system of these mice, with the exception of their downstream Ab-mediated inflammatory effector functions.

Biological systems have a strong tendency to bypass a blockade in development and functionality by adaptation (44). The complement system and FcγRs are redundant in the downstream effector pathways of IgG; however, the loss of FcγRs was not compensated for by increased activity of the complement system. We did not find indications for other compensation mechanisms in FcγRI/II/III/IV^{-/-} mice.

Surprisingly, in vivo cross-presentation of IgG IC-derived protein Ag, the adaptive immune response against *Salmonella*, and the Ab response against a model Ag were almost indistinguishable between FcγRI/II/III/IV^{-/-} and WT mice. This suggests strong redundancy in the involved adaptive immune pathways.

The role of FcγR in cross-presentation is still puzzling. We (11–13) and other investigators (14) have shown that the cross-presentation by DCs, loaded in vitro with IgG ICs, is dependent on FcR γ-chain. In contrast, we demonstrated more recently that the enhanced in vivo cross-presentation of protein Ag derived from injected preformed IgG ICs is partially, but not totally, dependent on FcR γ-chain (22), which is in line with previous data. den Haan and Bevan (45) showed that, in the absence of FcR γ-chain, the uptake of i.v. injected IgG ICs and enhanced cross-presentation by DCs were not impaired. An obvious explanation for this discrepancy is that an in vivo dominant FcR γ-chain-independent pathway, most likely a complement-dependent pathway, is bypassed by loading DCs with ICs in vitro. Our cross-presentation experiments with FcγRI/II/III/IV^{-/-} mice directly demonstrated that IgG-mediated enhanced cross-presentation by DCs, loaded with IgG ICs in vitro, is exclusively dependent on FcγR and not on other FcR γ-chain-associated receptor molecules. In vivo, in contrast, neither the inhibiting FcγRIIB nor the activating FcγRs are required. Besides FcγR, DCs display a large variety of other receptors that are involved in the uptake of exogenous Ag, such as C-type lectin receptors, TLRs, and complement receptors. We have found a pivotal role for C1q in the presentation of Ags derived from i.v. administered IgG ICs to CD8⁺ T cells in vivo (22), indicating that the complement system provides alternative pathways in IgG-dependent cross-presentation.

Remarkably, in comparison with WT control mice, the B cell responses of FcγRI/II/III/IV^{-/-} mice were not hampered; rather, they developed higher Ag-specific Ab titers upon immunization. This is not in agreement with the previous observation that FcR γ-chain-KO mice, lacking functional expression of all three activating receptors, develop lower Ag-specific Ab titers compared with WT mice (10). The discrepancy between these results might be explained by the use of different FcγR-deficient mouse strains. The FcR γ-chain is associated with at least nine other receptor complexes (18). Therefore, FcR γ-chain deficiency might cause a more pleiotropic effect in immunity compared with FcγRI/II/III/IV

deficiency. In contrast, the FcγRI/II/III/IV^{-/-} mouse lacks activating FcγR, as well as the regulatory inhibiting FcγRIIB. The higher IgG titers in FcγRI/II/III/IV^{-/-} mice suggest that the Ab response in these mice is affected by the deficiency of FcγRIIB on B cells but not by the deficiency of the three activating FcγRs expressed on APCs. These data indicate that activating FcγRs on APCs are not required for the development of a full-blown Ab response by facilitating presentation of IC-derived Ag, resulting in efficient priming of Th cell responses, as was suggested by previous results with FcR γ-chain-KO mice (10).

Altogether, our data suggest that, in vivo, the role of activating FcγR in the regulation of adaptive immunity by facilitating APC-mediated presentation of IgG IC-derived Ag is dispensable. The role of FcR has been implicated in enhancing an anti-tumor response by facilitating Ag presentation of IC-derived tumor Ag after anti-tumor Ab therapy (46, 47). In light of our findings indicating that other FcγR-independent mechanisms, most likely complement-associated ones, play a dominant role, we propose to study IgG IC-mediated immune modulation in more detail in our FcγRI/II/III/IV^{-/-} mice, because it is the first C57BL/6 model, to our knowledge, in which these questions can be answered without confounding factors. The use of Abs with a mutation in their Fc domain, which destroy FcγR binding without affecting interactions with complement, is limited to passive models (3), whereas our FcγRI/II/III/IV^{-/-} mouse enables the study of active models, such as vaccination and infection.

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Disclosures

The authors have no financial conflicts of interest.

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Body composition and plasma lipid levels		
	WT	FcγRI/II/III/IV ^{-/-}
Body weight (g)	24.6 ± 1.5	25.6 ± 1.7
Lean mass (g)	22.7 ± 1.6	23.7 ± 1.6
Fat mass (g)	1.14 ± 0.56	1.20 ± 0.20
TG (mM)	0.58 ± 0.10	0.51 ± 0.06
TC (mM)	2.67 ± 0.41	2.67 ± 0.48
FFA (mM)	0.97 ± 0.30	1.15 ± 0.15
Glucose (mM)	8.88 ± 0.88	7.88 ± 1.71
Insulin (ng/ml)	0.45 ± 0.09	0.43 ± 0.11
Indirect calorimetry		
	WT	FcγRI/II/III/IV ^{-/-}
FI day (g)	1.29 ± 0.12	1.12 ± 0.15
FI night (g)	2.34 ± 0.39	2.59 ± 0.33
EE day (kcal/h)	0.44 ± 0.03	0.46 ± 0.04
EE night (kcal/h)	0.55 ± 0.04	0.57 ± 0.05
PA day (A.U.)	107.8 ± 44.9	89.0 ± 43.9
PA night (A.U.)	372.0 ± 132.1	348.7 ± 139.4
RER day	0.88 ± 0.01	0.88 ± 0.02
RER night	0.95 ± 0.02	0.96 ± 0.02
FA _{ox} day (kcal/h)	0.16 ± 0.02	0.17 ± 0.04
FA _{ox} night (kcal/h)	0.07 ± 0.02	0.05 ± 0.03
CH _{ox} day (kcal/h)	0.29 ± 0.04	0.31 ± 0.04
CH _{ox} night (kcal/h)	0.50 ± 0.07	0.54 ± 0.07

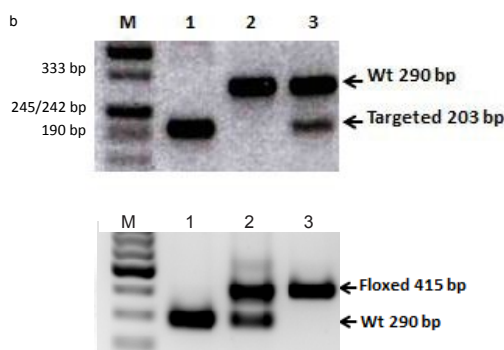
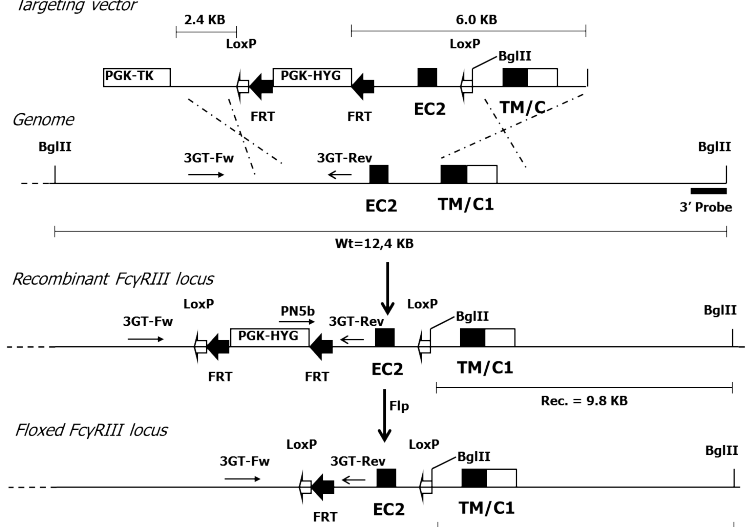
Table S1. Comparable metabolic phenotype of FcγRI/II/III/IV^{-/-} and WT control mice.

Body weight, body composition and plasma lipid levels were determined from male WT and FcγRI/II/III/IV^{-/-} mice. Plasma lipid levels were measured in plasma from 6 hour fasted mice. Indirect calorimetry was performed during the day (diurnal) and the night (nocturnal) time for WT and FcγRI/II/III/IV^{-/-} mice. 8 male mice per group.

TG=Triglycerides; TC=Total cholesterol; FFA=Free fatty acids; FI=Food intake; EE=Energy expenditure; PA=Physical activity; RER=Respiratory exchange ratio; FA_{ox}=Fatty acid oxidation; CH_{ox}=Carbohydrate oxidation

Supplementary figure 1

a Targeting vector

Fig. S1 Generation of a *FcγRIII* conditional KO mouse model.

A targeting vector was constructed based on a 8.4 kb genomic fragment containing exons 4, encoding extracellular domain 2 (EC2), and exon 5, encoding transmembrane and cytoplasmic domain (TM/C) of the *FcγRIII* gene, from BAC clone RPC123-87B18 of the RPC1 23 female (C57Bl/6J) mouse BAC genomic library (BACPAC Resources Center, Children's Hospital Oakland Research Institute, Oakland, California). A LoxP-FRT-Hygro-FRT cassette upstream of the EC2 exon was inserted. Gene targeting was performed in C57Bl/6-derived ES cells (Bruce4). Clones in which homologous recombination occurred were identified by Southern blotting and subsequently injected in C57Bl/6 blastocysts. The obtained chimeras were crossed with C57Bl/6J mice and the F1 offspring positive for the *FcγRIII* targeted allele was crossed with a C57Bl/6 Flp deleter strain resulting in mice with a floxed *FcγRIII* allele. Flp-mediated recombination was analyzed with PCR. a. From top to bottom schematic representation of the *FcγRIII* targeting vector, the relevant part of the WT mouse *FcγRIII* genomic locus, the targeted recombinant *FcγRIII* allele and the floxed *FcγRIII* allele after removal of the PGK-Hyg selection marker gene by Flp recombinase. The *FcγRIII* exon 4 and 5 (black boxes) are marked in accordance to the functional domains they encode: EC2, the extracellular immunoglobulin-like domain 2; TM/C, transmembrane-cytoplasmic tail region. Coding parts are depicted as closed boxes, non-coding parts as open boxes. Indicated are BglII restriction sites and location of a 3' probe used for the identification of the recombinant locus by Southern blot analysis (data not shown). Primers for PCR based genotyping are depicted as small arrows. b. PCR analysis of genomic DNA from tail biopsies. Top panel: chimeric mouse with targeted *FcγRIII* allele. Use of primer pair PN5b/3GT-Rev resulted in the amplification of a 203 bp fragment of the recombinant *FcγRIII* allele only (lane 1). Use of primer pair 3GT-Fw/3GT-Rev resulted in the amplification of a 290 bp fragment of the Wt *FcγRIII* allele only (lane 2). Lane 3 shows the PCR fragments when all three PCR primers were used. M: mol. weight marker Bottom panel: Use of primer pair 3GT-Fw/3GT-Rev resulted in the amplification of a 415 bp fragment of the floxed *FcγRIII* allele after Flp recombination. M: 100bp ladder. Primer sequences: 3GT-Fw: GAGGGCATCCGATTTCATTA; 3GT-Rev: GCTGTAGCTATCTCTCAGCAGAA; PN5b: CTAAAGCGCATGCTCCAGACT

Supplementary figure 2

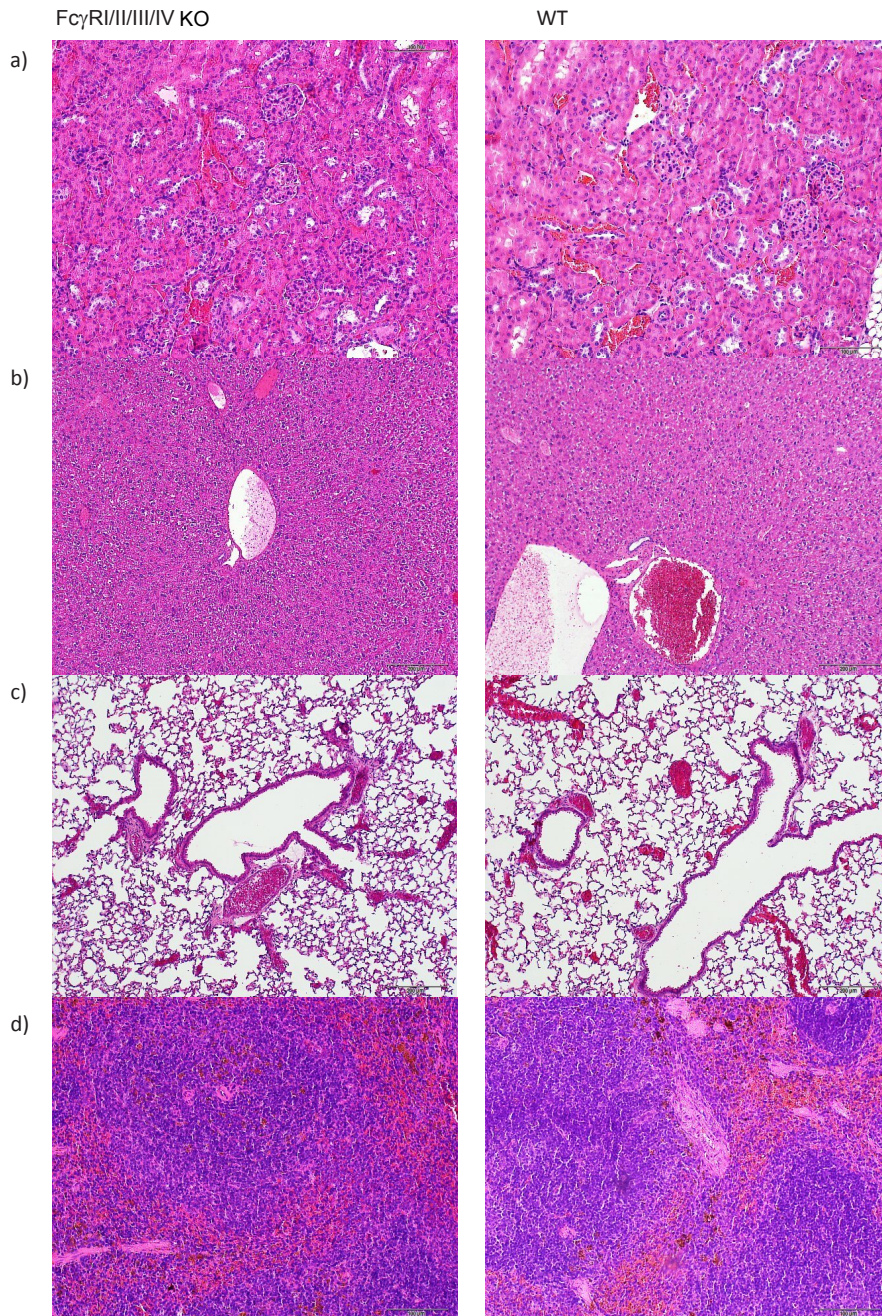


Fig. S2. No differences in the overall architecture of spleen, lung, liver and kidney between old FcγRI/II/III/IV KO and WT control mice. Representative pictures of histological HE stained sections of kidney (a), liver (b), lung (c) and spleen (d) of FcγRI/II/III/IV KO and WT control mice. Histological examination showed no morphological differences among the 2 groups of mice. Three 47 weeks old female mice per group.

Supplementary figure 3

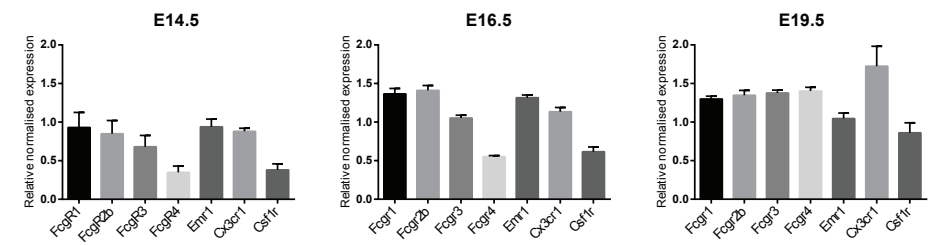


Fig.S3. FcγR expression during embryonic development.
Real-time PCR of mRNA from liver of WT mouse embryos at stage E14.5, E16.5 and E19.5. The relative normalized expression is shown of mRNA from all four FcγR as indicated. mCx3cr1, mEmr1 and mCsf1r are mouse macrophage specific reference genes.
Error bars= SEM from at least three embryos of each developmental stage.

CHAPTER 3

3

FcγRI expression on macrophages is required for antibody-mediated tumor protection by cytomegalovirus-based vaccines.

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FcγRI expression on macrophages is required for antibody-mediated tumor protection by cytomegalovirus-based vaccines

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ABSTRACT

Cytomegalovirus (CMV)-based vaccine vectors are promising vaccine platforms because they induce strong and long-lasting immune responses. Recently it has been shown that vaccination with a mouse CMV (MCMV) vector expressing the melanoma-specific antigen TRP2 (MCMV-TRP2) protects mice against outgrowth of TRP2-positive B16 melanoma tumors, and this protection was dependent on the induction of IgG antibodies. Here we demonstrate that, although mice lacking all receptors for the Fc part of IgG (FcγRs) develop normal IgG responses after MCMV-TRP2 vaccination, the protection against B16 melanoma was completely abrogated, indicating that FcγRs are indispensable in the downstream effector pathway of the polyclonal anti-TRP2 antibody response. By investigating compound FcγR-deficient mouse strains and by using immune cell type-specific cell ablation we show that the IgG antibody-mediated tumor protection elicited by MCMV-TRP2 mainly depends on FcγRI expression on macrophages, whereas FcγRIV plays only a modest role. Thus, tumor-specific antibody therapy might benefit from combination therapy that recruits FcγRI-expressing pro-inflammatory macrophages to the tumor micro-environment.

INTRODUCTION

Most vaccines against cancer exhibit less impressive results in comparison with the clinical results of immunomodulatory antibodies and adoptive T cell therapy (ACT) [1]. This could be attributed due to various immune evasion mechanisms and to the failure of inducing long-lasting sustainable functional T and B cell responses. With respect to the latter, cytomegalovirus (CMV)-based vaccines hold great potential because of the ability of CMV to elicit large adaptive immune responses that are

maintained without contracting [2]. This phenomenon, termed memory inflation, is observed for the increase in effector-memory T cell populations [3] as well as for IgG antibodies [4]. Other properties such as the ability of CMV to re-infect despite pre-existing immunity [5, 6] and the adaptability of CMV for genetic engineering [7], thereby even allowing large insertions of foreign DNA without losing viral replication potential, also contribute to the value of CMV as a vaccine vector.

The efficacy of CMV as a cancer vaccine vector has been demonstrated in several preclinical

models by using MCMV vectors containing prostate specific antigen (PSA), melanoma antigens (gp100, tyrosinase-related protein-2 (TRP-2)) or model antigens (ovalbumin) [8–11]. While the efficacy of most of these MCMV vaccine vectors was centred on the induction of large effector-memory T cell responses, the effectiveness of the MCMV-TRP2 vector was antibody dependent [10], but the mechanisms of actions of the TRP2 antibody-mediated tumor protection remain to be determined.

The B16 transplantable melanoma model is widely used to study underlying mechanisms of tumor-specific antibody-based therapy [12–19]. In most studies the IgG2c (the equivalent of IgG2a in C57BL/6 mice) monoclonal antibody TA99 [12–16, 19] or other IgG subclasses engineered from it [17, 18] specific for the B16-F10 antigen TRP1 (gp75) were used. In addition, active immunisation with gp75 protein was applied [15]. In contrast to anti-HER2/Neu antibody in breast cancer, TA99 does not interfere with cell signalling when bound to its tumor target and has no direct effect on growth or survival of tumor cells. Therefore, the therapeutic efficacy of TA99 depends on its ability to recruit effector cells of the immune system, which can kill the tumor cells by different mechanisms including antibody dependent cell cytotoxicity (ADCC), antibody dependent cell phagocytosis (ADCP), trogocytosis [20], complement dependent cell-mediated phagocytosis (CDCP), and cell-mediated cytotoxicity (CDCC) [21].

Four different FcγRs have been identified in the mouse. The IgG binding α-chains of the activating FcγRI, FcγRIII and FcγRIV are associated with the FcR γ chain, a signal transduction subunit that is also required for cell surface expression. The activating FcγR are counterbalanced by the inhibiting receptor FcγRIIb. The four FcγRs are expressed in different combinations on a variety of immune cells, mainly myeloid effector cells [22, 23]. Different laboratories reported contradictory results regarding the involvement of the individual activating FcγRs using one of the three B16-F10 tumor model variants and different panels of FcγR deficient mice and FcγR blocking antibodies [13, 14, 17–19].

Here we aim to decipher the underlying mechanisms of the anti-tumor effects of TRP2 polyclonal antibodies elicited by MCMV-TRP2 vaccination through investigating the individual role of the activating FcγRI, FcγRIII and FcγRIV and the different FcγR-expressing immune effector cells. We demonstrate that after immunization with the MCMV-TRP2 vector, the protection against tumor outgrowth mediated by the elicited polyclonal TRP2 antibodies is completely abrogated in FcγRI-deficient mice and partially diminished in FcγRIV^{-/-} mice. Cell subset depletion revealed that macrophages were the main innate effector immune cells involved.

RESULTS

Germinal center reactions and antibody responses during CMV infection are not FcγR dependent

To investigate the particular role of FcγRs in the antibody-mediated protection induced by MCMV-TRP2 vectors [10], we initially aimed to investigate whether FcγRs impact B cell responses including the development of germinal center (GC) B cells and plasma cells, T follicular helper (T_{FH}) cell responses, and antibody responses upon CMV infection.

Wild-type (WT) C57BL/6 mice and mice lacking all four FcγRs were infected with MCMV-Smith and analysed at day 14 post-infection. The GC B cell responses, and plasma cells were slightly increased in FcγRI/II/III/IV^{-/-} mice (Figure 1A and 1B) compared to WT mice, while the GC T_{FH} cell responses were comparable (Figure 1C and 1D). The IgG antibody responses specific to MCMV at day 14 post-infection with MCMV-Smith were comparable between WT and FcγRI/II/III/IV^{-/-} mice (Figure 2A). Consistent with natural infection, the IgG antibody responses (total IgG, IgG1, IgG2c) to MCMV after vaccination with MCMV-TRP2 were also similar in WT and FcγRI/II/III/IV^{-/-} mice (Figure 2B–2D). Thus, FcγRs have a minor impact on GC reactions and antibody formation, indicating that potential effects of FcγR-deficiency cannot be attributed to insufficiencies in antibody responses.

FcγRI is predominantly required for the antibody-mediated tumor protection induced by MCMV-TRP2 vaccination

Next, we aimed to analyse whether FcγRs play a role in the IgG-mediated effector mechanisms that are operational during tumor protection upon vaccination with MCMV-TRP2. To determine whether the MCMV-TRP2-induced anti-tumor immunity is FcγR dependent, WT mice and mice deficient in all four FcγRs were vaccinated with MCMV-TRP2, and two weeks later challenged with B16 tumor cells. In untreated WT and FcγRI/II/III/IV^{-/-} mice the outgrowth of B16 tumor was similar. In contrast, MCMV-TRP2 vaccination elicited a clearly delayed onset of B16 tumor outgrowth in WT mice but not in FcγRI/II/III/IV^{-/-} mice (Figure 3). Thus, the antibody-mediated protection induced by MCMV-TRP2 vaccination is FcγR dependent.

