



Published in final edited form as:

Cell. 2015 May 21; 161(5): 1035–1045. doi:10.1016/j.cell.2015.04.016.

Differential Fc-receptor engagement drives an anti-tumor vaccinal effect

David J. DiLillo¹ and Jeffrey V. Ravetch^{1,2}

¹The Laboratory of Molecular Genetics and Immunology, The Rockefeller University, 1230 York Ave, New York, NY 10065

Summary

Passively-administered anti-tumor mAbs rapidly kill tumor targets via Fc γ R-mediated cytotoxicity (ADCC), a short-term process. However, anti-tumor mAb treatment can also induce a vaccinal effect, in which mAb-mediated tumor death induces a long-term anti-tumor cellular immune response. To determine how such responses are generated, we utilized a murine model of an anti-tumor vaccinal effect against a model neoantigen. We demonstrate that Fc γ R expression by CD11c⁺ antigen-presenting cells is required to generate anti-tumor T cell responses upon ADCC-mediated tumor clearance. Using Fc γ R-humanized mice, we demonstrate that anti-tumor huIgG1 must engage hFc γ RIIIA on macrophages to mediate ADCC, but also engage hFc γ RIIA, the sole hFc γ R expressed by human DCs, to generate a potent vaccinal effect. Thus, while next-generation anti-tumor antibodies with enhanced binding to only hFc γ RIIIA are now in clinical use, ideal anti-tumor antibodies must be optimized for both cytotoxic effects as well as hFc γ RIIA engagement on DCs to stimulate long-term anti-tumor cellular immunity.

Introduction

Passive administration of anti-tumor antibodies is an important clinical tool for the management of a variety of cancers (Pincetic et al., 2014), and generally functions by targeting malignant cells through Fc-receptor for IgG (Fc γ R)-mediated antibody-dependent cellular cytotoxicity (ADCC) by myeloid effector cells (Clynes et al., 2000; Taylor and Lindorfer, 2008; Uchida et al., 2004) or possibly natural killer (NK) cells. Because of this Fc γ R-mediated mechanism of action, next-generation versions of anti-tumor mAbs that have been Fc-engineered for enhanced engagement of activating Fc γ Rs are now being used in the clinic or are under investigation (Goede et al., 2014). However, while ADCC-mediated tumor killing is rapid and relatively short-acting, patients with some malignancies see long-

© 2015 Published by Elsevier Inc.

²To whom correspondence should be addressed: ravetch@rockefeller.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Author Contributions

DJD and JVR designed experiments, analyzed the data, and wrote the manuscript. D.J.D. performed the experiments.

The authors declare no conflicts of interest.

term responses after cessation of antibody therapy; this has prompted the hypothesis that a vaccinal or auto-immunization effect is initiated, in which tumor targeting by a monoclonal antibody (mAb) primes the patient's immune system to generate an anti-tumor T cell memory response (Cartron et al., 2004). Thus, it has been demonstrated that cellular immune responses are generated in both mice and patients treated with anti-HER-2/neu mAb (Park et al., 2010; Taylor et al., 2007). Anti-MUC1 cellular immune responses have also been reported after the use of anti-MUC1 mAb in patients with MUC1⁺ tumors (de Bono et al., 2004). Evidence in lymphoma patients suggests that a vaccinal effect can be generated by anti-hCD20 mAb immunotherapy (rituximab), since a single course of treatment with mAb can result in long-lasting, durable responses (Cartron et al., 2004). In support of this, it has been reported that some patients treated with rituximab developed lymphoma-specific anti-idiotypic T cell responses after mAb treatment (Hilchey et al., 2009). Recent studies in mice have also demonstrated that passive administration of anti-CD20 mAbs can initiate anti-tumor cellular immune responses (Abes et al., 2010). Therefore, while the hypothesis of a tumor-specific antibody-induced anti-tumor vaccinal effect has persisted for more than a decade, an experimentally-derived mechanistic explanation is lacking.