The expression profiles vary between FcγR classes as well as the downstream antibody effector functions they facilitate. To gain insight in the contribution of the individual activating FcγRs, the protection against outgrowth of the B16 tumor after vaccination with MCMV-TRP2 was analysed in mice deficient for one

or two FcγR classes. The generation of FcγRIV^{-/-} and FcγRIII/IV^{-/-} mouse strains is described in the Supplementary Data. FcγRI^{-/-}, FcγRIII^{-/-}, FcγRIV^{-/-} and FcγRIII/IV^{-/-} mice were vaccinated with MCMV-TRP2 or left unvaccinated, and two weeks later challenged with B16 tumor cells. All unvaccinated WT and FcγR^{-/-} mice developed progressive tumors within 2–3 weeks after

challenge. Whereas vaccinated WT and FcγRIII^{-/-} mice showed a substantial increased survival, MCMV-TRP2 vaccinated FcγRI^{-/-} mice did not (Figure 4A and 4B). FcγRIV^{-/-} and FcγRIII/IV^{-/-} mice showed a delay in tumor outgrowth, albeit significantly less compared to MCMV-TRP2 vaccinated WT mice (Figure 4C). Taken together these data suggest that FcγRI plays a dominant role in the

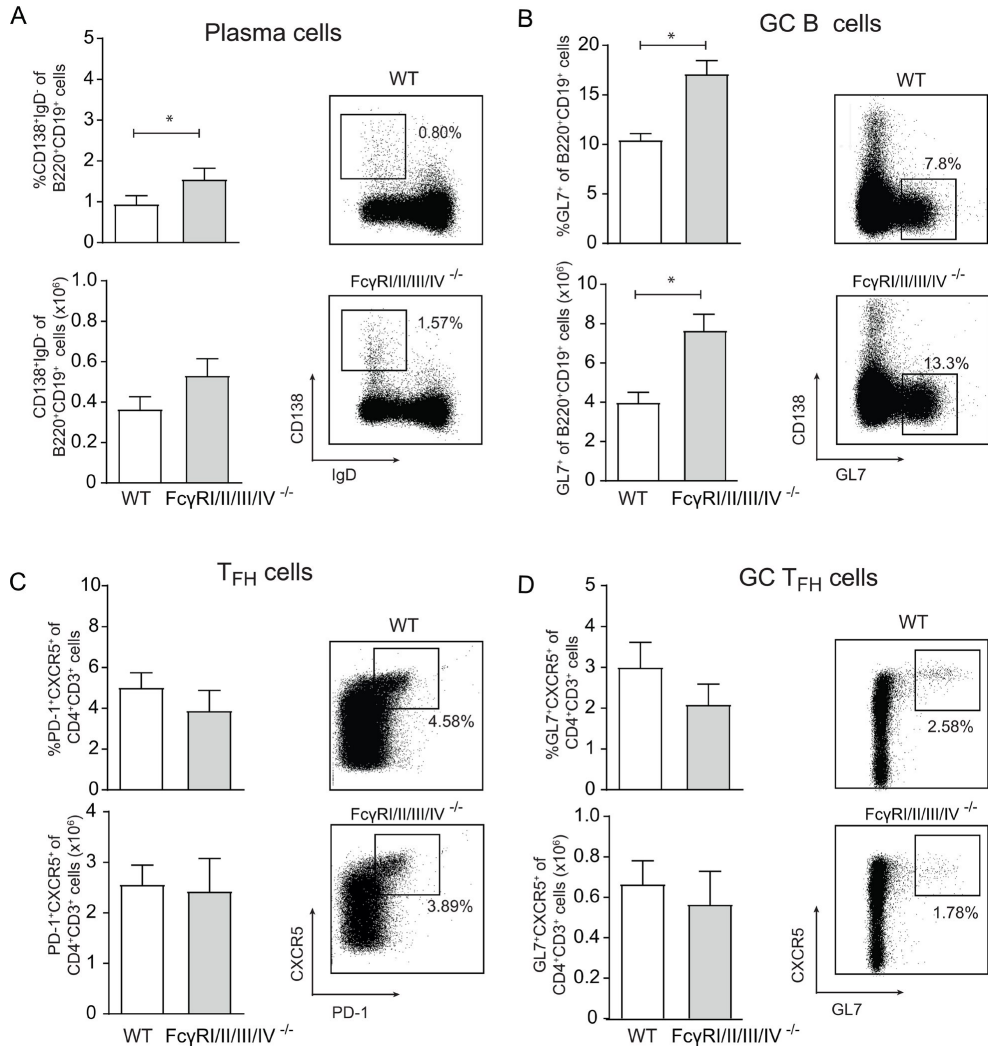


Figure 1: Germinal center reactions upon CMV infection are not abrogated in mice lacking all FcγRs. (A–D) WT and FcγRI/II/III/IV^{-/-} mice were infected with MCMV-Smith and at day 14 post-infection the B cell and T cell germinal center (GC) responses in the spleen were examined. Representative flow cytometry plots are shown. (A–B) Percentage and absolute number of plasma cells (CD138⁺IgD⁺) and GC B cells (GL7⁺) within splenic CD19⁺ B220⁺ B cells in WT and FcγRI/II/III/IV^{-/-} mice. (C–D) Percentage and total cell numbers of (C) T_{FH} (CXCR5⁺PD1⁺) cells and (D) GC T_{FH} (CXCR5⁺GL7⁺) cells within CD4⁺CD3⁺ T cells are shown. Results from one representative experiment out of three performed are shown (**p* < 0.05, *n* = 5).

antibody-mediated tumor protection induced by MCMV-TRP2 vaccination whereas FcγRIV has moderate impact.

Tumor protection by MCMV-TRP2 vaccination predominantly requires macrophages

FcγRs are variably expressed by different cell types including phagocytic myeloid cells, neutrophils and NK cells [22, 23]. To determine which FcγR-expressing

effector cell types are involved in the antibody mediated tumor protection induced by MCMV-TRP2 vaccination, the outgrowth of B16 cells was monitored after s.c. transplantation, followed two days later by depletion of the following cell types: macrophages (depleted by clodronate liposomes), neutrophils (depleted by anti-Ly6G antibody) and NK cells (depleted by anti-NK1.1 antibody). After the depletion of macrophages, the protection against B16 tumor challenge upon MCMV-TRP2 vaccination was

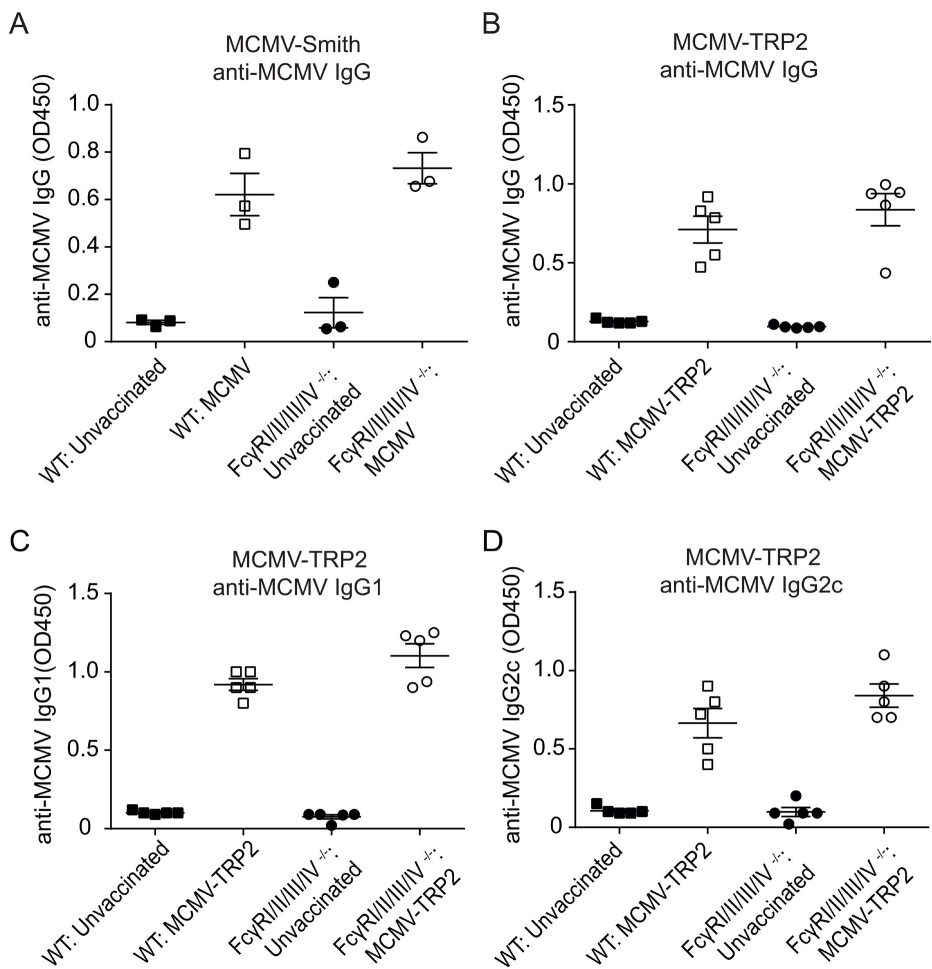


Figure 2: FcγR deficiency does not alter the anti-MCMV antibody response after MCMV-TRP2 vaccination. (A) Levels of MCMV-specific total IgG in serum of WT and FcγR1/II/III/IV^{-/-} mice at day 14 post-infection with MCMV-Smith. Measurement at OD450 of 1:400 diluted serum. (B) Levels of MCMV-specific total IgG in serum of WT and FcγR1/II/III/IV^{-/-} mice at day 14 post vaccination with MCMV-TRP2. Measurement at OD450 of 1:100 diluted serum. (C) Levels of MCMV-specific IgG1 in serum of WT and FcγR1/II/III/IV^{-/-} mice at day 14 post vaccination with MCMV-TRP2. Measurement at OD450 of 1:100 diluted serum. (D) Levels of MCMV-specific IgG2c in serum of WT and FcγR1/II/III/IV^{-/-} mice at day 14 post vaccination with MCMV-TRP2. Measurement at OD450 of 1:100 diluted serum.

abrogated while depletion of NK cells had a slight effect. In addition, depletion of neutrophils did not alter tumor protection (Figure 5A and 5B).

The dominant importance of macrophages is in line with the expression of the FcγRs on these cells. Notably,

FcγRI is highly expressed on inflammatory macrophages (Ly6C⁺ F4/80⁺) residing in the tumor and spleen (Figure 5C). Neutrophils express FcγRIII and FcγRIV, while NK cells only express low levels of FcγRIII. Thus, macrophages expressing high levels of FcγRI are the

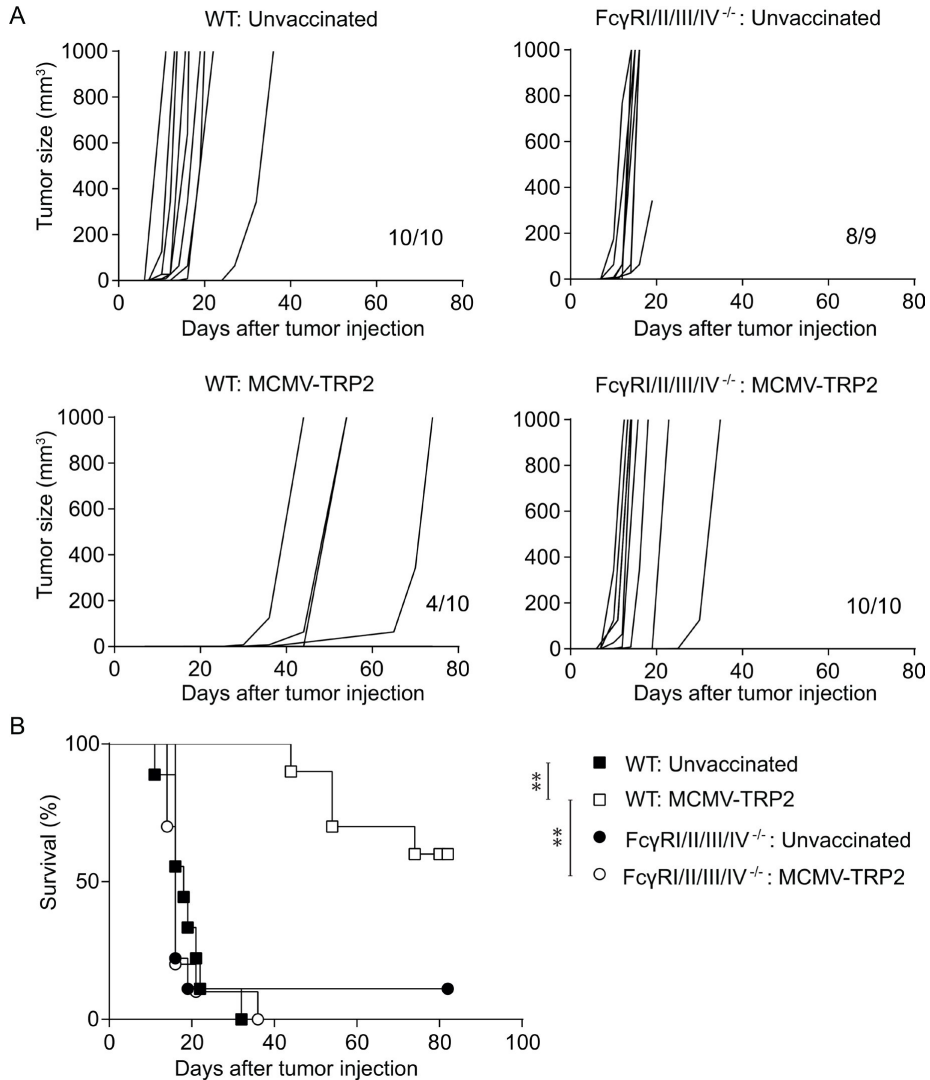


Figure 3: Protection against B16 tumor outgrowth by MCMV-TRP2 vaccination is FcγR dependent. WT and FcγRI/II/III/IV^{-/-} mice were vaccinated with MCMV-TRP2 or left unvaccinated, and at day 14 post-vaccination challenged with B16 tumor cells. **(A)** Tumor growth curves of B16 tumor challenged WT and FcγRI/II/III/IV^{-/-} mice following MCMV-TRP2 vaccination. Numbers at the bottom right of each panel represent the number of mice from total that succumbed to the tumor challenge. **(B)** Survival of B16 tumor-bearing WT and FcγRI/II/III/IV^{-/-} mice following vaccination with MCMV-TRP2. Results are shown from two pooled experiments (* $p < 0.05$, ** $p < 0.01$, $n = 10$).

dominant cell type involved in the tumor protecting effects mediated by polyclonal antibodies elicited via MCMV-TRP2 vaccination.

Macrophages mediate antibody-dependent killing of tumor cells

To gain insight into the mechanisms of macrophage-dependent killing of the B16 tumor cells, we first performed cell surface stainings of B16 tumor cells with serum from MCMV-TRP2 vaccinated WT and FcγRI/II/III/IV^{-/-} mice followed by an Alexa488-conjugated anti-mouse IgG specific antibody (Figure 6A and 6B). The cell surface of the B16 tumor cells were positively stained with serum from MCMV-TRP2 vaccinated WT mice while serum from unvaccinated WT mice or serum from WT mice that were infected with MCMV not expressing TRP2 resulted in negative staining. Staining with serum from MCMV-TRP2 vaccinated FcγRI/II/III/IV^{-/-} mice resulted also in a positive cell surface staining whereas serum from unvaccinated FcγRI/II/III/IV^{-/-} mice did not.

To show that macrophages can kill B16F10 cells via serum antibodies that are able to bind to the cell surface of the B16F10 cells, we co-cultured these tumor cells with macrophages (RAW264.7, a murine macrophage cell line) in presence and absence of serum of vaccinated and

non-vaccinated mice. We observed that only serum from MCMV-TRP2 vaccinated mice resulted in lysis of the tumor cells (Figure 6C). Thus, macrophages can mediate killing of the B16F10 cells via serum antibodies elicited by the MCMV-TRP2 vaccine, indicating that ADCP is the main effector mechanism involved.

DISCUSSION

This study shows that a MCMV-based vaccine vector, expressing the full native protein of the melanoma specific antigen TRP2, induced a strong polyclonal antibody response independent of FcγRs. However, these tumor-specific antibodies required FcγRs for their protective effects against the outgrowth of the TRP2 expressing B16 melanoma cells. In particular, FcγRI and to a lower extend FcγRIV expressed on macrophages was required for the tumor protection.

A previous study showed that protection against tumor outgrowth by vaccination with MCMV-TRP2 was not dependent on CD8 or CD4 T cells but rather on antibodies [10]. Other MCMV-based vaccinations in mice have shown to be dependent on the induction of tumor-specific CD8 T cell responses [8, 9, 11, 24]. The most effective anti-tumor CD8 T cell responses were induced with MCMV vectors expressing altered versions or short

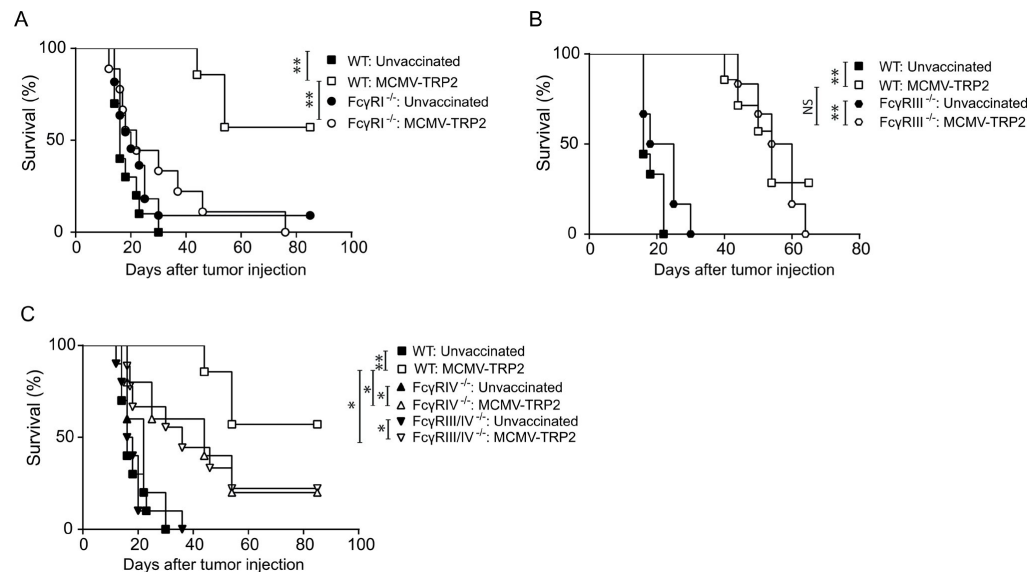


Figure 4: Protection against B16 tumor outgrowth by MCMV-TRP2 vaccination is mainly FcγRI dependent. WT mice and mice deficient in one or more FcγRs were vaccinated with MCMV-TRP2 or left unvaccinated, and at day 14 post-vaccination challenged with B16 tumor cells. (A) Survival of B16 tumor challenged WT and FcγRI^{-/-} mice. (B) Survival of B16 tumor challenged WT and FcγRIII^{-/-} mice. (C) Survival of B16 tumor challenged WT, FcγRIV^{-/-} and FcγRIII/IV^{-/-} mice. Results shown are from two pooled experiments (*p* < 0.05, ***p* < 0.01 *n* = 5–11).

peptides derived from tumor antigens instead of the full native protein. The MCMV vector used in this study, MCMV-TRP2, expresses the full native TRP2 protein, which might explain the elicitation of antibody responses rather than CD8 T cell responses.

We explored the underlying mechanisms of the therapeutic effect of the polyclonal IgG response induced by MCMV-TRP2. We found that the therapeutic efficacy depended mainly on FcγRI and partially on

FcγRIV. Macrophages, expressing high levels of FcγRI and FcγRIV, were in particular crucial for the efficacy of MCMV-TRP2, indicating that FcγRI expression on macrophages is crucial. Most likely crosslinking of the high affinity FcγRI on macrophages induces ADCP of the B16 tumor cells located subcutaneously. Consistent with this are the results of experiments using intravital microscopy in which it was shown that ADCP by macrophages is prominent in the removal of circulating

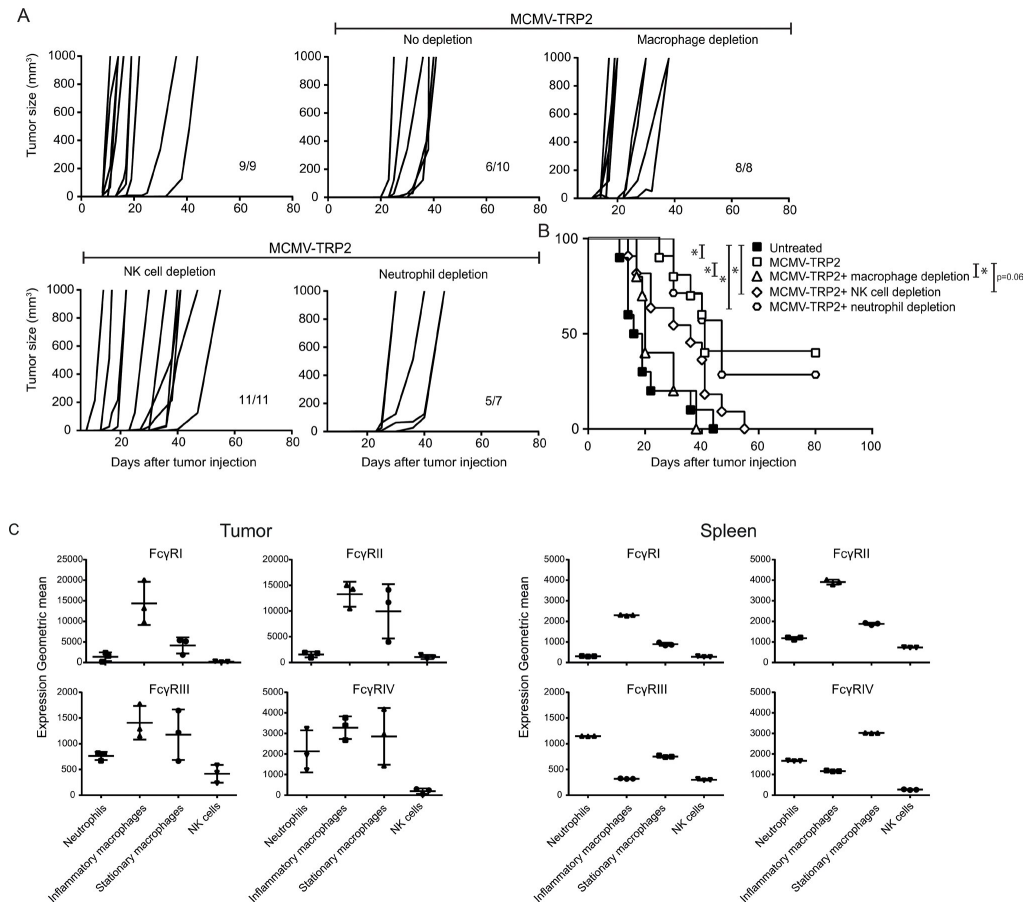


Figure 5: Protection against B16 melanoma outgrowth by MCMV-TRP2 vaccination requires macrophages. WT mice were vaccinated with MCMV-TRP2 or left unvaccinated, and at day 14 post-vaccination challenged with B16 tumor cells. Macrophages, NK cells or neutrophils were depleted right before and during tumor challenge. (A) Tumor growth curves of B16 tumor challenged WT mice in which the indicated cell subsets have been depleted following vaccination with MCMV-TRP2. Numbers at the bottom right of each panel represent the number of mice from total that survived the treatment. Results are shown from two pooled experiments. (B) Survival of B16 tumor-bearing WT mice in which the indicated cell subsets have been depleted following vaccination with MCMV-TRP2. Results are shown from two pooled experiments ($p < 0.05$, $**p < 0.01$, $n = 9-11$). (C) Expression of FcγRI, FcγRII, FcγRIII and FcγRIV on inflammatory macrophages (F4/80⁺ Ly6C⁺), stationary macrophages (F4/80⁺ Ly6C⁻), neutrophils (Ly6G⁺) and NK cells (NK1.1⁺) in tumor and spleen of unvaccinated mice at day 14 post tumor challenge.

tumor cells through FcγRI and FcγRIV [16]. ADCP may thus be important for cancer vaccines inducing antibody responses and for antibody therapy of cancer. NK cells seem to play a minor role in the anti-tumor protection by the MCMV-TRP2 vaccine. Since NK cells only express FcγRIII and this FcγR is not important in the anti-tumor effects elicited by the MCMV-TRP2 vaccine vector it is expected that the minor anti-tumor effect of NK cells is not mediated via FcγRIII and antibodies.

MCMV-TRP2 induced a polyclonal IgG antibody response of different subclasses that are known to mediate different downstream effector functions. Most tumor-specific antibodies currently used in clinics are monoclonal antibodies. These antibodies have mostly been humanized for their usage in patients. It is known that entirely human antibodies are longer lasting as they have a lower likelihood to induce anti-antibody responses [25]. Essentially, vaccination should be able to induce fully human antibodies against the tumor target. Moreover, polyclonal antibodies initiate responses against multiple epitopes thereby minimizing tumor escape. Furthermore, it has been reported that antibodies recognizing multiple epitopes of epidermal growth factor receptor (EGFR) were able to induce complement-dependent tumor cell killing while a monoclonal antibody was not [26]. As such polyclonal antibodies against multiple epitopes could thus also be better at inducing complement-dependent lysis. A mixture of specificities in serum has been shown to induce increased complement activation [27]. Although, in this study we found a prominent role for FcγR-dependent mechanisms, we do not rule out that complement induced lysis could also play a role.

In conclusion our study showed that MCMV-based vaccine vectors can induce strong IgG antibody responses against tumors involving FcγRI expressing macrophages. We anticipate that additional treatments, such as those counter-acting the immunosuppressive tumor micro-environment, could act in synergy in order to improve the therapeutic efficacy of antibodies. Thus, CMV-based vectors are promising anti-cancer vaccines and may become a potent component of an effective combination therapy in cancer.

MATERIALS AND METHODS

Mice and antibodies

Mice were housed in the SPF animal facilities of the Central Animal Facility (PDC) 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 FELASA (Federation of European Laboratory Animal Science Associations) guidelines for SPF mouse colonies. 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'). C57BL/6J mice were purchased from Charles River laboratories. FcγR^{-/-} mice were generated in the transgenic mouse facility of the LUMC (i.e., FcγRI^{-/-} [28], FcγRII/II/III/IV^{-/-} [29]. The generation of FcγRIV^{-/-} and FcγRIII/IV^{-/-} mouse strains is described in the Supplementary Data (Supplementary Figures 1 and 2).

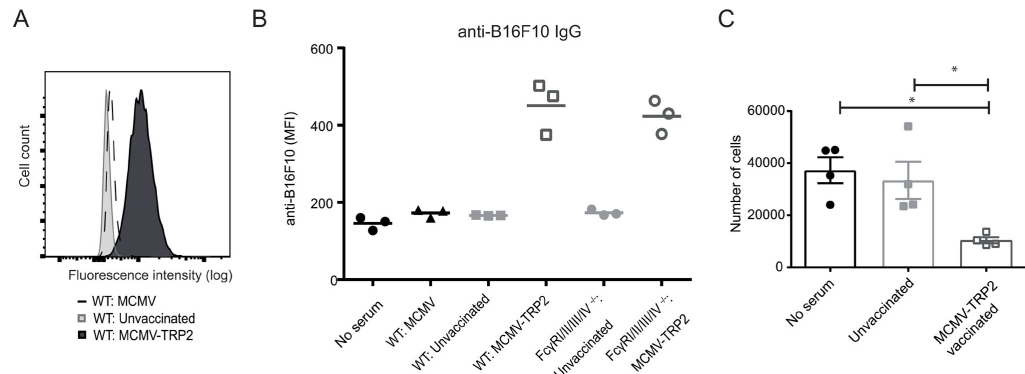


Figure 6: Macrophages mediate antibody-dependent killing of tumor cells. (A) Histogram showing cell surface staining of B16-F10 tumor cells with 1:100 diluted serum from MCMV infected mice (day 14 post infection), MCMV-TRP2 vaccinated mice (day 14 post-vaccination), and unvaccinated mice. Tumor cells were first incubated with serum and subsequently stained with an Alexa488-conjugated anti-mouse IgG specific antibody. (B) B16-F10 tumor-specific staining as described above with no serum and with serum from MCMV infected mice (day 14 post infection), unvaccinated WT and FcγRI/II/III/IV^{-/-} mice, and MCMV-TRP2 vaccinated WT and FcγRI/II/III/IV^{-/-} mice (day 14 after vaccination). (C) B16-F10 tumor cells were incubated with macrophages in absence of serum or in presence of serum from non-vaccinated mice and MCMV-TRP2 vaccinated mice (**p* < 0.05). The number of tumor cells is indicated.

The EIIaCre deleter strain (>20 generations backcrossed on a C57BL/6J background), was a kind gift of Dr. Heiner Westphal. The Flp deleter strain C57BL/6-Tg(CAG-flpe)36Ito/ItoRbrc (>20 generations backcrossed on a C57BL/6J background) was purchased from Jackson Laboratories (Bar Harbor, Me). Mice were routinely checked for their genotype by PCR analysis.

Vaccination, tumor challenge, and cell subset depletion

WT C57BL/6J, FcγRI^{-/-}, FcγRIII^{-/-}, FcγRIV^{-/-} and FcγRIII/IV^{-/-} mice were intraperitoneally (i.p.) inoculated with 2×10^6 pfu MCMV-TRP2 [10] (kindly provided by Ann Hill, Department of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, Oregon, USA). Mice were 14 days post vaccination subcutaneously (s.c.) challenged with 2×10^5 B16-F10 melanoma cells in 100 μl PBS. Tumor growth was measured two or three times a week in three dimensions (width, length and height) using a caliper. Mice were euthanized when tumor size reached >2,000 mm³ in volume or when mice lost >20% of their total body weight (relative to initial body mass). Mice were bled for serum samples at day 7, 14 and 21 days after infection.

To examine germinal center (GC) responses, WT C57BL/6J and FcγRI/II/III/IV^{-/-} mice were infected with 5×10^4 pfu MCMV-Smith or 2×10^6 pfu MCMV-TRP2 and at day 14 post-infection spleens were harvested and the plasma cells, GC B cells, T follicular helper (T_{FH}) cells, GC T_{FH} cells were analyzed by flow cytometry.

For specific cell subset depletion, vaccinated and unvaccinated mice were injected with either 200 μl clodronate liposomes intravenously (Liposoma B.V.), 100 μg anti-NK1.1, or 100 μg anti-Ly6G (1A8) (Bioxcell) intraperitoneally once a week starting two days before tumor inoculation to deplete phagocytic macrophages, NK cells or neutrophils, respectively. Blood samples were analysed by flow cytometry to determine the efficacy of the cell subset depletion.

Flow cytometry

Blood cells, tumor cells and splenocytes were phenotyped by flow cytometry as described [4, 30]. Briefly, single cell suspensions of spleens were prepared by mincing the spleens through 70-μm cell strainers (BD Biosciences). For FcγR cell surface staining of tumor-infiltrated immune cells, tumors were isolated from mice that were transcardially perfused with PBS-EDTA. Subsequently, tumors were mechanically disrupted in small pieces, incubated for 15 minutes at 37° C in Iscove's modified Dulbecco's medium (IMDM)–containing Liberase (Roche), minced through 70 μm cell strainers and resuspended in staining buffer (PBS + 2% FSC + 0.05% sodium azide) containing antibodies for

30 minutes at 4° C. Erythrocytes were removed by hypotonic lysis.

Fluorochrome-labelled antibodies specific for CD3, CD4, CD8, CD11b, CD95, CD138, GL7, Ly6C, Ly6G, F4/80, FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), FcγRIV (CD16.2), NK1.1 and IgD, were purchased from BD Biosciences, Biolegend, eBioscience and R&D Systems. Samples were acquired with LSRII flow cytometers (BD Biosciences) and data was analyzed with FlowJo software (TreeStar).

To stain B16-F10 tumor cells with serum, cells were plated in 48 well plates and incubated with serum obtained from MCMV-TRP2 and MCMV vaccinated mice at day 14 post-infection. Subsequently, cells were stained with Alexa488-conjugated goat anti-mouse as a secondary antibody, and subjected to flow cytometry as described above.

ELISA

MCMV-specific antibodies in serum were detected by ELISA as described [31]. Briefly, 96-well plates (Nunc maxisorp) were coated overnight 4° C with NIH-3T3 derived MCMV-Smith in bicarbonate buffer. Plates were then blocked at 37° C with blocking buffer (5 % milk in PBS) for 1 hour. Sera were diluted in 1% milk PBA and incubated in the blocked wells for 1 hour. HRP conjugated anti-IgG antibodies (Southern biotech, Birmingham, USA) were diluted 1:4000 in 1% milk PBS and incubated 1 hour at 37° C. To develop the plates 100 μl of TMB (sigma Aldrich) was added to each well and incubated for 15 min at room temperature after which 100 μl of stop solution (1 M H₂SO₄) was added. Plates were measured using a microplate reader.

ADCP assay

Macrophages (RAW264.7) were incubated with B16-F10 cells in a 2:1 ratio in 96 well plates. Serum from MCMV-TRP2 vaccinated mice, unvaccinated or no serum was added to the wells. 48 hours later the number of tumor cells were determined by flow cytometry.

Statistics

Mann–Whitney tests were used to check statistical significance between groups in antibody titers and number of positive cells. Mantel-Cox rank tests were used to check significance between survival of mice between groups from Kaplan Meier plots.

Author contributions

Performed experiments: HB, HSS, CB, JC, CB, ML, AR, MFF; Participated in designing research: HB, TvH, FO, RA, SV; Wrote the paper: HB, RA, SV.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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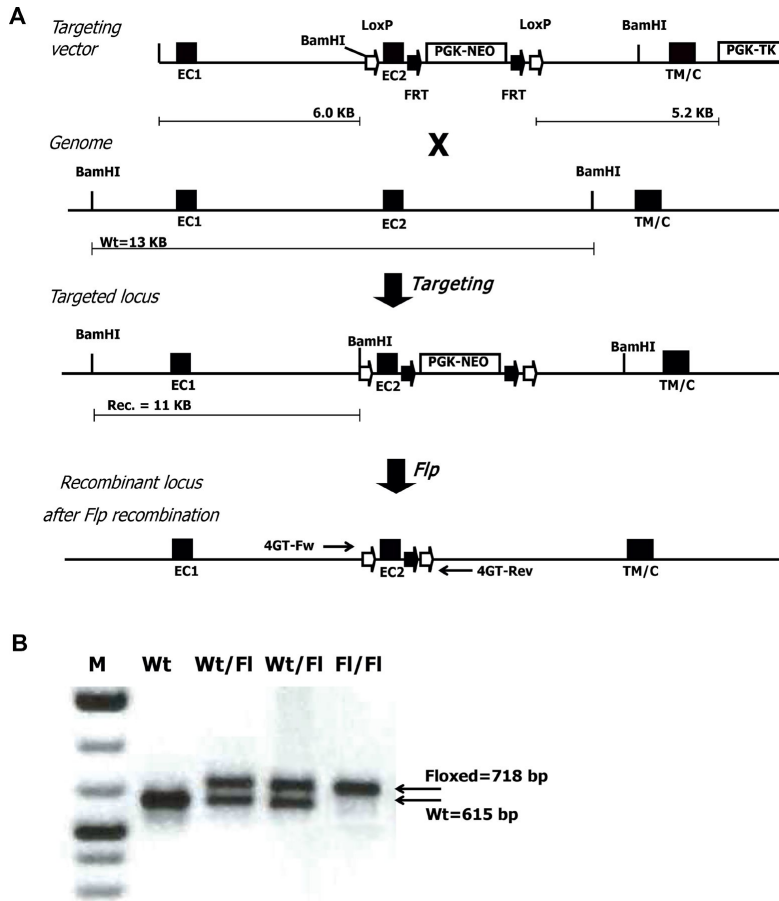
FcγRI expression on macrophages is required for antibody-mediated tumor protection by cytomegalovirus-based vaccines

SUPPLEMENTARY MATERIALS

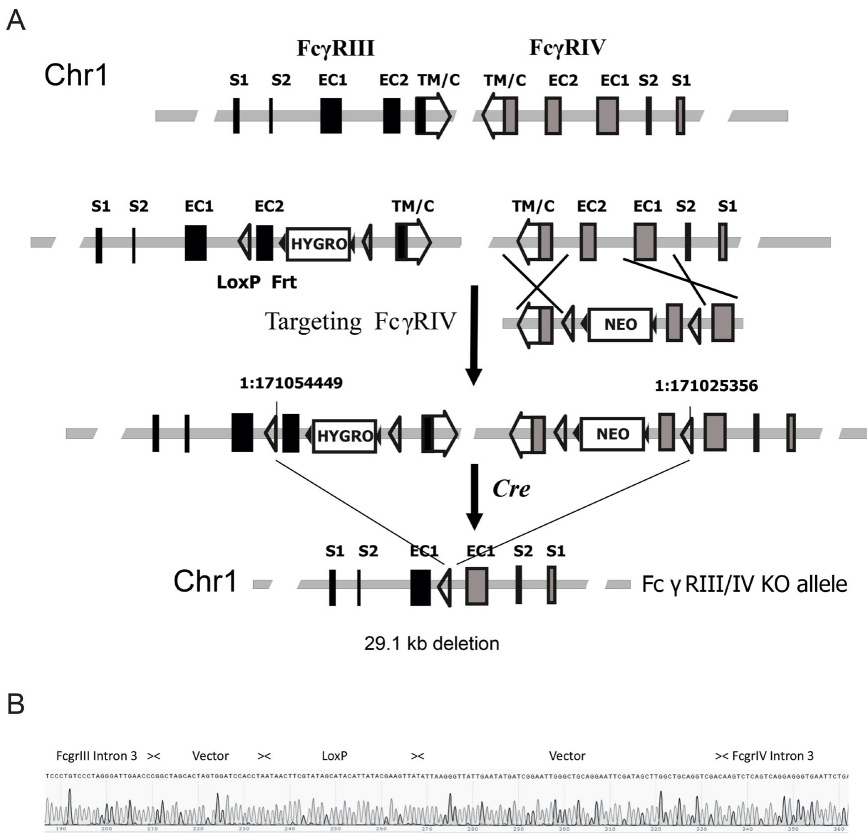
Generation of a novel FcγRIV and FcγRIII/IV^{-/-} mouse models

In the mouse the FcγRIII and FcγRIV genes are located next to each other on chromosome 1. For the generation of the FcγRIII/IV double knock-out (KO) mouse on C57BL/6 background a previously generated Bruce4 ES cell clone with a floxed FcγRIII allele (Fransen MF *et al.*, J Immunol. 2018; 200:2615–26) was targeted with a FcγRIV specific targeting vector that was based on a 11.2 kb genomic fragment containing exon 3, encoding extracellular domain 1 (EC1), exon 4, encoding extracellular domain 2 (EC2), and exon 5, encoding transmembrane and cytoplasmic domain (TM/C) of the FcγRIV gene. This fragment was subcloned by means of *Recombineering* from BAC clone RPCI23-87B18 of the RPCI 23 Female (C57BL/6J) mouse BAC genomic library (BACPAC Resources Center, Children's Hospital Oakland Research Institute, Oakland, California). Recombineering was also used to insert a single LoxP site downstream of the EC2 exon as well as a LoxP-FRT-NEO-FRT cassette upstream of the EC2 exon (Supplementary Figure 1). Clones in which homologous recombination occurred were identified by Southern

blotting (not shown) and subsequently injected in C57BL/6 blastocysts. Recombinant ES cell clones targeted in trans were used to generate a single FcγRIV conditional KO strain (Supplementary Figure 1A) whereas the ES cell clones targeted in cis were used to generate a conditional FcγRIII/IV double KO strain. The obtained chimeras were crossed with WT C57BL/6J mice and the F1 offspring positive for the FcγRIV targeted allele was crossed with a Flp deleter strain to remove the selection marker cassette, resulting in mice with a floxed FcγRIV allele. Flp-mediated recombination was analyzed with PCR (Supplementary Figure 1B). Crossing these mice with the C57BL/6J EIIACre deleter strain resulted in a full FcγRIV deficiency (FcγRIV^{-/-}) and a full FcγRIII/IV double KO strain (FcγRIII/IV^{-/-}) with a deletion of 29.1 kb between the two most distant loxP sites, respectively (Supplementary Figure 2A). The presence of the deletion was confirmed by a specific PCR and DNA sequencing (Supplementary Figure 2B). The absence of FcγRIII and FcγRIV on the surface of immune cells was confirmed by flow cytometry using FcγRIII and FcγRIV specific antibodies (data not shown). FcγRIII/IV^{-/-} mice developed normally and showed normal breeding characteristics.



Supplementary Figure 1: Generation of a floxed FcγRIV allele by gene targeting in embryonic stem cells. (A) Exons are represented by closed rectangles. EC1 and EC2: extra cellular domain1 and 2 encoding exons; TM/C: transmembrane and cytoplasmic domain encoding exon. Open rectangles: selection marker genes required for gene targeting. **(B)** PCR analysis of genomic DNA from tail biopsies of offspring from an inter-cross of heterozygous floxed FcγRIV mice. Use of primer pair 4GT-Fw and 4GT-Rev results in the amplification of a 718 bp fragment of the floxed FcγRIV allele and the amplification of a 615 bp fragment of the wild-type (Wt) FcγRIV allele. Lane FI/FI: homozygous floxed FcγRIV mouse, lane Wt: Wt mouse, lanes Wt/FI: heterozygous floxed FcγRIV mice. Primer sequences: 4GT-Fw: GGAGGCCAGAAGACTTTTA 4GT-Rev: GGAAGTGTGTTTGAAGGATT.



Supplementary Figure 2: Generation of the *FcγRIII/IV*^{-/-} mouse model. (A) The *FcγRIII/IV*^{f/f} mice were crossed with *EllaCre* deleter mice and *FcγRIII/IV* KO offspring with a 29.093 bp deletion was selected. The locus is shown in reverse orientation in relation to the chromosomal nucleotide numbering. The exact location of the borders of the deletion (NC_000067.6:g.171054449_1:171025356del according to HGVS nomenclature) on chromosome 1 is depicted according to the mouse reference genome build GRCm38.p3 (C57BL/6J) provided by the Genome Reference Consortium. Exons are represented by closed rectangles. S1 and S2: signal peptide 1 and 2 encoding exons; EC1 and EC2: extra cellular domain1 and 2 encoding exons; TM/C: transmembrane and cytoplasmic domain encoding exon. Open rectangles: selection marker genes required for gene targeting. Top line: WT locus; second line: *FcγRIII* floxed locus; third line: *FcγRIII/IV* double floxed locus, bottom line: *FcγRIII/IV*^{-/-} locus. (B) Core sequence of a unique 477 bp PCR fragment flanking the remaining LoxP site and bridging the 29.1 kb deletion in *FcγRIII/IV*^{-/-} mice. Primer sequences: *FcγRIII* Geno Fw: GAGGGCATCCGATTTCATTA *FcγRIV* Null Fw TAGACTAAAGGTCATGTGTGATC

CHAPTER 4

4

High FcγR Expression on Intratumoral Macrophages Enhances Tumor-Targeting Antibody Therapy.

Hreinn Benonisson, Heng Sheng Sow, Cor Breukel, Jill W. Claassens, Conny Brouwers, Margot M. Linssen, Marieke F. Fransen, Marjolein Sluijter, Ferry Ossendorp, Thorbald van Hall, and J. Sjef Verbeek

High FcγR Expression on Intratumoral Macrophages Enhances Tumor-Targeting Antibody Therapy

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Therapy with tumor-specific 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⁺Ly-6C⁺ 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. *The Journal of Immunology*, 2018, 201: 3741–3749.

Tumor-targeting Ab therapy is an established treatment in cancer, and Abs such as anti-Her2/neu (ERBB) (trastuzumab), 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/neu (ERBB) 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 complement-dependent 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 C region of the Ab to FcγR. FcγR binding initiates downstream effector functions directed toward tumor cells, including Ab-dependent cell-mediated cytotoxicity, Ab-dependent 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 tumor-targeting 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 *t*_{1/2} 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

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Abbreviations used in this article: ADCP, Ab-dependent cell phagocytosis; KO, knockout; LUMC, Leiden University Medical Center; WT, wild type.

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^{-/-} mice (29), FcγRIII/IV^{-/-} mice (30), and the novel generated FcγRIII/IV^{fl/fl} (Supplemental Fig. 1). FcγRIII/IV^{-/-} were generated by crossbreeding FcγRI^{-/-} and FcγRIII/IV^{-/-} mice. The EllaCre deleter strain ($n > 20$ on C57BL/6J background) was a kind gift of Dr. H. Westphal. The Flp deleter strain C57BL/6-Tg(CAG-loxP)36It0/ItoRbr, 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. Pasagüé, and the Ncr1-Cre strain was a kind gift of Dr. V. Sexl. Both strains were backcrossed into C57BL/6J 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'-TGATAGGAGAATCAGGAGTCAAGGTTAGCTTCAACTATAGTGCCTTCCAATTCCAGGCCGCTAGCATATACTTCGTATAGCATACATTATACGAAGTTATAACCTGGCTTTGTGAGACCCCTGTGAAAAAAGAGATTTCTAGAGCTGGGTGTGGTGATCG-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'-GGTTTAGAAACCGGGAGAG-3', F3_cr_AB reverse: 5'-GACAGCCTTTCAGGGAAGT-3'; F4_genotype null forward: 5'-TAGACTAAAGGTCATGTGTGATC-3', F4_genotype forward: 5'-GGAGGCCAGAGAAGACTTTTA-3', F4_genotype reverse: 5'-GGAAGCTGTTTGGAGGAT-3'; MRP8 forward: 5'-CGGTGATGCAACGAGTGATGAGG-3', MRP8 reverse: 5'-CCAGAGAGCGAAATCCATCGCTCG-3'; Ncr1 forward: 5'-GACCATGATGCTGGGTTGGCCCATGATG-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^{-/-}, FcγRIII/IV^{fl/fl}, 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 (6×10^7 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 mm³ 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 aminocaproic acid-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 XMGI.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 TLR7/8 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.

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.

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.

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

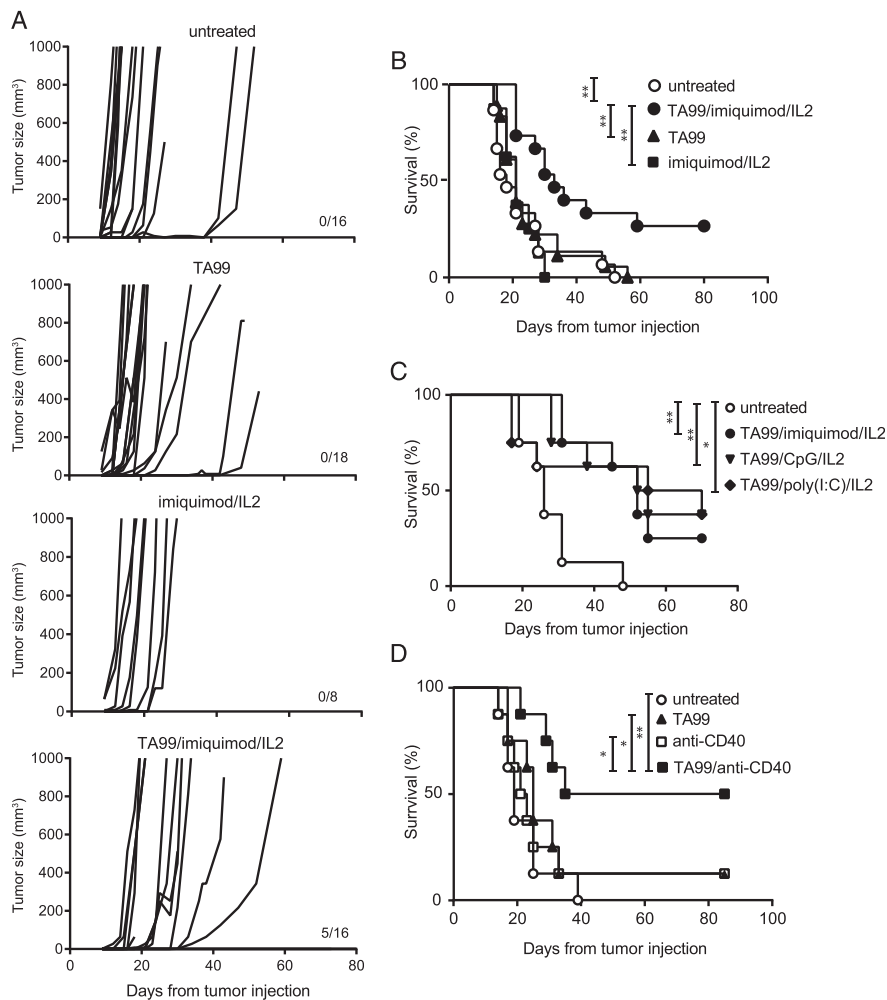


FIGURE 1. Imiquimod cream and IL-2 enhances the therapeutic efficacy of TA99 in B16F10 mouse model of melanoma **(A)**. Tumor growth curves of B16F10 melanoma treated with TA99 alone or combinations of imiquimod/IL-2 or TA99/imiquimod/IL-2. Numbers in each graph represent fraction of surviving mice at 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.

these myeloid cells displayed the highest Fc γ R 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 Fc γ Rs, including the involved high-affinity Fc γ RI (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 Fc γ R on intratumoral inflammatory macrophages.

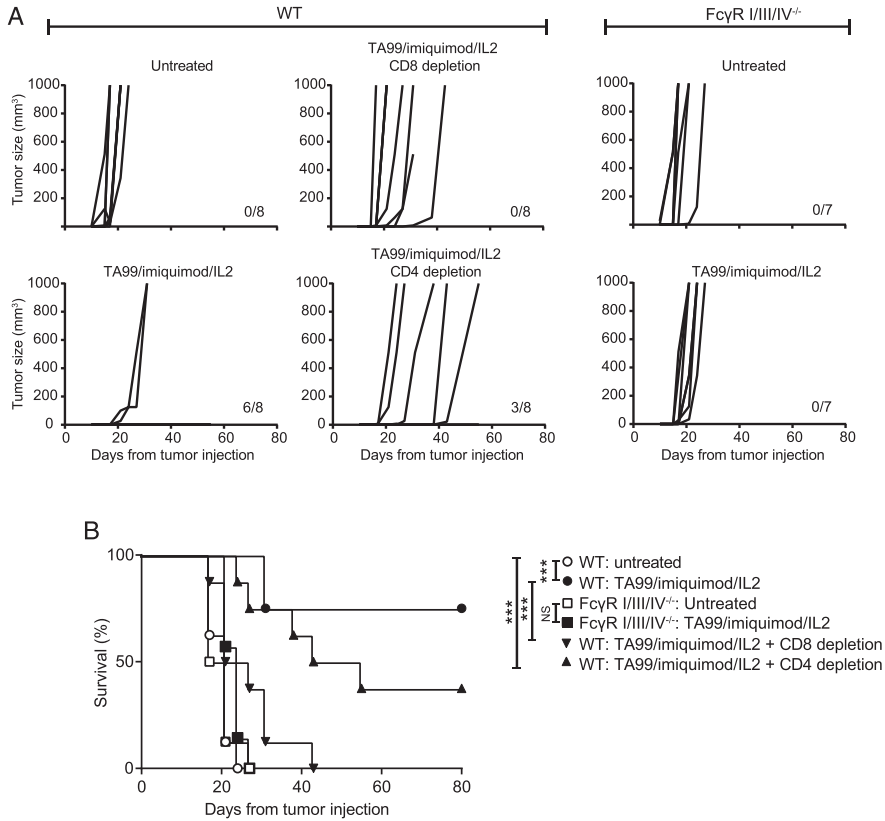


FIGURE 2. The efficacy of TA99/imiquimod/IL-2 combination therapy depends on FcγR and CD8⁺ T cells. **(A)** Tumor growth curves of B16F10 melanoma in WT mice (left panels) or in FcγRI/III/IV^{-/-} mice (right panels). WT mice were also depleted for CD4⁺ or CD8⁺ T cells before the start of the TA99/imiquimod/IL-2 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.

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 macro-

phages, 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

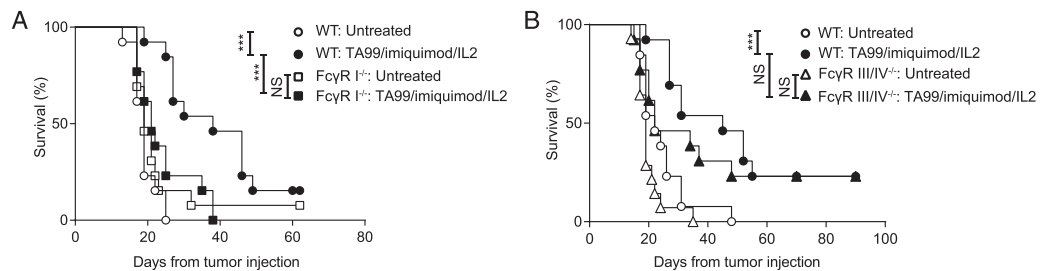


FIGURE 3. TA99/imiquimod/IL-2 combination therapy is abrogated in FcγRI^{-/-} mice and diminished in FcγRIII/IV^{-/-} mice. **(A)** Kaplan–Meier survival curves of B16F10 melanoma in WT mice and FcγRI^{-/-} mice. Mice were treated with TA99/imiquimod/IL-2. **(B)** Kaplan–Meier survival curves of B16F10 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.

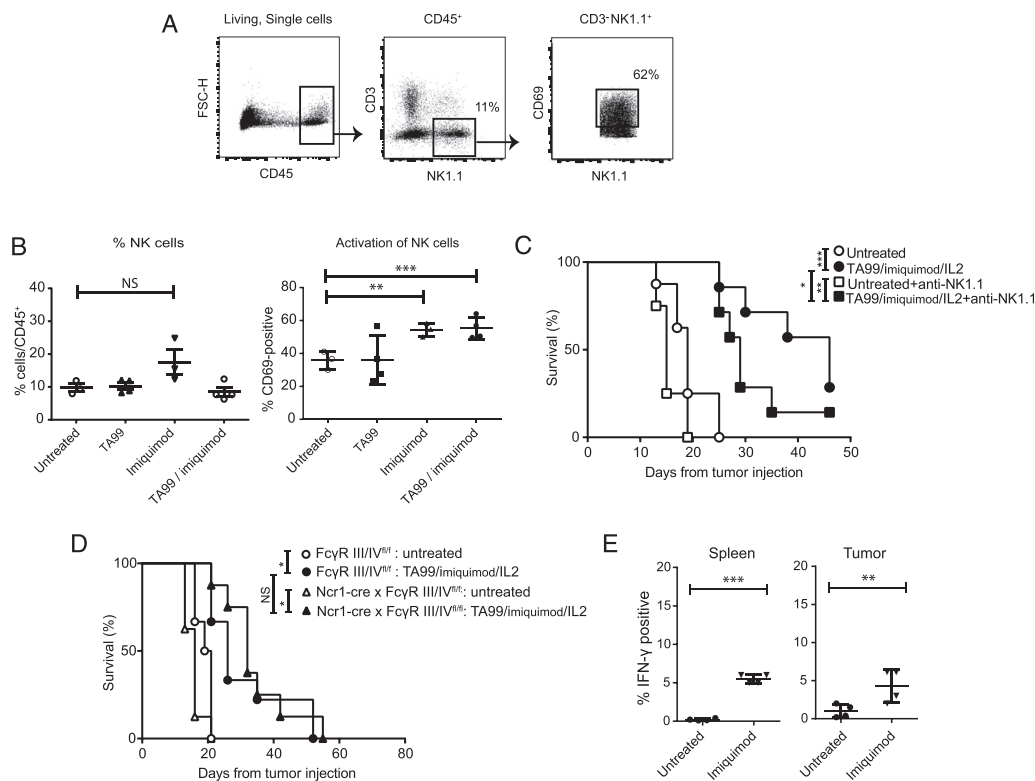


FIGURE 4. NK cells contribute to the efficacy of TA99/imiquimod/IL-2 therapy independent of FcγRIII (**A**). B16F10 tumors were harvested from mice and enzymatically dispersed before analysis by flow cytometry. Gating strategy is shown for NK cells. (**B**) Tumor infiltration frequency and activation 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 $\times$ FcγRIII/IV^{fl/fl} mice) and FcγRIII/IV^{fl/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.

tumor was transplanted. Different laboratories reported contradictory results from their analysis of the role of activating FcγR using different panels of FcγR^{-/-} 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

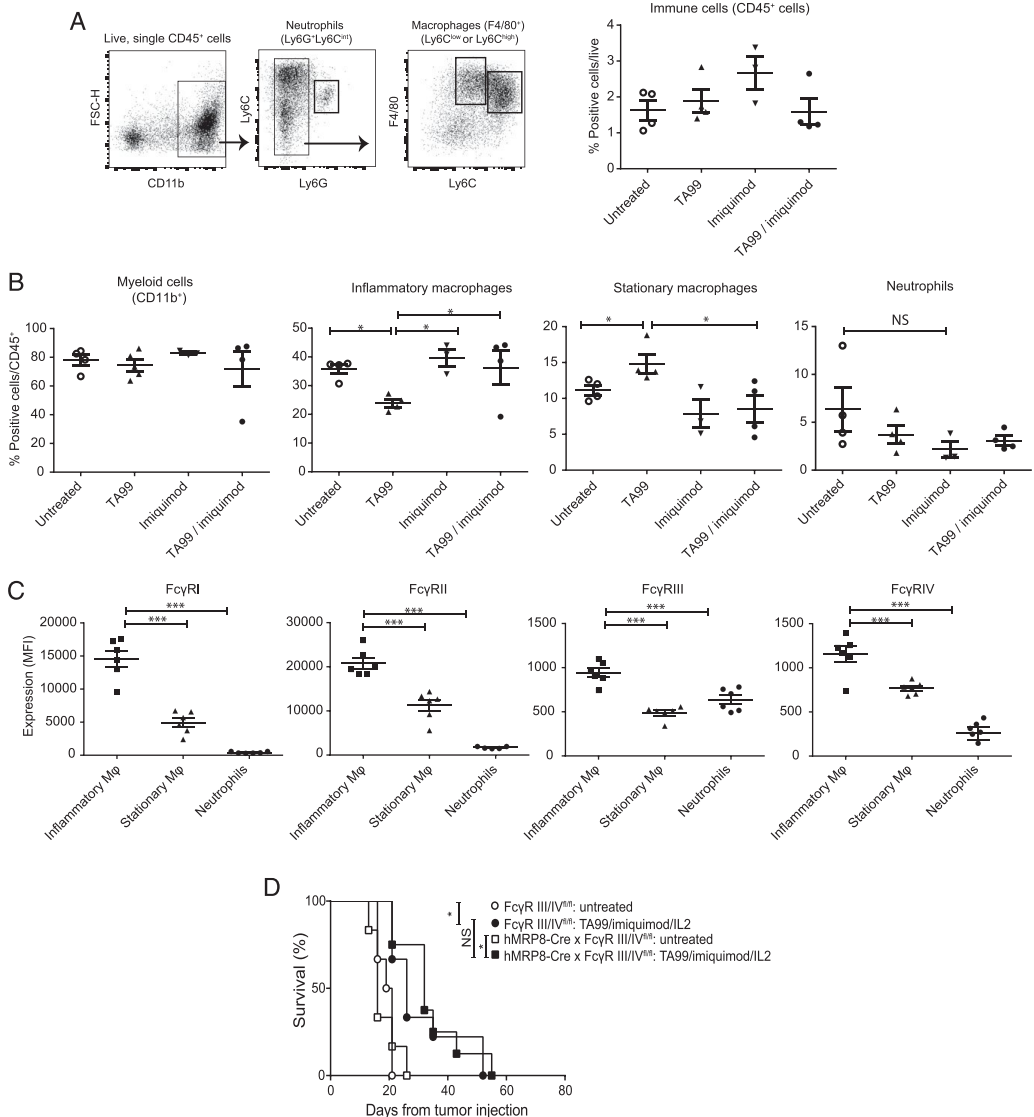


FIGURE 5. Imiquimod sustains the infiltration of highly FcγR-positive inflammatory macrophages. **(A)** B16F10 tumors were harvested from mice and enzymatically dispersed before analysis by flow cytometry. Gating strategy is shown for myeloid cells, and frequencies of all CD45⁺ cells out of live cells are shown. **(B)** B16F10 melanomas were treated with imiquimod (3 d before) or with TA99 (2 d 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-6C^{high}), stationary macrophages (CD45⁺CD11b⁺F4/80⁺Ly-6C^{low}), and neutrophils (CD45⁺CD11b⁺Ly-6G⁺Ly-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 FcγRI, FcγRII, FcγRIII, and FcγRIV on tumor-infiltrating 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 FcγRIII and IV on neutrophils (hMRP8Cre × FcγRIII/IV^{−/−} mice) and FcγRIII/IV^{fl/fl} control mice. Experiment performed twice with similar outcomes, shown are six to eight animals per group. **p* < 0.05, log-rank analysis.

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

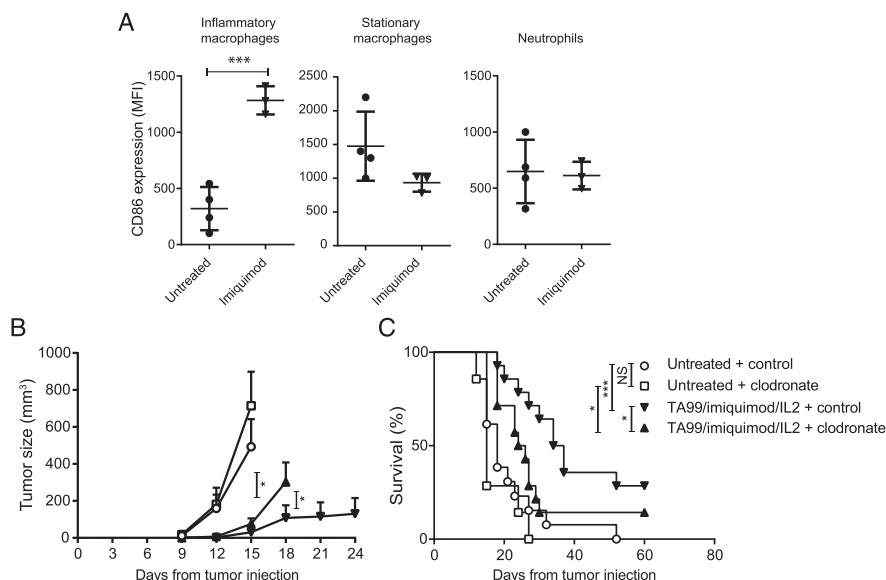


FIGURE 6. Macrophages are indispensable for TA99/imiquimod/IL-2 therapy in B16F10 melanoma model. **(A)** Expression of CD86 by tumor-infiltrating inflammatory macrophages, stationary macrophages, and neutrophils in WT mice measured by flow cytometry. Means and SEM shown from four animals per group. *** $p < 0.001$, 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 mm³. Results are shown from two pooled experiments, 14–16 mice per group. One-way ANOVA (B) and log-rank analysis (C). * $p < 0.05$, *** $p < 0.001$.

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.

Disclosures

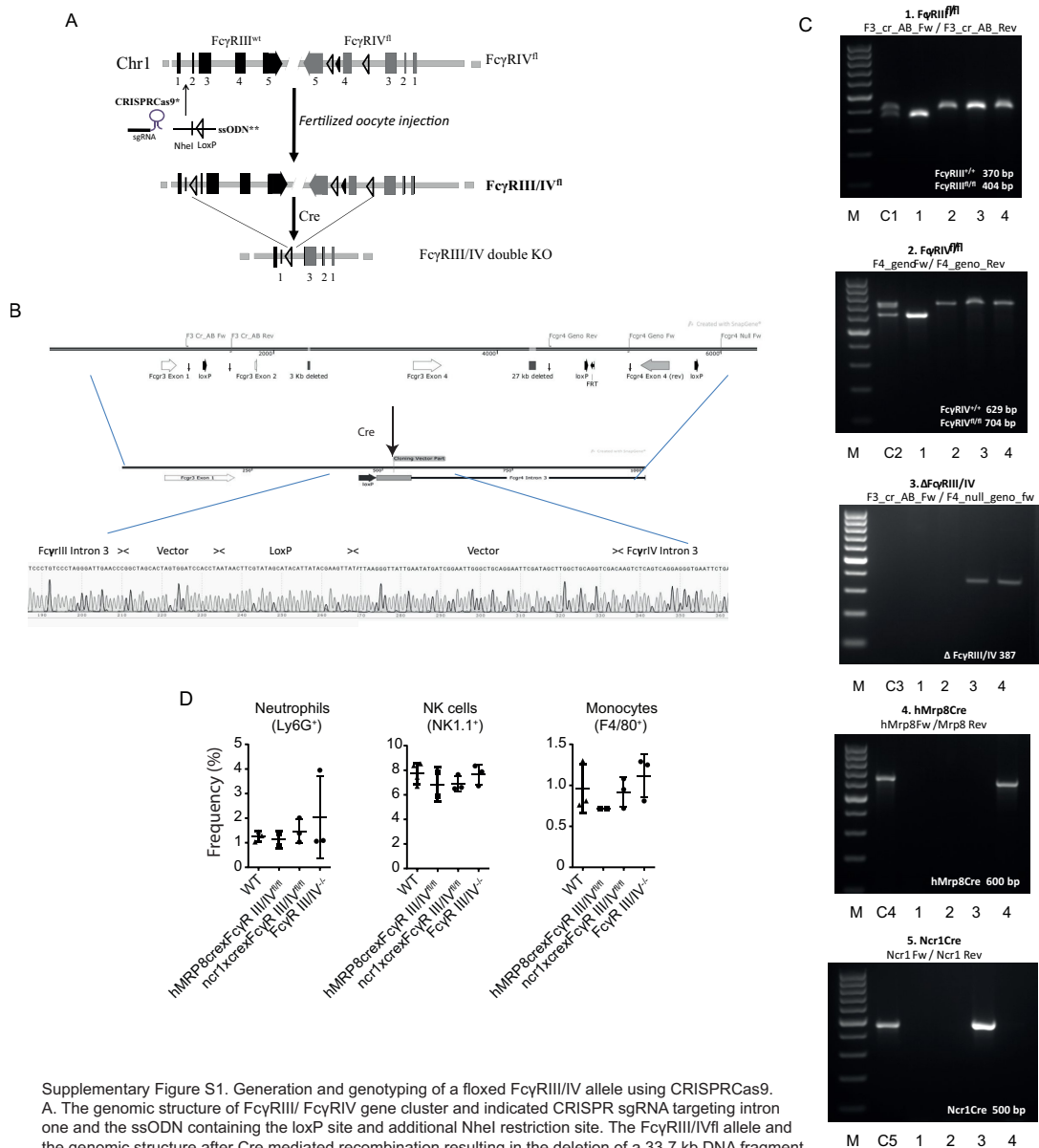
The authors have no financial conflicts of interest.

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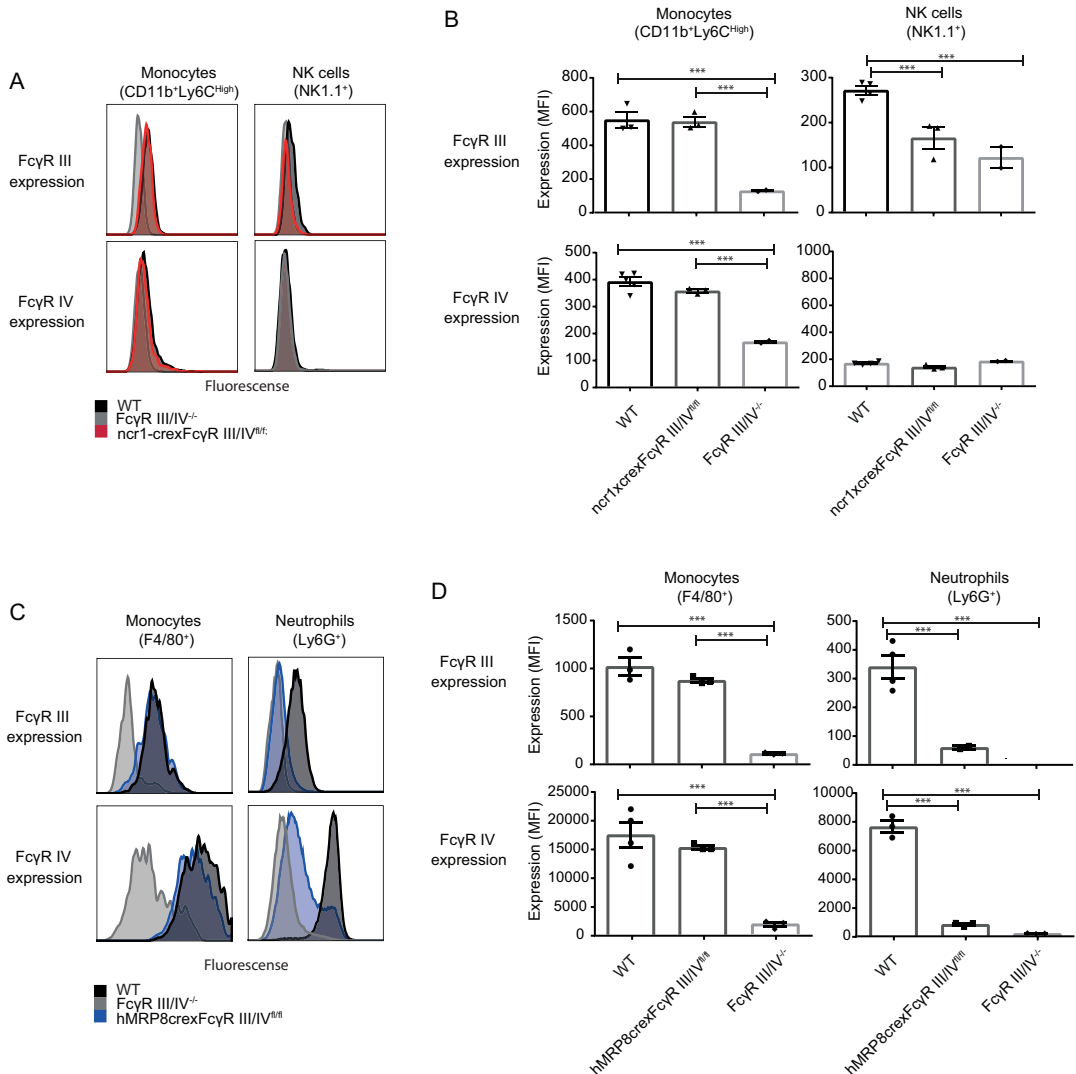
Supplementary Figure S1



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/IV^{fl} 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: Ncr1Cre;FcyRIII/IV^{fl/fl}; 4: hMrp8Cre;FcyRIII/IV^{fl/fl}. Control DNA was isolated from C1: FcγRIII^{fl/fl}; C2: FcγRIV^{fl/fl}; C4: hMrp8Cre; C5: Ncr1Cre. C3: H₂O 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

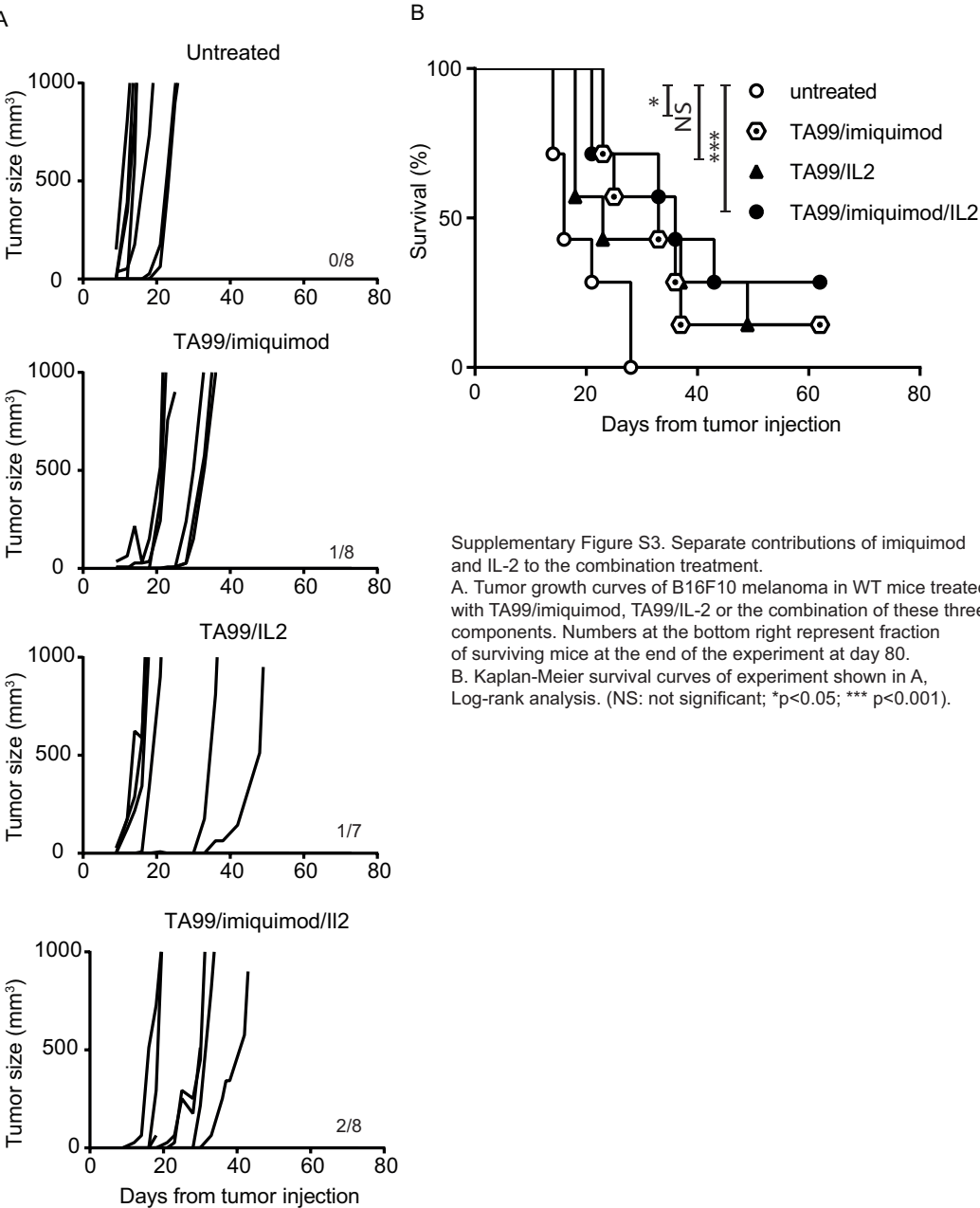


Supplementary Figure S2. Expression analysis of FcγRs in the conditional knockout mice.

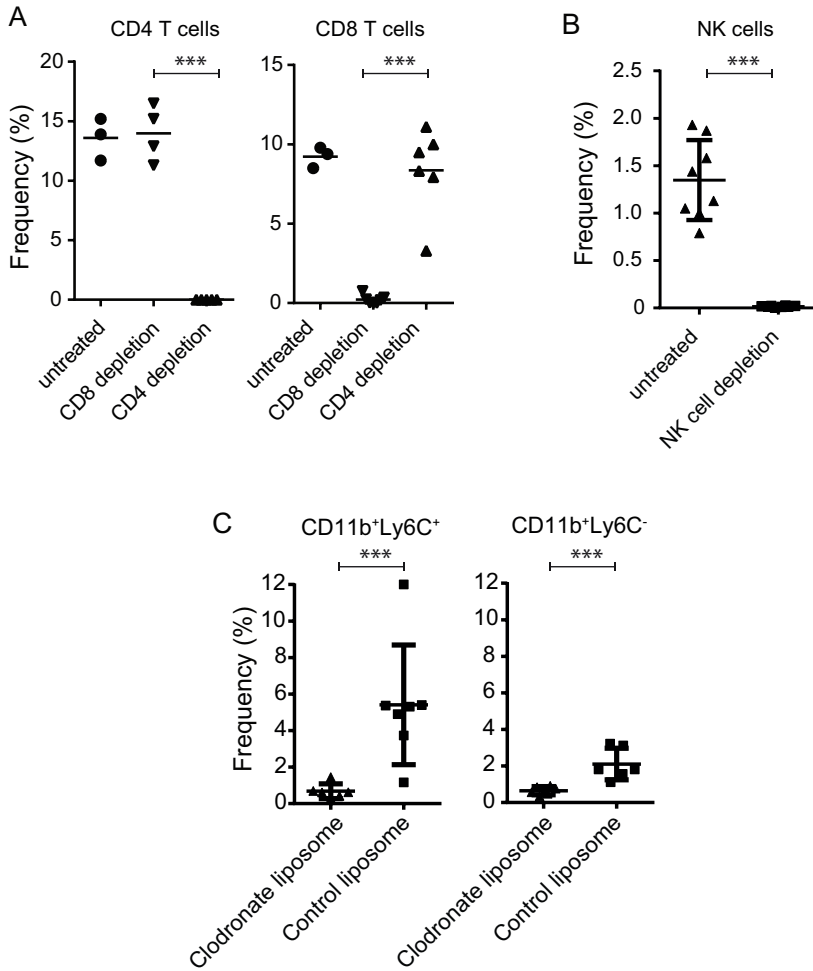
A-B. Expression of FcγRIII (stained with monoclonal antibody clone AT154-2) and FcγRIV measured by flow cytometry on monocytes and NK cells in blood of WT C57BL/6, Ncr1-cre x FcγRIII/IV^{fl/fl} and FcγRIII/IV^{-/-} mice. Overlapping histograms (A) and average mean fluorescence (MFI) depicted in bar graphs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

Supplementary Figure S3



Supplementary Figure S4



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⁻.

CHAPTER 5

5

CD3-Bispecific Antibody Therapy Turns Solid Tumors into Inflammatory Sites but Does Not Install Protective Memory.

Hreinn Benonisson, Işıl Altıntaş, Marjolein Sluijter, Sandra Verploegen, Aran F. Labrijn, Danita H. Schuurhuis, Mischa A. Houtkamp, J. Sjef Verbeek, Janine Schuurman and Thorbald van Hall

CD3-Bispecific Antibody Therapy Turns Solid Tumors into Inflammatory Sites but Does Not Install Protective Memory

Hreinn Benonisson¹, Işıl Altıntaş², Marjolein Sluijter³, Sandra Verploegen², Aran F. Labrijn², Danita H. Schuurhuis², Mischa A. Houtkamp², J. Sjef Verbeek¹, Janine Schuurman², and Thorbald van Hall³

Abstract

Immunotherapy of cancer with CD3-targeting bispecific antibodies (CD3 bsAb) is a fast developing field, and multiple tumor-associated antigens (TAA) are evaluated for hematologic and solid malignancies. The efficacy of these CD3 bsAb is usually examined in xenograft mouse tumor models with human T cells or in genetically engineered mouse models, where human TAA are introduced. These models often fail to fully recapitulate the natural tumor environment, especially for solid cancers, because of interspecies differences. Here, we investigated the systemic and intratumoral effects of a mouse CD3 bsAb in a fully immune-competent mouse melanoma model. Systemic administration of 0.5 mg/kg antibody induced a brief overall T-cell activation that was selectively sustained in the tumor microenvironment for several days. A

fast subsequent influx of inflammatory macrophages into the tumor microenvironment was observed, followed by an increase in the number of CD4⁺ and CD8⁺ T cells. Although the capacity to directly kill melanoma cells *in vitro* was very modest, optimal tumor elimination was observed *in vivo*, even in the absence of CD8⁺ T cells, implying a redundancy in T-cell subsets for therapeutic efficacy. Finally, we took advantage of the full immune competence of our mouse model and tested immune memory induction. Despite a strong initial immunity against melanoma, treatment with the CD3 bsAb did not install protective memory responses. The observed mechanisms of action revealed in this immune-competent mouse model might form a rational basis for combinatorial approaches.

Introduction

The idea of using the cytotoxic capacity of T cells through CD3-targeting bispecific antibodies (CD3 bsAb) to kill tumor cells dates back to 1985 (1), and it took 30 years before both the FDA and European Medicines Agency approved blinatumomab, a CD19-directed T-cell engager (BiTE), for the treatment of refractory B-cell precursor acute lymphoblastic leukemia (ALL) patients (2). The first clinically registered CD3 bsAb was actually catumaxomab, binding the epithelial adhesion molecule EpCAM on carcinomas (3, 4), which was recently voluntarily withdrawn from the market for commercial reasons. Bridging T cells and tumor cells by immunoglobulin (Ig)-based biologicals can induce dramatic regression of advanced-stage malignancies and even lead to complete remission (2, 5–7). CD3 bsAb trigger the CD3 surface receptor on T cells by binding to their second target protein

expressed on tumors. Consequently, all available T cells can bind to target-expressing cells via the CD3 bsAb, irrespective of the peptide/MHC specificity of their T-cell receptor (TCR).

Currently, more than 25 different CD3 bsAb are in clinical development for the treatment of hematologic malignancies, for example, by targeting CD19, CD20, CD33, and CD123, or solid cancers, for example, by targeting EpCAM, HER2, PSMA, and CEA (8). A multitude of other tumor-associated targets are explored in preclinical investigations. Many different bispecific formats have been developed over the years, ranging from Ab fragment-based bispecifics lacking an immunoglobulin constant region (Fc), to IgG-like molecules, in which additional Ab fragments are fused to regular antibody molecules, or bsAbs with regular IgG architecture (9, 10). The CD3 bsAbs in clinical trials either lack an Fc region, such as blinatumomab, or contain an engineered Fc domain to minimize interaction with Fc receptors (8, 11). The presence of an Fc domain increases the *in vivo* half-life through binding to the neonatal Fc receptor (FcRn; refs. 12–15).

The activity and sensitivity of CD3 bsAbs are commonly evaluated *in vitro* using human cancer cell lines or 3D spheroid cultures (16). Further efficacy studies are subsequently performed in small laboratory animals, often xenograft mouse models, in which established human tumor cell lines or fresh tumor samples from patients are transplanted into immune-deficient mice (12, 13, 16–18). Coengraftment of human peripheral blood mononuclear cells or purified T cells is required in these models to supply a source of immune effector cells. Alternatively, immune-deficient mice can be transplanted with human hematopoietic progenitor cells, which generate human leukocytes, the so-called human immune system mice (19). Although these models

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have been valuable for the study of tumor growth inhibition and for comparison of the potency of various bsAb clones, they fail to integrate the complex interplay between tumor cells and immune cells, which is of particular importance in the microenvironment of solid cancers. Moreover, long-term effects of CD3 bsAb cannot be evaluated due to induction of graft-versus-host responses in these immune-deficient mice (20). Recently, transgenic mice that coexpress human CD3 ϵ (hCD3 ϵ) on their T cells, in addition to mouse CD3 ϵ , were exploited in this research field, enabling the use of endogenous T cells in immune-competent mice (21, 22). Although hCD3 ϵ transgenic mice harbor a relatively normal immune system and allow analysis of immune–tumor cell interaction, they still lack a natural distribution of the selected TAA in mouse tissues, hampering examination of “on-target, off-tumor” effects via target expression in host tissues (21–24). Moreover, induction of neutralizing anti-human IgG antibodies prevents long-term evaluation in these mouse models.

Here, we report, for the first time, the immunologic effects of a CD3 bsAb in a completely syngeneic mouse tumor model. We performed immunotherapy of B16F10 melanomas in fully immune-competent wild-type C57BL/6 mice with an effector function silenced (inert) mouse IgG2a bsAb, targeting mouse CD3 ϵ and mouse tyrosinase-related protein 1 (TRP1 or gp75), that is expressed on the surface of transformed melanoma cells and healthy melanocytes of the skin, just like in humans (25). We generated these mouse bsAbs with regular IgG architecture using controlled Fab-arm exchange (cFAE) which, apart from applications for human antibody therapeutics, can also be applied to efficiently generate rodent bispecifics (26–28).

In this tumor model, we observed a systemic T-cell activation that was selectively sustained in the tumor microenvironment for at least 4 days, coinciding with influx of inflammatory macrophages. Strikingly, the strong initial elimination of melanomas was not accompanied by induction of protective long-term immunity, as surviving mice were still vulnerable to a rechallenge with B16F10 melanoma cells. Interestingly, our syngeneic mouse model revealed a fast tumor influx of innate immune cells such as inflammatory macrophages before the arrival of additional CD4 $^{+}$ and CD8 $^{+}$ T-cell subsets. Our data demonstrate that antitumor activity of CD3 bsAb *in vivo* is associated with direct tumor kill and a complex interplay between immune cell subsets.

Materials and Methods

Mice

C57BL/6J mice were purchased from The Charles River Laboratories. All mouse experiments were performed at the animal facility of the Leiden University Medical Center (LUMC), the Netherlands. The health status of the animals was monitored over time, and all animals were tested negative for agents listed in the Federation of European Laboratory Animal Science Associations (FELASA) guidelines for specific pathogen-free mouse colonies (29). All mouse studies were approved by the Dutch animal ethics committee (CCD) and the local Animal Welfare Body of the LUMC on the permit number AVD116002015271. 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

Chimeric antibodies were produced as described previously (26). In short, the VH and VL regions of Armenian hamster mAb

145-2C11 (mouse CD3 ϵ -specific; ref. 30), murine mAb TA99 (human and mouse TYRP1/gp75-specific) (25), or human mAb b12 (HIV-1 gp120-specific; ref. 31) were cloned into mouse IgG2a and kappa constant region backbones, that contained the appropriate mutations to enable controlled Fab-arm exchange (cFAE; either F405L-R411T or T370K-K409R; ref. 26) and silence effector functions (L234A-L235A; LALA; refs. 32, 33). Exact amino acid sequences of the 2C11xTA99 bsAb are provided in Supplementary Fig. S1a. All antibodies were transiently produced under serum-free conditions in FreeStyle 293-F or Expi293F cells (Life Technologies), purified by protein A affinity chromatography (MabSelect SuRe; GE Health Care), dialyzed to phosphate-buffered saline (PBS) and sterilized over 0.2- μ m filters. The purity was determined by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or capillary electrophoresis sodium-dodecyl sulfate (CE-SDS), and the concentration was measured by absorbance at 280 nm. Aggregates and degradation products were measured by high-performance size-exclusion chromatography (HP-SEC). Purified antibody batches with low endotoxin levels (<0.1 units/mg IgG) were stored at 2–8°C.

Controlled Fab-arm exchange (cFAE)

Equimolar amounts of relevant mouse IgG2a-LALA-F405L-R411T and IgG2a-LALA-T370K-K409R antibodies were mixed and incubated with 2-mercaptoethylamine (2-MEA; Sigma) at a final concentration of 1 mg/mL per antibody. The final concentration of 2-MEA was 75 mmol/L. The mixtures were typically incubated at 31°C for 5 hours. To remove 2-MEA, the mixtures were buffer-exchanged against PBS using PD-10 desalting columns (5 kDa molecular weight cutoff; GE Healthcare) or dialysis using Slide-A-Lyzer cassettes (10 kDa molecular weight cutoff; Pierce). Samples were stored at 4°C overnight, to allow for the reoxidation of the disulfide bonds. The efficacy of cFAE was assessed by hydrophobic interaction chromatography and depicted in Supplementary Fig. S1b.

Cell lines

The B16F10 melanoma cell line was obtained from the American Type Culture Collection (CRL-6475) and maintained in tissue culture as described (34). KPC3 is a cell line isolated from the genetic pancreatic ductal adenocarcinoma “KPC” mouse model with K-ras^{G12D/+}, p53^{R172H/+} and pancreatic and duodenal homeobox 1 (Pdx-1)-Cre transgenes (35). KPC3-TRP1 cells were generated by transfection of the *TRP1/gp75*-coding plasmid using lipofectamine (Invitrogen), as described (36). This plasmid was provided by Gestur Vidarsson (Academic Medical Center, Amsterdam) and optimized by exchanging the zeocin selection gene with a neomycin selection gene and by replacing the cytomegalovirus (CMV) promoter with the CMV enhancer, chicken beta-actin promoter and rabbit beta-globin splice acceptor site (CAG) promoter. Transfected cells were selected with 400 μ g neomycin for 7 days and cell sorting by fluorescence-activated cell sorting on TRP1/gp75 expression using the TA99 antibody and a secondary Alexa Fluor-labeled anti-mouse IgG (BioLegend).

For every mouse tumor experiment, liquid nitrogen stored vials from the same cell batch were used and passaged once before injection. *Mycoplasma* testing was performed every 2 months by PCR on *in vitro* propagated cultures, which were maintained for maximal 6 months. No additional authentication method was performed. All cells were cultured in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 8%

fetal calf serum (Gibco), 2 mmol/L L-glutamine (Gibco), and 100 µg/mL penicillin and 100 µg/mL streptomycin (Gibco). All cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

In vitro killing assay

B16F10, KPC3, and KPC3-TRP1 cells were irradiated at 6,000 rad and plated in 48-well plates with 30,000 cells per well in 100 µL. CD4⁺ or CD8⁺ T cells were isolated from naïve C57BL/6J mice spleens by nylon wool passage and MACS purification columns (Miltenyi Biotec). The T-cell populations were more than 95% pure, as validated by flow cytometry, and were added to the wells in 50 µL and then bsAb were added in 50 µL. Triton X-100 (20 µL) to wells containing tumor cells alone served as positive control. After 48 hours, 50 µL of supernatant was harvested and transferred to 50 µL of lactate dehydrogenase reaction mix (LDH, Life Technologies). After gently mixing, the plates were incubated at room temperature (RT) in the dark for 30 minutes, and 50 µL stop solution was added before absorbance was measured at 490 and 655 nm. Another 50 µL supernatant from these tests was subjected to interferon gamma (IFNγ) sandwich ELISA according to the manufacturer's instructions (BD Biosciences). Activation of T cells from these tests was measured by flow cytometry.

In vivo tumor treatment

C57BL/6J mice were subcutaneously (s.c.) injected with 1×10^5 B16F10, KPC3, or KPC3-TRP1 cells in 200 µL PBS and treated with i.p. administered 12.5 µg (0.5 mg/kg) of bsAb. CD8⁺ TCR-transgenic T cells specific for the gp100 tumor antigen ("pmel") were transferred into tumor-bearing mice in combination with gp100 long synthetic peptide (human variant) and Aldara adjuvant (3M Pharmaceuticals) and IL2 (Proleukin, Novartis), as described previously (34). Tumor sizes were measured by caliper twice weekly, and mice were euthanized when tumors reached a volume of 1,000 mm³. To rechallenge animals, 1×10^5 B16F10 tumor cells were injected s.c. at the opposite flank.

For depletion of immune cell subsets, C57BL/6J mice were injected i.p. with 100 µg of anti-NK1.1 antibody [mouse IgG2a, clone PK136 (own stock)] to deplete NK cells, 50 µg of anti-CD8 antibody [rat IgG2b, clone 2.43 (own stock)] to deplete CD8⁺ T cells, or 50 µg anti-CD4 [rat IgG2b, clone GK1.5 (own stock)] to deplete CD4⁺ T cells. Depletions were started at days 2 and 5 after tumor inoculation and continued weekly up to the end of the experiment.

Analysis of immune cell composition

When tumors reached a volume of 50 to 250 mm³, mice were treated with bsAb. Tumors and spleens were harvested and analyzed with IHC and flow cytometry. Single-cell suspensions for flow cytometry were made by physically fragmentation and 30 µg/mL Liberase (Roche) for 30 minutes, after which the cells were minced through a cell strainer (BD Biosciences). LIVE-DEAD Fixable yellow dead cell stain kit (Life Technologies) was included in all stainings together with antibodies to Ly6C (HK1.4), Ly6G (1A8), F4/80 (Bm8), NK1.1 (PK135), CD25 (PC61.5) CD45.2 (104), CD11b (M1/70), CD4 (RM4-4), CD8 (53-6.7), CD69 (H1.2F3), and CD3 (145-2C11). Intracellular FoxP3 (FJK-16s) staining was performed according to the manufacturer's instructions (eBioscience). All antibodies were purchased from BD Biosciences, BioLegend/Sony or eBioscience. Samples were mea-

sured in a Fortessa cytometer (BD Bioscience) and analyzed with FlowJo software (Treestar).

IHC of tumors

Harvested tumors were fixed in 4% neutral buffered formalin and after 24 hours dehydrated in 70% alcohol and embedded in paraffin. Formalin-fixed paraffin-embedded (FFPE) 3- to 4-µm sections were stained for CD3e (SP7, rabbit anti-mouse, Abcam), CD4 (EPR19514, rabbit anti-mouse, Abcam), and CD8α (D4W2Z, rabbit anti-mouse, Cell Signaling Technology) using the Ventana Discovery autostainer platform (Ventana Medical Systems). Slides were deparaffinized, subjected to antigen retrieval buffer CC1 (Ventana) at 95°C for 32 minutes, and blocked for endogenous peroxidase using Discovery Inhibitor (Ventana) at 37°C for 8 minutes. All primary antibodies were incubated in Discovery Ab Diluent (Ventana) at 37°C for 32 minutes, and subsequently incubated with Discovery OmniMap-anti-Rabbit horseradish peroxidase (HRP; Ventana) at 37°C for 16 minutes. For the anti-CD8 antibody, signal amplification was applied by subsequent incubations with Discovery AMP HQ (Ventana) and Discovery Amplification anti-HQ HRP (Ventana) at 37°C for 16 minutes. HRP was visualized with Discovery purple (Ventana) at 37°C for 4 minutes. Nuclei were counterstained with Hematoxylin II (Ventana) at 37°C for 16 minutes and Bluing reagent (Ventana) at 37°C for 8 minutes. Slides were mounted in glycerol (DAKO, Code C0563). Macrophages were manually stained by incubation with rat anti-mouse macrophage mAb F4/80 (clone CL:A3-1, IgG2b; Serotec) at RT for 30 minutes, followed by incubation with biotinylated rabbit anti-rat IgG Ab (code number E0467; DakoCytomation) at RT for 30 minutes and alkaline phosphatase-streptavidin (code number K0391; DakoCytomation) at RT for 20 minutes. The alkaline phosphatase reaction was developed using Fast Red (Scytek) in a naphthol-phosphate buffer (Scytek) with 50 mmol/L levamisole (DakoCytomation). After 20 minutes, this reaction was blocked in distilled water. The slides were counterstained with Mayer's hematoxylin and mounted in Kaiser's glycerin.

Slide scanning and image analysis

All immunostained slides were scanned on AxioScan Z1 (Zeiss), and computerized image analysis was performed by OracleBio (Biocity Scotland, UK) using the Indica Labs HALO platform. A customized classifier algorithm was developed within HALO to exclude necrotic areas and any prominent artifacts. Numbers of CD3, CD4, and CD8 cells were quantified using a customized cellular multiplex algorithm and F4/80 staining was quantified as percentage area.

Statistical analysis

Survival between groups was compared using Kaplan-Meier curves and statistical log-rank test (Mantel-Cox). Additional statistical methods are stated in the figure legends. All $P < 0.05$ were considered statistically significant. The different levels of significance were labeled with asterisks, where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

Immunotherapy of mouse melanoma with CD3 bsAb outperforms adoptive T-cell therapy

Previously, we showed that treatment of B16F10 melanoma with the bsAb 2C11xTA99, targeting the mouse CD3 with one arm

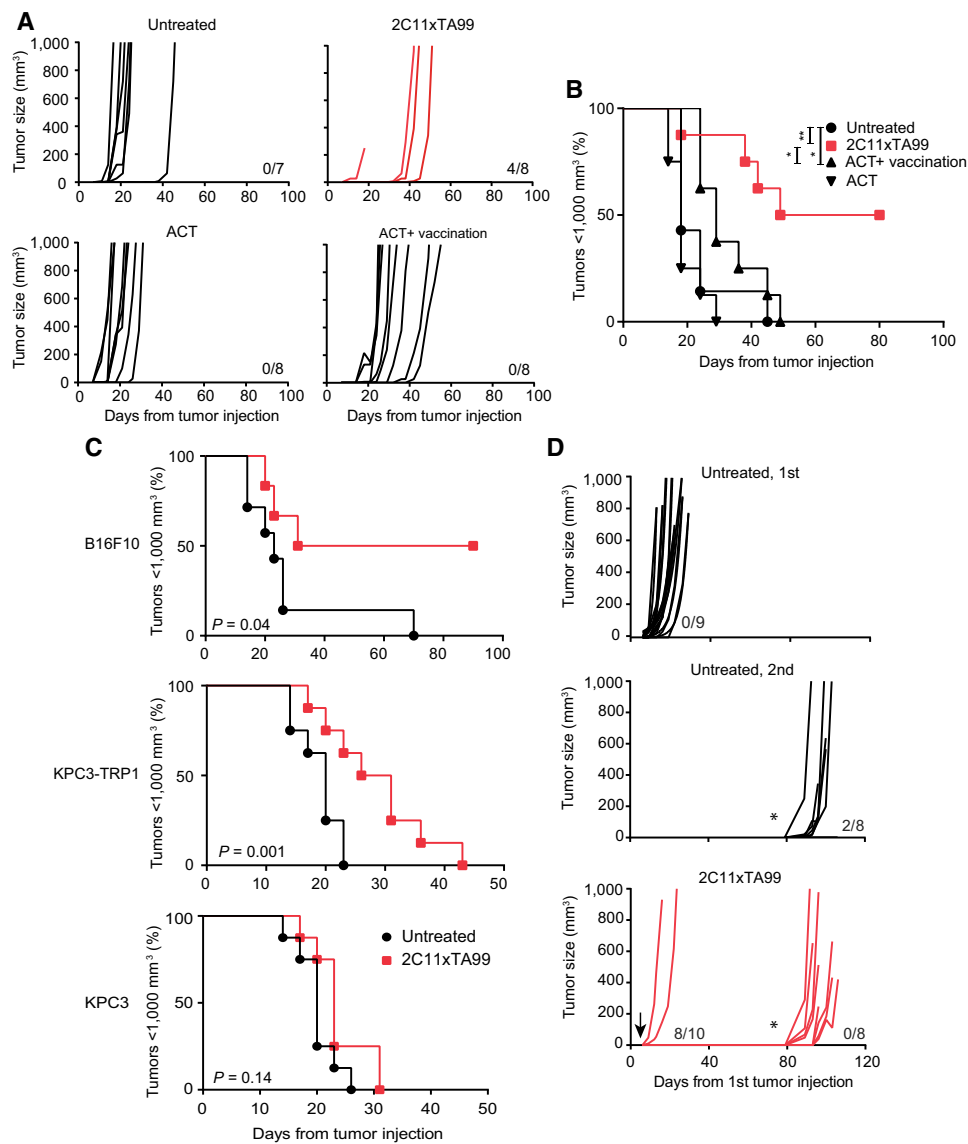


Figure 1. The CD3 bsAb eradicates melanoma in the syngeneic B16F10 model. **A**, Individual tumor growth curves of B16F10 melanomas that were left untreated or were treated with bsAb 2C11xTA99 at days 6 and 9; adoptive T-cell transfer (ACT) with melanoma-specific pmel CD8⁺ T cells alone, at day 5; or with adoptive transfer of pmel T cells, at day 5, activated by gp100 peptide vaccination, at days 6 and 13. Numbers represent the fraction of mice with no tumor outgrowth at the end of the experiment at day 80. **B**, Kaplan-Meier curves of the experiment shown in **A**, with at least 7 mice per group. Shown is one representative experiment out of 2. Log-rank test was used for statistical analysis. **C**, Mice bearing B16F10 melanoma, KPC3-TRP1 pancreas carcinoma, and parental KPC3 pancreas carcinoma treated with bsAb 2C11xTA99 at days 6 and 9. Log-rank test was used for statistical analysis, $n = 8$ in each group. **D**, Individual tumor growth curves of untreated mice (first) or mice treated with bsAb 2C11xTA99 at days 6, 9, and 12 (indicated by the arrow). Successfully treated mice and a second cohort of untreated mice (second) were injected with B16F10 at day 70, indicated by asterisks. Numbers indicate the fraction of mice without tumors.

and the surface melanocyte differentiation protein TRP1 with the other arm, resulted in significant survival rates (26). This mouse IgG2a-based bsAb contains a full Fc region with silenced (inert) effector function. We now expanded on these data by directly comparing the therapeutic efficacy with a previously established cellular immunotherapy protocol, consisting of transfer of naïve TCR-transgenic, gp100-specific, CD8⁺ T cells into melanoma-bearing animals in combination with a long synthetic peptide vaccine (34). This cellular immunotherapy protocol induced delayed tumor outgrowth, but no long-term survival of mice (Fig. 1A and B). In contrast, 3 injections of bsAb 2C11xTA99 at days 6, 9, and 12 at a dose of 0.5 mg/kg resulted in delayed tumor outgrowth in all mice and long-term survival in 50% of the group (Fig. 1A and B). In a previous study, we showed that control antibodies lacking the TA99 tumor-targeting arm failed to control tumor outgrowth (26). These results highlight the potency of CD3 bsAb for immunotherapy of solid cancers even in a T-cell-poor tumor microenvironment such as the B16F10 model (37).

To further substantiate these results, the efficacy of the CD3 bsAb was tested in a syngeneic pancreatic adenocarcinoma model, as these lesions are notoriously enriched in fibroblastic microen-

vironment with little T-cell infiltration (38). Stable expression of TRP1 by KPC3 pancreatic tumor cells was achieved after transfection (Supplementary Fig. S2a). The KPC3 line was derived from the tumor-prone KPC mouse with activated *K-ras* and mutant *p53* genes (35). CD3 bsAb therapy indeed induced a significant delay in tumor outgrowth of KPC3-TRP1 tumors, but not of parental KPC3 tumors, while half of the mice with B16F10 tumors displayed long-term survival in this 2 injection protocol (days 6 and 9; Fig. 1C), showing therapeutic efficacy in a second, T-cell-poor, tumor model (38).

We then took advantage of the fact that our mouse tumor model is completely syngeneic, including immune cells, tumor cells, and therapeutic compound, and investigated long-term immunity. Long-term surviving mice that successfully eliminated B16F10 melanomas after treatment with the CD3 bsAb were rechallenged with the same tumor cells in the opposite flank at day 70. These secondarily injected melanomas grew progressively and at a similar pace as in a second cohort of naïve mice, implying that successful treatment with CD3 bsAb did not install protective immunity after tumor clearance (Fig. 1D). Together, these results indicate that CD3 bsAb induce a potent antitumor response, but do not induce immunologic memory in this particular mouse tumor model.

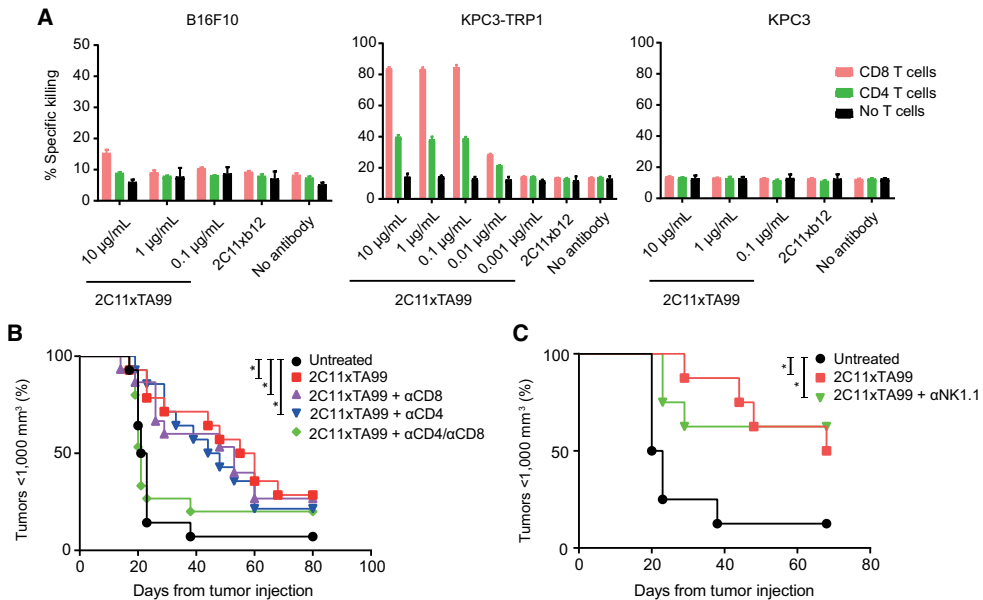


Figure 2.

CD8⁺ as well as CD4⁺ T cells are capable of controlling tumor growth upon bsAb treatment. **A**, *In vitro* killing of B16F10, KPC3, and KPC3-TRP1 tumor cells by T-cell subsets (E:T ratio of 5:1) purified from naïve mouse splenocytes (CD8⁺ or CD4⁺) mediated by titrated concentrations of the bsAb 2C11xTA99 was measured using an LDH release assay. Control bsAb 2C11xb12, and no antibody were included as control. Data shown are means and SD of triplicate wells from one of 2 experiments with comparable results. **B**, Kaplan-Meier plots of B16F10 melanoma-bearing mice treated with bsAb 2C11xTA99 at days 6 and 9 after tumor injection. Mice were depleted for CD4⁺ T cells, CD8⁺ T cells, or both by injection of depleting Abs. Compiled data from 2 experiments with similar outcome are shown. Log-rank analysis on Kaplan-Meier curves, $n = 14$ to 15 per group. **C**, Kaplan-Meier curves of mice bearing B16F10 tumors treated with bsAb 2C11xTA99 at days 6 and 9 and depleted of NK cells, by injection of depleting Ab. Log-rank test was used as statistical analysis. *, $P < 0.05$.

CD3 bsAb is effective by redirecting both CD4⁺ and CD8⁺ T cells

The rationale behind CD3 bsAb is the activation of T cells residing in conjunction with tumor cells, irrespective of their peptide/MHC specificity. Direct cytotoxic killing is predominantly expected from CD8⁺ T cells, which classically express perforin and granzymes, but a subset of cytotoxic CD4⁺ T cells exists as well (39, 40). We therefore tested tumor cell killing capacity of purified CD8⁺ and CD4⁺ T cells from spleens of naïve mice in coculture assays. Surprisingly, even though the bsAb 2C11xTA99 induced strong antitumor effects against the B16F10 melanoma *in vivo*, it was not so potent in mediating cell lysis of B16F10 cells *in vitro* (Fig. 2A). High numbers of effector cells isolated from the splenocytes were required (E:T ratio of 20:1) in combination with high bsAb concentration (10 µg/mL) to reach killing percentages around 35% (Supplementary Fig. S2b). When the CD8⁺ T cells were analyzed at the end of the assay by flow cytometry, clear signs of activation were detectable as measured by increased expression of CD25 and CD69 molecules on the T cells (Supplementary Fig. S2c). In addition, high concentrations of IFN γ were observed in the supernatants of these cocultures (Supplementary Fig. S2d), indicating that CD8⁺ T cells were activated by bsAb 2C11xTA99, despite the lack of efficient melanoma cell killing.

KPC3-TRP1 cells displayed much higher levels of TRP1 protein on their surface than B16F10 cells (Supplementary Fig. S2a) and were accordingly more efficiently killed (Fig. 2A). Parental KPC3 tumor cells were not lysed, showing dependency on the tumor antigen. Control bsAb 2C11xb12 lacking the tumor-binding arm also failed to induce cytotoxicity. Finally, purified CD4⁺ T cells induced some killing of KPC3-TRP1 tumor cells, but not of B16F10 (Fig. 2A), in line with earlier findings that report inefficiency of this subset (41).

This prompted us to examine the contribution of the 2 T-cell subsets to tumor clearance in the B16F10 mouse model. T-cell subsets were successfully removed from B16F10-bearing mice by depleting antibodies (Supplementary Fig. S3a). Importantly, depletion of CD8⁺ T cells did not diminish the treatment effect of bsAb 2C11xTA99, indicating that this subset was redundant and suggesting that CD4⁺ T cells could replace the role of CD8⁺ T cells *in vivo* (Fig. 2B). Vice versa, CD4⁺ T cells were also redundant. Depletion of both T-cell populations, however, completely neutralized the antitumor response induced by the CD3 bsAb (Fig. 2B; Supplementary Fig. S3b). NK cells were not required, because depletion of these innate lymphocytes did not change the percentage of surviving mice and the NK cells in the T-cell-deficient animals were incapable to delay tumor outgrowth (Fig. 2C). Thus, the presence of CD4⁺ or CD8⁺ T cells is sufficient to eradicate

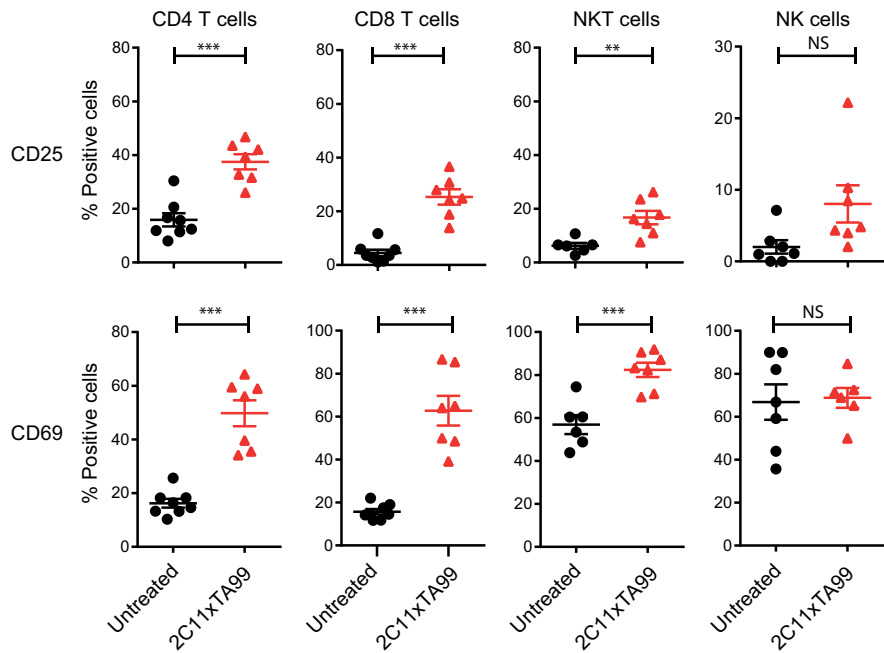


Figure 3. Activation of intratumoral T-cell subsets by systemic administration of bsAb. Surface expression of early activation markers CD25 and CD69 on intratumoral CD4⁺ T cells, CD8⁺ T cells, NKT cells, and NK cells. B16F10 tumor-bearing mice were treated with bsAb 2C11xTA99 via i.p. injection at an average tumor volume of 100 mm³. Six hours later, intratumoral lymphocytes were examined by flow cytometry. Percentages indicate frequencies of positive cells out of the respective lymphocyte subset. Data shown are means and SEM from 2 independent experiments. Student *t* test was used for statistical analysis. **, *P* < 0.01; ***, *P* < 0.001.

B16F10 tumors using CD3 bsAb in this syngeneic mouse model, despite the fact that their killing capacity, as tested in coculture assays, was very low. This demonstrates that *in vitro* cytotoxicity assays may be poor predictors of tumor control *in vivo* and that activation of T cells and release of IFN γ should be regarded as well.

Systemic administration of bsAb 2C11xTA99 induces an instant activation of intratumoral T cells

We then studied T-cell activation *in vivo*. Already 6 hours after injection of the CD3 bsAb, the frequencies of activated CD4 $^{+}$ and CD8 $^{+}$ T cells in the tumor microenvironment more than doubled, as measured by expression of CD25 and CD69 markers by flow cytometry (Fig. 3). CD3-positive, NK1.1-positive NKT cells in

the tumor were also activated, whereas the CD3-negative, NK1.1-positive NK cells were not (Fig. 3). Strong activation of intratumoral CD4 $^{+}$ T cells was sustained for at least 2 days and started to decline after 4 days, as observed by a decrease in CD69-expressing cells (Fig. 4A). Importantly, a high frequency of intratumoral CD8 $^{+}$ T cells still displayed the CD69 activation marker at 4 days. These results indicated that the CD3 bsAb was able to sustain the T-cell response in the tumors for at least 4 days.

Interestingly, analyses of activation markers on T cells in the spleens revealed a transient response, detected only at the 6-hour time point, but not thereafter, as determined by CD69 expression (Fig. 4A). The control antibody that lacked the tumor antigen targeting arm TA99 (2C11xb12) seemed to also induce T-cell

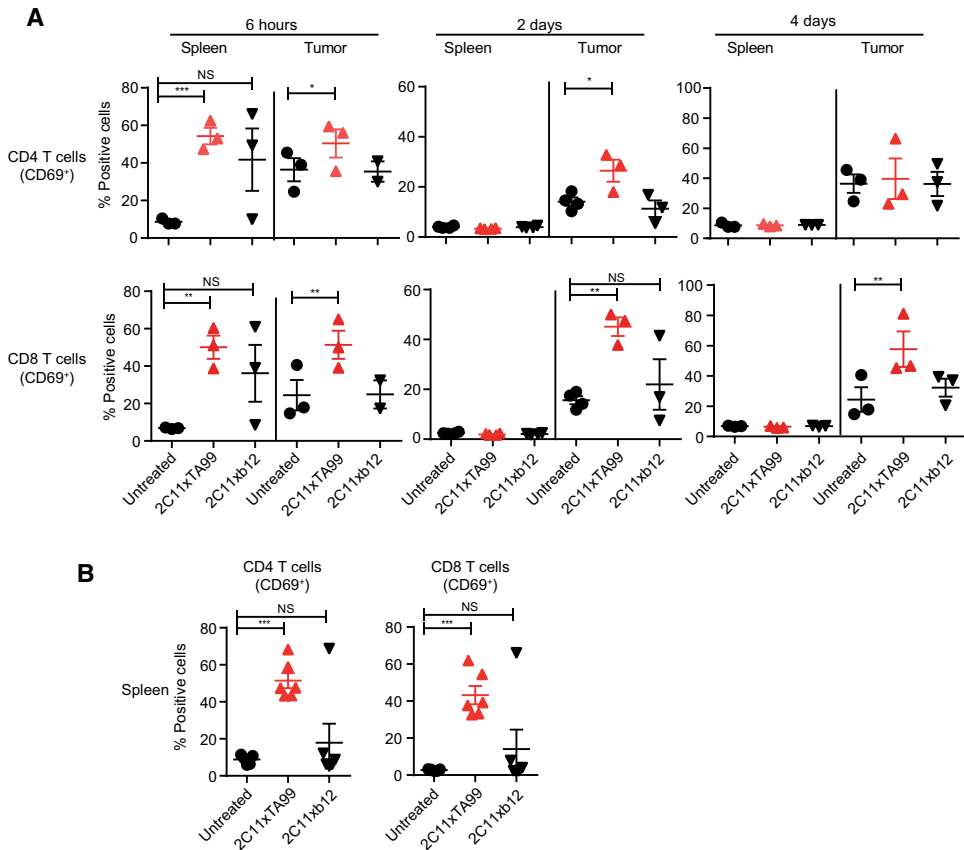


Figure 4.

Kinetics of T-cell activation in bsAb-treated mice in spleen and tumor. **A**, Surface expression of CD69 activation marker on CD8 $^{+}$ and CD4 $^{+}$ T cells in spleens and tumors at 6 hours, 2 days, and 4 days after systemic treatment with bsAb2C11xTA99. Mice were injected with B16F10 melanoma cells and treated at an average tumor volume of 100 mm ^{3} . Spleens and tumors were analyzed by flow cytometry. Data shown are from one representative experiment out of 3 experiments with similar outcome. **B**, CD69 expression by CD4 $^{+}$ T cells and CD8 $^{+}$ T cells in spleens of mice without B16F10 melanoma, at 6 hours after injection of bsAb 2C11xTA99 or control bsAb 2C11xb12. Results shown are of 2 pooled experiments with similar outcome. One-way ANOVA and Tukey multiple comparison test were used for statistical analysis. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

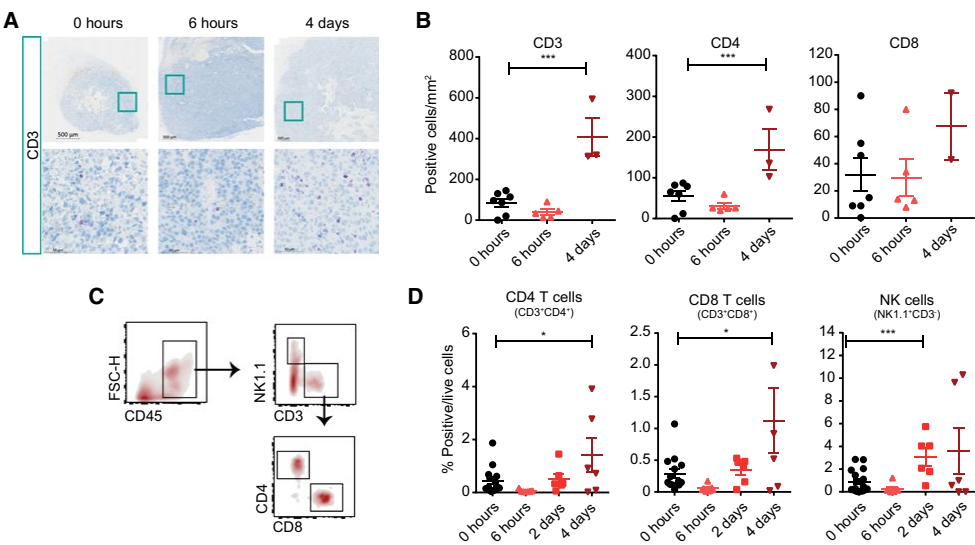


Figure 5. Treatment with bsAb induces increased numbers of intratumoral T cells. Infiltration of T-cell subsets in response to bsAb 2C11xTA99 treatment of B16F10 tumors was analyzed by IHC and flow cytometry at different time points after treatment. **A**, Representative examples of CD3 staining at different time points after bsAb injection. Bottom, higher magnification pictures from the inlets. **B**, Quantification of computer-assisted counts of cells stained for CD3, CD4, and CD8 on the tissue slide. **C–D**, Flow cytometry analysis of T-cell infiltrate in B16F10 melanomas in mice treated with bsAb 2C11xTA99. Gating strategy is shown (**C**), and percentages of CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells, and CD3⁺NK1.1⁺ NK cells were calculated out of all live cells (**D**). Results are shown from 2 pooled experiments with similar outcome. One-way ANOVA and Tukey multiple comparison test was used for statistical analysis. *, $P < 0.05$; ***, $P < 0.001$.

activation in spleens of some animals, but this was not statistically significant compared with untreated mice. These results prompted us to analyze T-cell activation in the absence of B16F10 melanomas (Fig. 4B). Interestingly, 6 hours after i.p. injection, bsAb 2C11xTA99 induced systemic activation to comparable extent as was observed in tumor-bearing animals, suggesting that the presence of the tumor was not related to this transient response (Fig. 4B compared with Fig. 4A; Supplementary Fig. S4a). Administration of the control bsAb 2C11x12 in tumor-free animals did not result in T-cell activation, suggesting that TRP1 expression on normal melanocytes might have caused this transient response. The bsAb-activated CD4 T cells expressing CD25 and CD69 at 6 hours after the injection in tumor-free mice were FoxP3-negative, indicating that these cells represent effector T cells and not regulatory CD4 T cells (Supplementary Fig. S4b). Together, these results demonstrated that after a very transient systemic T-cell response, a sustained T-cell activation in the tumor microenvironment is induced by CD3 bsAb treatment. Of note, mice did not experience a cytokine release syndrome in these first hours, nor was autoimmune reactivity, like overt depigmentation, observed during long-term experiments.

Immunotherapy with CD3 bsAb results in an influx of T cells and macrophages

Finally, we examined the cascade of immune events after the initial T-cell activation in the B16F10 melanoma model. T-cell and myeloid cell numbers in the tumors upon treatment with

CD3 bsAb were evaluated (Figs. 5 and 6). Frequencies of T-cell subsets first seemed to decrease 6 hours after injection of the antibody, before strong increases were observed, as measured by tissue slide staining and flow cytometry. Four days after bsAb injection, the number of CD3-positive cells increased 4 times (Fig. 5A–5B). This increase was caused mainly by CD4⁺ cells (Fig. 5B; Supplementary Fig. S5A–S5B). Multiparameter staining and analysis by flow cytometry confirmed the findings from tissue sections and, furthermore, showed an increase of NK cells from day 2 onward (Fig. 5C and D), even though these cells were not direct targets of the bsAb (Fig. 3). The temporal decrease of intratumoral T cells at 6 hours after treatment was also observed in lymph nodes (Supplementary Fig. S5c). This T-cell redistribution has been described in patients as well and might be explained by adhesion to the vasculature via activation-induced integrins (42).

We then investigated whether the CD3 bsAb induced recruitment of myeloid cells (Fig. 6). Staining for the common macrophage marker F4/80 was evaluated as a percentage positive area, by IHC, because myeloid cells were difficult to exactly enumerate due to their veiled morphology. A significant increase in macrophages was observed in the tumor area, whereas their high numbers at the surrounding tumor rim did not alter in time (Fig. 6A and B). Flow cytometry allowed us to discern inflammatory macrophages from tumor-associated stationary macrophages and neutrophilic granulocytes using the markers CD45, CD11b, F4/80, Ly6G, and Ly6C. Interestingly,

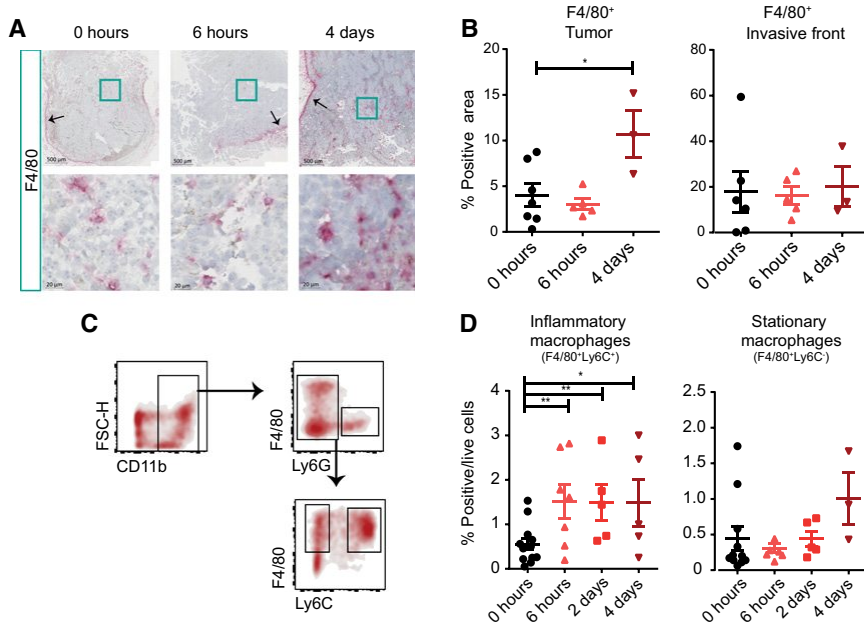


Figure 6.

Early influx of inflammatory macrophages induced by CD3 bsAb. Infiltration of immune cells in B16F10 tumors was analyzed by IHC and flow cytometry at the indicated time points after systemic treatment with bsAb 2C11xTA99. **A**, Representative tissue slide staining for the common macrophage marker F4/80. **B**, Quantification of positively stained area for F4/80 within the tumor and surrounding the tumor at the invasive front, as indicated with the arrows in **A**. **C**, Flow cytometry analysis of myeloid cells in treated B16F10 tumors using markers CD45, CD11b, F4/80, Ly6G, and Ly6C. **D**, Composition of the immune infiltrate delineated for inflammatory monocytes (F4/80⁺, Ly6C⁻) and stationary macrophages (F4/80⁺, Ly6C⁺). Results are shown from 2 pooled experiments with similar outcome. One-way ANOVA and Tukey multiple comparison test was used for statistical analysis. *, $P < 0.05$; **, $P < 0.01$.

the frequencies of neutrophils (Ly6G⁺, Ly6C^{low}, F4/80⁻) and stationary macrophages (F4/80⁺, Ly6C⁺) were not significantly altered, whereas numbers of inflammatory macrophages (F4/80⁺, Ly6C⁻) were increased, already 6 hours after start of treatment (Fig. 6C and D; Supplementary Fig. S5d). The fact that the early increase of this macrophage subset was not detected in tissue slide staining might be due to the broader tissue area we resected for flow cytometry.

In summary, systemic injection of the bsAb 2C11xTA99 leads to a fast activation of intratumoral T cells and a fast and continued influx of inflammatory macrophages. Within a few days, this culminates in an additional increased number of CD4⁺ T cells, CD8⁺ T cells, and NK cells. The examination of immune cell composition in the tumor microenvironment was possible because we applied a completely syngeneic mouse model system and this might form the basis for studying combinatorial approaches to further improve CD3 bsAb therapies.

Discussion

Here, we show immunologic effects of immunotherapy with a CD3 bsAb in a completely immune-competent mouse melanoma model. Treatment with the TRP1-targeting bsAb resulted in a

strong and sustained activation of intratumoral T-cell subsets (CD4⁺ and CD8⁺ T cells, as well as NKT cells), but not of CD3-lacking NK cells. From day 2 onward, a strong increase of innate and adaptive immune cells was observed in the tumor microenvironment. This remodeling was especially pronounced for NK cells, inflammatory macrophages, and CD4⁺ T cells. A brief systemic T-cell activation in lymphoid organs was observed only up to 6 hours. Although the capacity of T cells to kill melanoma cells *in vitro* was very modest, melanoma control *in vivo* was rather efficient and mediated by both CD4⁺ and CD8⁺ T cell subsets. These data suggest that cytokine-mediated effector functions of CD4⁺ T cells, which rarely contain cytolytic granules, might be important contributors to tumor control, as previously observed in other systems (43–46). Finally, the CD3 bsAb led to a fast and strong induction of antitumor immunity, but failed to install long-term protective memory responses, indicating a lack of memory T-cell formation in the B16F10 tumor model.

Most of the immune features observed in our study could be detected due to the presence of a fully intact immune system in our immune-competent mouse model, in which we applied mouse bsAb targeting the mouse CD3 protein and the mouse TAA to treat mouse melanoma in a syngeneic host. Most PDX interspecies models have used bispecifics targeting human CD3

and human tumor antigens, not expressed on mouse host tissues or immune cells (12, 13, 16–18) and thus fail to monitor the full so-called on-target, off-tumor effects. Even the more elegant models in this research field, in which human CD3E is transgenically expressed in mouse T cells and human TAA are expressed in mouse tumor cell lines under heterologous promoters, fall short, due to artificial expression levels of the CD3 and TAA (21, 22). Indeed, the expression levels and frequency of CD3E in these transgenic mouse models are lower than those in wild-type mice (24). Recently, a triple knock-in mouse was reported in which human CD3E, -D, and -G genes replace the mouse homologous genes CD3 ϵ , - δ , and - γ . In this knock-in mouse, T-cell numbers and CD3 expression levels are comparable to those in wild-type mice and treatment with CD3 bsAb binding to human CD3 can representatively be evaluated (23, 24). In our syngeneic mouse model, we report a strong increase of innate immune cells, such as inflammatory macrophages and natural killer cells, and a fast intratumoral activation of CD4⁺ and CD8⁺ T-cell subsets (Figs. 5 and 6).

An initial systemic activation of T cells was observed up to 6 hours after bsAb administration, as witnessed by the early activation markers CD25 and CD69 on T cells in the spleen (Fig. 4). This transient pulse of activation was also observed in tumor-free animals, suggesting that this might be caused by binding of the CD3 bsAb to endogenous expressed TRP1 on healthy melanocytes of the host. The fact that control bsAb 2C11xb12 did not induce this brief T-cell activation in most tumor-free mice further substantiated this notion. TRP1 expression indeed has been detected on melanocytes in the skin in humans and mice (25, 47, 48). In addition, trace amounts of monospecific parental CD3 antibodies might have been present in the product as a result of incomplete cFAB reaction. However, less than 0.3% monospecific anti-CD3 Ab were present in our bsAb (Supplementary Fig. S1b), and it is very unlikely that these small amounts would explain the observed transient systemic T-cell activation. Alternatively, low-level residual interactions between Fc γ R or complement and the mIgG2a-LALA backbone could exist (26), allowing for residual cross-linking of CD3. A combination of these factors might contribute to the early peak of systemic T-cell activation.

The analyzed surface markers CD69 and CD25 are early indicators of T-cell activation and might merely reflect a brief and reversible TCR triggering and do not guarantee full-blown differentiation to memory cells. Lack of protective memory responses in our model might be explained by lack of tumor specificity of the targeted pool of T cells or lack of costimulatory signals during the treatment of the CD3 bsAb. Most likely, higher expression levels of TRP1 on the tumor or different environmental cues in tumor tissue influencing the trafficking of CD3⁺ cells contribute to the observed tumor eradication via CD3 bsAb in the tumor microenvironment.

In conclusion, our findings show that a CD3 bsAb treatment in the B16F10 mouse solid tumor model can result in antitumor efficacy and remodeling of the tumor microenvironment. The B16F10 melanoma model is known for its low immune infiltration and can be considered as immunologically cold (37, 49). The fact that bsAb treatment leads to increased numbers of intratumoral inflammatory macrophages, natural killer cells, and T cells within a few days marks this therapeutic as a remodeler of the tumor microenvironment. The TCR specificity of these expanded T cells in the tumor microenvironment and their contribution to tumor reduction remains to be investigated.

Disclosure of Potential Conflicts of Interest

I. Altuntaş has ownership interest (including stock, patents, warrants, etc.). A. Labrijn is Principal Scientist at Genmab and has ownership interest (including stock, patents, etc.) in the same. D.H. Schuurhuis has ownership interest (including stock options, patents, etc.). M.A. Houtkamp has ownership interest (including stock, patents, warrants, etc.). J. Schuurman has ownership interest (including stock, patents, etc.) in Genmab. T. van Hall reports receiving a commercial research grant from Genmab and is a consultant/advisory board member for the same. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: H. Benonissou, I. Altuntaş

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Benonissou, S. Verploegen, M.A. Houtkamp, J.S. Verbeek

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Benonissou, I. Altuntaş, S. Verploegen, A.F. Labrijn, M.A. Houtkamp, J.S. Verbeek, T. van Hall

Writing, review, and/or revision of the manuscript: H. Benonissou, I. Altuntaş, S. Verploegen, A.F. Labrijn, D.H. Schuurhuis, M.A. Houtkamp, J.S. Verbeek, J. Schuurman, T. van Hall

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Sluiter, S. Verploegen

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Supplementary Figure legends

Suppl Figure S1. Amino acid sequences of the 2C11xTA99 bsAb and percentages of monospecific Ab in the products

A. Amino acid sequences of the 2C11 anti-CD3 antibody chains and the TA99 anti-Trp1 antibody chains containing the LALA mutations and Fab arm exchange mutations. B. Quality of the Fab arm exchanged bsAb. Percentages indicated contaminations with monospecific Ab in the products.

Suppl Figure S2. TRP1 expression and bsAb-mediated killing assays *in vitro*

A. Flow cytometry histograms of TRP1 expression at the cell surface of B16F10 melanoma cells, KPC3 pancreas carcinoma cells and TRP1 gene transfected KPC3 cells, as determined by staining with TA99. B. *In vitro* killing of B16F10 melanoma cells with higher E:T ratios of spleen-purified CD8⁺ T cells from naïve mice using bsAb 2C11xTA99 and the control bsAb 2C11xb12. Specific killing was measured by LDH release. Cell lysis induced by Triton X-100 was set at 100% cell kill. Data shown are means of triplicate wells from one of two experiments with comparable results. C. Expression of CD25 and CD69 on CD8⁺ T cells as measured by flow cytometry from the experiment shown in panel B with bsAb concentration of 10 µg/mL. D. T cell activation was measured by IFN γ release (pg/mL) in supernatants of the experiment shown in panel B, at an E:T ratio of 20:1.

Suppl Figure S3. Individual tumor growth curves of treatment experiment with depletion of T cell subsets

A. Efficiency of depletion of T cell subsets using anti-CD8 and/or anti-CD4 cell depleting antibodies, as measured by flow cytometry in blood samples at day 19. B. Tumor outgrowth curves of B16F10 melanomas treated with bsAb 2C11xTA99 at days 6 and 9. Mice were depleted of CD8⁺ T cells, CD4⁺ T cells or both. Numbers reflect the fraction of

surviving mice. Kaplan-Meier curves are shown in Figure 2. All groups are from two pooled experiments.

Suppl Figure S4. Ex vivo analysis of T cell activation in spleens of tumor-free mice

A. Expression of CD25 on CD4⁺ T cells and on CD8⁺ T cells in spleens of mice without tumor, analyzed by flow cytometry at 6 hours after injection of bsAb 2C11xTA99 or control bsAb 2C11xb12. Results are shown from two pooled experiments with similar outcome. One-way ANOVA and Tukey's multiple comparison test was used for statistical analysis. B. bsAb 2C11xTA99 was administered to tumor-free mice and T cell activation in spleens was analyzed after 6 hours by flow cytometry. Regulatory T cells were defined as CD3⁺CD4⁺FoxP3⁺ cells and regular T cells were defined as CD3⁺CD4⁺FoxP3⁻ cells. Percentages and geometric means of positive cells expressing the indicated activation markers within these two subsets were calculated.

Suppl Figure S5. Analysis of infiltrated immune cells

A-B. Examples of tissue slide stainings for T cells. A. CD4 staining of treated B16F10 tumors with in the lower panel the higher magnifications of the inlets. B. CD8 staining of the same tumors. C. Frequencies of CD4 and CD8 T cells in tumor-draining lymph nodes (DLN) and non-draining LN (nDLN) were determined by flow cytometry and expressed as percentage out of all CD45⁺ cells. D. Flow cytometry analysis of tumor infiltration by neutrophilic granulocytes. Results are shown from two pooled experiments with similar outcome. One way ANOVA and Tukey's multiple comparison test was used for statistical analysis.

Supplementary Figure S1

A

IgG2amm-TA99-AAKR

Heavy chain sequence:
EVQLQQSGAELVRPGALVKLSCKTSGFNIKDYFLHWVRQRPDQGLEWIGWINPDNGNTVYDPKFQGT
ASLTADTSSNTVYVLQSLGTLSEDTAVYFCRRDYTEKAALDYWGQASVIVSSAKTTAPSVYPLAPV
CGDTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNV
AHPASSTKVDDKIEPPRGPTIKPCPPCKCPAPNAAGGPSVFIFPPKIKDVLMSLSPMVTCVVVDVSEDDP
DVQISWVFNVEVLTAQTQTHREDYNSTLRVVSALPIQHQQDWMMSGKEFKCKVNNKALPAPIERTISKP
KGSVRAPQVYVLPPEEEMTKKQVTLTCMVKDFMPEDIYVEWTNNGKTELNYKNTEPVLDSGGSYFM
YSRLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGK

Light chain sequence:
AIQMSQSPASLSASVGETVTITCRASGNIYNYLAWYQQKQGKSPHLLVYDAKTLADGVPSRFSGSGSG
TQYSLKISSLQTEDSGNYYCQHFWSLPFTFGSGTKLEIKRADAAPTVSIFPPSSEQLTSGGASVVCFLNMF
YPKDINVKWKIDGSRQNGVLNSWTDQDSKDYSTYSMSSTLTCLKDEYERHNSYTCEATHKSTSPIVK
SFNRNEC

IgG2amm-145-2C11-AALT

Heavy chain sequence:
EVQLVESGGGLVQPGKSLKLSCEASGFTFSGYGMHWVRQAPGRGLESVAYITSSSINKYADAVKGRF
TVSRDNAKNLLFLQMNILKSEDTAMYCARFDWDKNYWGQGTMTVTVSSAKTTAPSVYPLAPVCGDT
TGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPA
SSTKVDDKIEPPRGPTIKPCPPCKCPAPNAAGGPSVFIFPPKIKDVLMSLSPMVTCVVVDVSEDDP
DVQISWVFNVEVLTAQTQTHREDYNSTLRVVSALPIQHQQDWMMSGKEFKCKVNNKALPAPIERTISKPKGSVR
APQVYVLPPEEEMTKKQVTLTCMVTDMPEDIYVEWTNNGKTELNYKNTEPVLDSGSYLMYSKLT
VEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGK

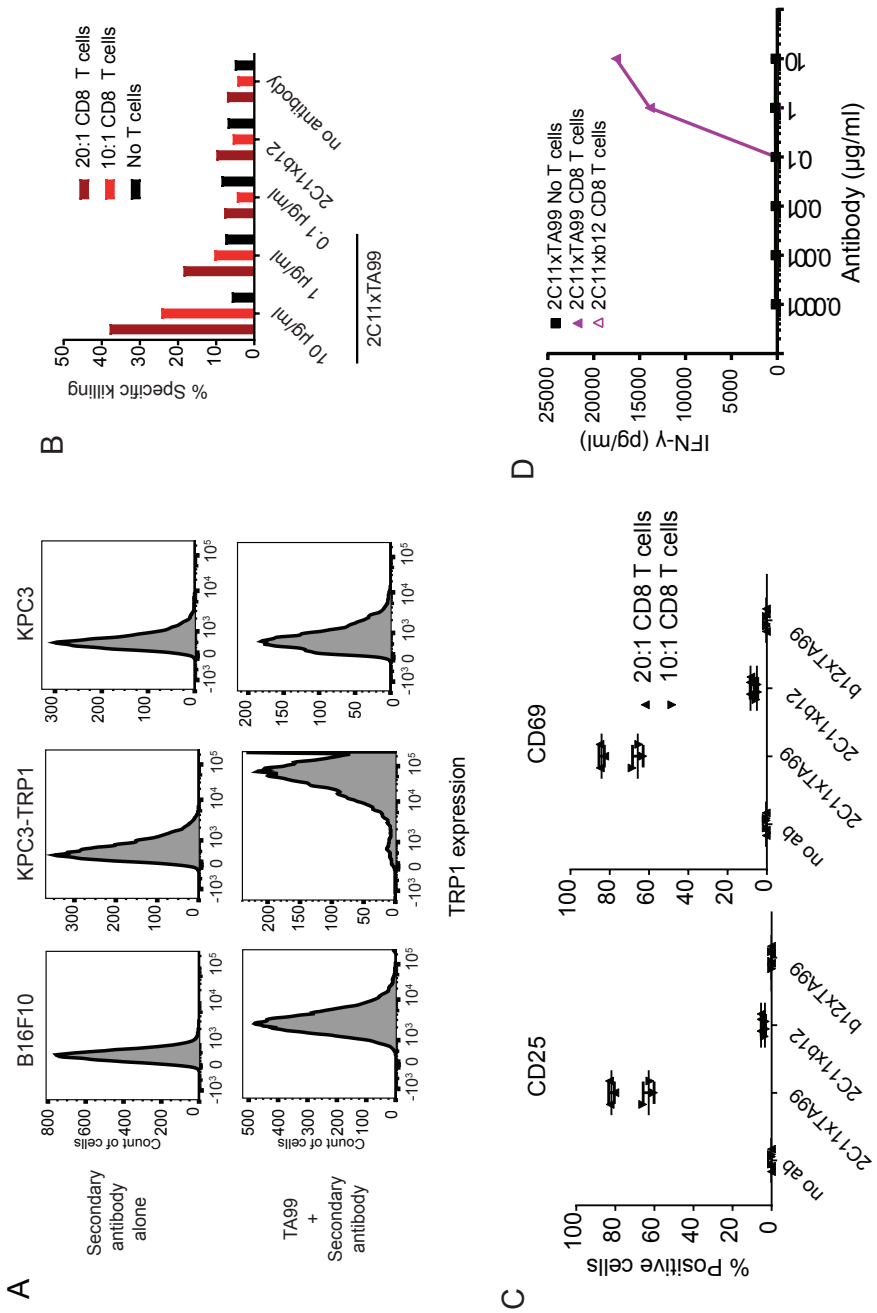
Light chain sequence:
DIQMTQSPSSLPASLGDRVTINCQASQDISNYLNWYQQKPGKAPKLLIYYTNKLADGVPSRFSGSGSGR
DSSFTISSLESEDIGSYCYQYYNPWTFGPGTKLEIKRADAAPTVSIFPPSSEQLTSGGASVVCFLNMFY
PKDINVKWKIDGSRQNGVLNSWTDQDSKDYSTYSMSSTLTCLKDEYERHNSYTCEATHKSTSPIVKSF
NRNEC

B

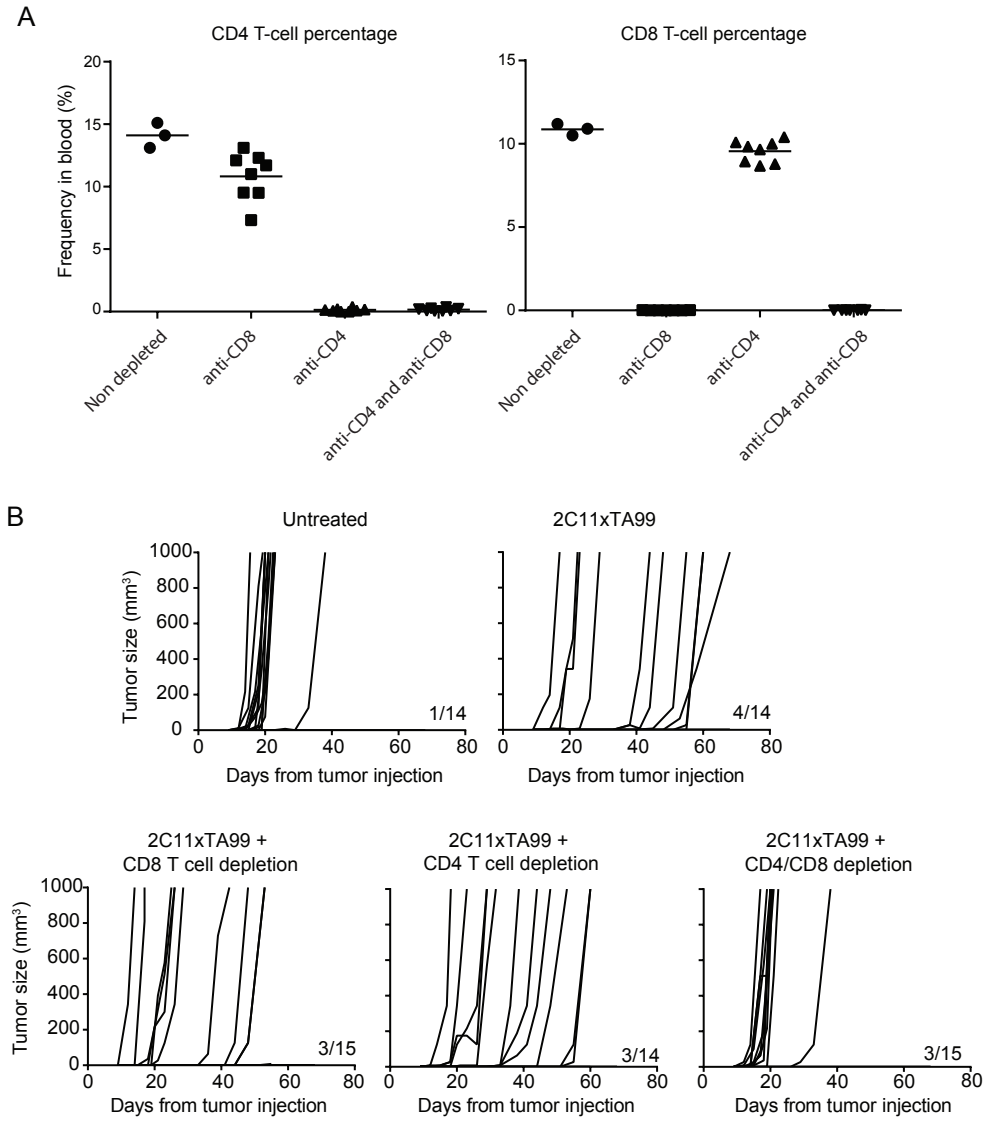
bsAb	b12 ^a xTA99 ^b	2C11 ^a xb12 ^b	(2C11 ^a xTA99 ^b) ¹	(2C11 ^a xTA99 ^b) ²
% of total	92.6	95.8	92.3	99.7
Homodimer A	2.1	1.0	3.8	0.3
Homodimer B	5.3	3.2	3.9	0

^a: homodimer A, ^b: homodimer B, ¹: first batch, ²: second batch

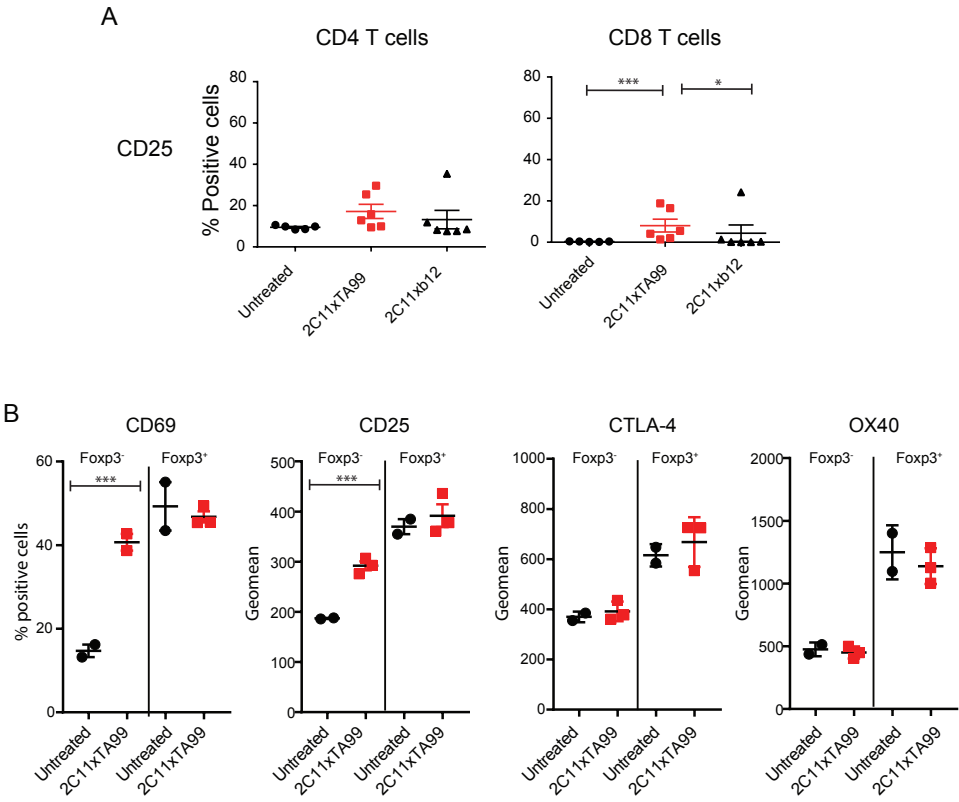
Supplementary Figure S2



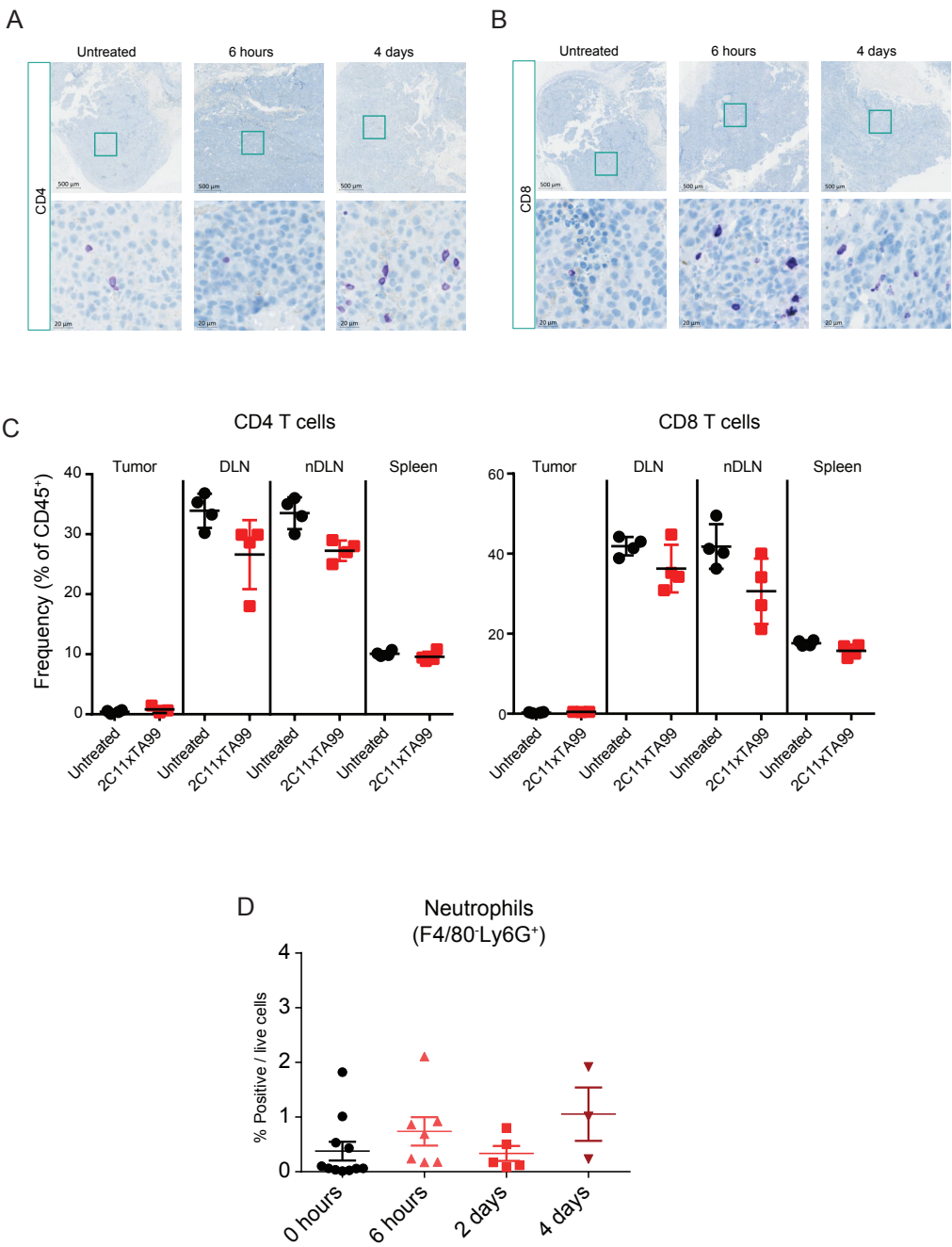
Supplementary Figure S3



Supplementary Figure S4



Supplementary Figure S5



CHAPTER 6

6

General Discussion

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General Discussion

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

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 Trp2 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 IFN γ , 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 FcγRIII 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: Kupfer cells "Metastatic"
(17)	Skin s.c	TA99 day 0	FcγRI KO FcγRIII KO FcγRIV KO FcγRI/IIII 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/IIII KO FcγRII/IIII/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/IIII/IV KO FcγRI KO; FcγRIII 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/IIII/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 FcγRIV on Mφ FcγR independent small contribution NK cells No role (FcγR on) neutrophils T cell dependent	Effect of Aldara and IL-2 "Therapeutic"

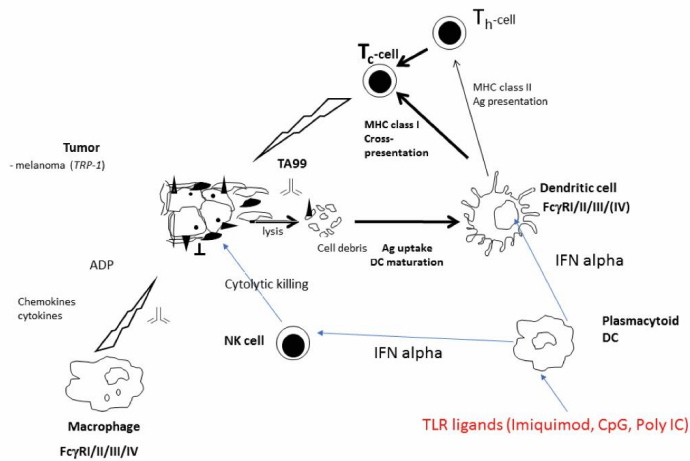


Figure 1: Schematic overview of the mode of action of the combination therapy of imiquimod and TA99 and how the mechanism could be when using previous studies to fill in the gap (31-35, 51).

CHAPTER 7



Nederlandse samenvatting
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Samenvatting in het Nederlands

Eind jaren negentig van de vorige eeuw werden de eerste antistoffen die aan eiwitten op het oppervlakte van tumorcellen (tumor antigenen) kunnen binden, toegelaten voor gebruik in de kliniek om lymfomen en borstkanker te bestrijden. De tumorantigenen waren zodanig gekozen dat binding van het antistof aan de tumorcel de groei van die cel remt ofwel de cel direct doodt. Meer recent is echter duidelijk geworden dat tumor specifieke antistoffen ook cellen van onze afweer kunnen activeren wat een substantiële bijdrage kan leveren aan het therapeutische effect van de antistoffen. Antistoffen bestaan uit een variabel uniek deel dat aan het antigeen bindt en een constant deel (Fc domein) dat kan binden aan receptoren (Fc receptoren, FcR) op het oppervlak van verschillende soorten afweercellen zoals macrofagen, neutrofielen en NK cellen. Wanneer antilichamen die gebonden zijn aan een tumorcel met hun Fc domein aan FcR op afweercellen binden worden in die cellen mechanismen geactiveerd die de tumorcel kunnen doden. Een macrofaag zal de tumorcel opslokken (fagocyteren) en een NK cel zal (cytotoxische) stoffen uitscheiden die de tumorcel lek prikken. Dit is een natuurlijke afweerreactie die ook optreedt wanneer lichaamscellen worden geïnfecteerd door een virus.

Het doel van het in dit proefschrift beschreven onderzoek was om beter inzicht te verkrijgen in de rol van de verschillende afweercellen en de verschillende FcR op hun celoppervlak, in antistof therapie en te onderzoeken of de effectiviteit van de door de antistoffen geactiveerde tumor-dodende mechanismen kan worden verbeterd. Allereerst hebben we de rol van FcR in uiteenlopende afweerreacties onderzocht met behulp van een genetisch veranderde muis waarin alle FcR ontbraken. Nadat die rol in kaart was gebracht hebben we op tweeërlei wijzen antistof therapie in een muismodel voor pigmentcel tumoren bestudeerd. Enerzijds na vaccinatie met een virale vector die een tumor antigeen tot expressie bracht. Anderzijds door het inspuiten van een tumor specifieke antistof in combinatie met andere stoffen die de afweer activeren. Vaccinatie resulteerde in een antistof respons die goede bescherming bood tegen de uitgroei van de tumoren, welke afhankelijk was van FcR. Door gebruik te maken van genetisch veranderde muizen waarin een enkel type FcR ontbrak en door selectief bepaalde typen afweercellen uit de muis te verwijderen kon worden vastgesteld dat de FcγRI op macrofagen essentieel was voor de bescherming tegen uitgroei van deze pigmentcel tumoren. Echter reeds uitgegroeide tumoren waren ongevoelig voor deze vaccinatie therapie. De oorzaak hiervan is dat tumoren zijn ingebed in een micro-omgeving vol andere cellen die afweer reacties onderdrukken en tumorgroei bevorderen. Daardoor is de tumor niet vatbaar voor de aangemaakte antistoffen en kunnen antistoffen niet de juiste afweercellen met FcR activeren om de tumor te doden. Daarom hebben we onderzocht of het toedienen van stoffen die de afweer stimuleren de effectiviteit van antistof therapie kon verbeteren. We hebben kunnen aantonen dat het toedienen van een signaalstof, het cytokine IL-2, en een andere stof genaamd imiquimod, die receptoren op macrofagen kan stimuleren, het therapeutisch effect van antistoffen substantieel versterkt. Een groot percentage muizen met een uitgegroeide pigmentcel tumor was na behandeling met deze combinatie therapie volledig genezen. Het bleek dat imiquimod ervoor zorgt dat er meer actieve macrofagen met meer FcR op hun oppervlak in de micro-omgeving van de tumor aanwezig zijn. Deze

bevindingen openen de weg naar de ontwikkeling van effectievere antistof therapie voor de bestrijding van kanker. In de laatste studie werd onderzoek gedaan naar een antistof dat aan twee verschillende antigenen kan binden: het CD3 eiwit dat op killer cellen zit en daarnaast hetzelfde tumorantigeen dat in voorgaande studies werd gebruikt. Met zo'n bi-specifiek antistof is men in staat om de killer cellen te activeren in de microomgeving van de pigmentcel tumor. Er is nog weinig bekend over het mechanisme van afweerreacties die optreden met zo'n type behandeling. De meeste eerdere studies werden uitgevoerd in zogenaamde xenotransplantaatmodellen, waarin menselijke tumorcellen worden ingebracht bij muizen zonder goed functionerend afweersysteem. Die modellen zijn ongeschikt om werkingsmechanismen van zulke antistoffen te bestuderen, omdat afweercellen ontbreken in deze muizen. Toediening van het bi-specifieke antistof veroorzaakte onmiddellijk activatie van killer cellen in de tumor en deze activatie hield een aantal dagen aan. Secundaire influx van macrofagen en andere afweercellen trad 2-4 dagen na toediening op. Opvallende vinding bij dit onderzoek was dat de afweer geen langdurig geheugen ontwikkelde waarmee de muizen beschermd zouden zijn tegen terugkeer van de tumoren. Toekomstig onderzoek kan deze resultaten gebruiken om antistof-gebaseerde therapieën verder te verbeteren.

Summary

At the end of the 1990s, the first antibodies that can bind proteins on the surface of tumor cells (tumor antigens) were approved for use in the clinic to combat lymphomas and breast cancer. The tumor antigens were chosen such that binding of the antibody to the tumor cell inhibits the growth of that cell or kills the cell directly. More recently, however, it has become clear that tumor-specific antibodies can also activate cells of our immune system, which can make a substantial contribution to the therapeutic effect of the antibodies.

Antibodies consist of a variable unique part that binds to the antigen and a constant part (Fc domain) that can bind to receptors (Fc receptors, FcR) on the surface of various types of immune cells such as macrophages, neutrophils and NK cells. When antibodies bound to a tumor cell bind with their Fc moiety to FcR on immune cells, mechanisms are activated in those cells that can kill the tumor cell. A macrophage will swallow the tumor cell (phagocytosis) and an NK cell will secrete (cytotoxic) substances that puncture the tumor cell. This is a natural immune response that also occurs when cells in the body are infected by a virus.

The aim of the research described in this thesis was to gain a better understanding of the role of the different immune cells and the different FcR on their cell surface, in antibody therapy and to

investigate whether the effectiveness of the tumor-killing mechanisms, activated by the antibodies, can be improved. First, we investigated the role of FcR in various immune responses using a genetically modified mouse, in which all FcR were missing. After defining that role, we studied antibody therapy in a mouse model for melanoma in two different ways: on the one hand, after vaccination using a viral vector that expressed a melanoma antigen, on the other hand, by injecting a melanoma-specific antibody in combination with other substances that activate the immune system. Vaccination resulted in an antibody response that offered good protection against melanoma outgrowth but not in the absence of FcR. By using mice lacking a single type of FcR and by selectively removing certain types of immune cells from the mice, it could be determined that FcγRI on macrophages was essential for the protection against outgrowth of a melanoma. However, vaccination had no effect in a mouse with an already established tumor. The reason for this is that tumors are embedded in a micro-environment full of other cells, including those that suppress immunity and those that support tumor cell growth. As a result, the tumor is not easily accessible for the antibody and antibodies cannot properly activate the right immune cells with FcR to kill the tumor. Therefore, we investigated whether adding substances that stimulate the immune system could improve the effectiveness of antibody therapy. We then demonstrated that administration of a survival substance, the cytokine IL-2, and the substance imiquimod that stimulates macrophages, improved the therapeutic effect of a melanoma-specific antibody. A large percentage of mice with a developed melanoma was completely cured after treatment with this combination therapy. It appeared that in the presence of imiquimod more active macrophages with more FcR on their surface were present in the micro-environment of the tumor. Moreover, killer immune cells were created, as depletion of CD8 T cells negated the effect. These findings open the way for the development of more effective antibody therapy for the fight against cancer and show as well the importance of initiating T cell response against tumors. In the last study, a bispecific antibody has been applied that binds the CD3 molecule on killer immune cells and at the same time the melanoma antigen used in the previous studies. We investigated the functional and mechanistic aspects of this antibody. Most prior functional studies on tumor and T cell targeting bispecific antibodies were done in xenograft models. Those models fail to recapitulate the normal mechanism which would occur in syngeneic models, due to the absence of mouse immune cells. Systemic administration of the bispecific antibody induced activation of T cells in the tumor that continued for several consecutive days. The secondary influx of macrophages, CD4 T and CD8 T cells occurred 2-4 days after administration. Even though T cells were needed for the initial efficacy, the treatment did not induce long term immune memory. Future research will benefit from these results aiming at the improvement of therapeutic efficacy of antibody-based therapy of cancer.

Curriculum vitae

Hreinn Benonisson was born on November 28, 1986 in Reykjavik, Iceland. He finished high school in 2005 and received his University Bachelor's degree in Biology in 2009 from the University of Iceland in Reykjavik. In 2010 he started the research project for his master's degree on the generation of T cell-dependent antibody responses and the impact of T cell-independent antigens on the antibody memory, at the University of Iceland and the National University Hospital (Landspítali) under the guidance of professor Ingileif Jonsdottir. He finished his master's degree in Biomedical Science at the University of Iceland in 2013. Hreinn started his PhD studies in October 2013 in the department of Human Genetics (head professor Silvere van der Maarel) under the supervision of associate professor Sjef Verbeek and associate professor Thorbald van Hall (Department of Medical Oncology) of the Leiden University Medical Center, the Netherlands. His PhD project focused on different cancer immunotherapy approaches exploiting tumor-targeting antibodies. The project was part of the EU funded Marie-Curie Initial Training network (ITN) TIMCC (Tumor Infiltrating Myeloid Cell Compartment) coordinated by Sjef Verbeek.

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I started my study in October 2013 and since then a lot of people have contributed to and helped me with the research presented in this thesis.

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My project was part of the EU Marie Curie Initial Training Network (ITN) on the Tumor Infiltrating Myeloid Cell Compartment (TIMCC) initiated by Sjef. This multi-disciplinary network offered me training in state-of the-art approaches in molecular genetics, genomics and molecular and cellular immunology to the functional *in vivo* analysis of many different aspects of immune - tumour interactions. I am grateful to the EU for developing such international training programs in which both Academia and private sector participate and that equipped me with multidisciplinary biomedical knowledge and skill-sets, and key insights into the complexity of translational medicine preparing me to meet the new challenges in medical research and the development of translational medicine. I would like to thank everyone involved in the TIMCC network for their contribution to my research presented in this thesis

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List of publication

1. CD3-Bispecific Antibody Therapy Turns Solid Tumors into Inflammatory Sites but Does Not Install Protective Memory. **Hreinn Benonisson**, Işıl Altıntaş, Marjolein Sluijter, Sandra Verploegen, Aran F. Labrijn, Danita H. Schuurhuis, Mischa A. Houtkamp, J. Sjeff Verbeek, Janine Schuurman and Thorbald van Hall *Mol Cancer Ther.* 2019 Feb;18(2):312-322.
2. FcγR interaction is not required for effective anti-PD-L1 immunotherapy but can add additional benefit depending on the tumor model. Heng Sheng Sow, **Hreinn Benonisson**, Cor Breukel, Remco Visser, Onno JHM Verhagen, AEH Bentlage, Conny Brouwers, Jill WC Claassens, Margot M Linssen, Marcel Camps, Thorbald van Hall, Ferry Ossendorp, Marieke F. Fransen, Gestur Vidarsson and J. Sjeff Verbeek. *Int J. Cancer.* 2019. Jan 15: 144 (2): 345-354.
3. FcγRI expression on macrophages is required for antibody-mediated tumor protection by cytomegalovirus-based vaccines. **Hreinn Benonisson**, Heng Sheng Sow, Cor Breukel, Jill W. Claassens, Conny Brouwers, Margot M. Linssen, Anke Redeker, Marieke F. Fransen, Marjolein Sluijter, Thorbald van Hall, Ferry Ossendorp, Ramon Arens and J. Sjeff Verbeek *Oncotarget.* 2018 Jun 29;9(50):29392-29402.
4. High FcγR Expression on Intratumoral Macrophages Enhances Tumor-Targeting Antibody Therapy. **Hreinn Benonisson**, Heng Sheng Sow, Cor Breukel, Jill W. Claassens, Conny Brouwers, Margot M. Linssen, Marieke F. Fransen, Marjolein Sluijter, Ferry Ossendorp, Thorbald van Hall, and J. Sjeff Verbeek *J. Immunol.* 2018 Dec 15 ;201 (12): 3741-3749.
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7. The adjuvant LT-K63 can restore delayed maturation of follicular dendritic cells and poor persistence of both protein- and polysaccharide-specific antibody-secreting cells in neonatal mice. Stefania P Bjarnarson, Brenda C Adarna, **Hreinn Benonisson**, Giuseppe Del Giudice and Ingileif Jonsdottir. *J. Immunol.* 2012. Aug 1;189(3):1265-73.

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