New technologies have enabled the identification of tumor mutational signatures, some common across multiple cancer types while others are restricted to specific malignancies (Alexandrov et al., 2013). Thus, mutation-induced, developmentally-restricted, or over-expressed tumor neoantigens are a major target of tumor-infiltrating lymphocytes in patients (Fritsch et al., 2014; Tran et al., 2014). Neoantigen-specific CD4⁺ and CD8⁺ T cells have been identified, showing that such antigens are indeed processed and presented (Gros et al., 2014; van Rooij et al., 2013). Further, new immune-checkpoint blockade therapies function in patients by amplifying neoantigen-specific responses (van Rooij et al., 2013). However, although studies analyzing antibody responses to tumor neoantigens are lacking, antibody:antigen immune complexes can stimulate cellular immunity by engaging activating FcγRs on antigen-presenting cells, such as dendritic cells (DCs), to induce DC maturation, traditional antigen presentation and cross-presentation, co-stimulatory molecule upregulation, and stimulate cellular immune responses in both mice (Kalergis and Ravetch, 2002; Rafiq et al., 2002) and humans (Boruchov et al., 2005; Dhodapkar et al., 2005). Often, antibody:antigen immune complex immunization results in more potent cross-presentation and CD4 or CD8 T cell responses than antigen immunization alone. Thus, a logical approach to boosting cellular immune responses involves passive administration of antibodies reactive with tumor antigens or tumor neoantigens. Therefore, in this current study, we utilize a tumor model expressing a model tumor neoantigen to test whether and how passive anti-tumor antibody treatment stimulates an anti-tumor vaccinal effect and cellular immune response.

Three activating FcγRs are expressed in mice (mFcγRI, mFcγRIII, and mFcγRIV) and humans (hFcγRI, hFcγRIIA and hFcγRIIIA), and a single inhibitory FcγR, FcγRIIB, is expressed in both species. The cellular outcome of IgG interactions with FcγRs is governed by the affinity of an antibody's Fc for the specific receptor and the expression pattern of those receptors on effector cells (Nimmerjahn and Ravetch, 2008). Since most effector cells co-express activation and inhibitory FcγRs, it is the ratio of the binding affinities of a specific IgG Fc to these receptors that will determine the outcome of the IgG-FcγR

interaction. These binding affinities are determined by the amino acid sequences of the IgG Fc subclasses and the IgG Fc's N-linked glycan. The IgG Fc composition can dramatically influence the in vivo outcome of engaging a tumor antigen by directing the antibody-antigen immune complex or opsonized cell into either a pro- or anti-inflammatory response. For example, mouse (m)IgG2a antibodies trigger cytotoxicity by virtue of this Fc having a 2-log greater affinity for the activating mFc γ RIV receptor as compared to the inhibitory mFc γ RIIb receptor, while mIgG1 preferentially engages the inhibitory mFc γ RIIb receptor.

Mice and humans differ regarding the specific Fc γ Rs expressed on various APCs and the relative affinities of each IgG Fc subclass for each Fc γ R (Nimmerjahn and Ravetch, 2007). Thus, human (h)IgG1 Fc does not preferentially engage a single hFc γ R, as occurs in mice. Two low-affinity activating Fc γ Rs are expressed on monocytes and macrophages in both mice (mFc γ RIII and mFc γ RIV) and humans (hFc γ RIIA and hFc γ RIIIA). Murine NK cells express only mFc γ RIII, while human NK cells express only hFc γ RIIIA. Importantly, while mice express two low-affinity activating Fc γ Rs, mFc γ RIII and mFc γ RIV, on DCs, humans only express one low-affinity activating Fc γ R on DCs, hFc γ RIIA. Because of these species differences, we have generated Fc γ R-humanized mice, which express the full array of hFc γ Rs on a background lacking all murine Fc γ Rs (Smith et al., 2012). Appropriate cell type-specific expression of all hFc γ Rs is observed in the Fc γ R-humanized mice, allowing the characterization of hIgG1 antibody-mediated effector function in the context of human Fc γ Rs.

Here, using a murine lymphoma expressing a model tumor neoantigen (human CD20; hCD20), we mechanistically dissect how an anti-tumor cellular immune response is generated after passive treatment of lymphoma-bearing mice with anti-hCD20 mAb. We demonstrate that not only are activating Fc γ Rs required for macrophage-mediated ADCC, but activating Fc γ R expression specifically on CD11c⁺ antigen-presenting cells is required for the generation of an anti-tumor T cell memory immune response and long-term survival. Because of the complexity of the Fc γ R system, with multiple genes expressed and regulated differentially on the diverse cells of the immune system, designing an anti-tumor antibody for optimal activity requires compatible model systems. We therefore assessed the generation of an anti-tumor vaccinal effect for a hIgG1 anti-hCD20 mAb in Fc γ R-humanized mice. We show that anti-tumor mAb must engage hFc γ RIIIA on CLOD-sensitive macrophages to mediate immediate ADCC, as well as hFc γ RIIA (the sole Fc γ R expressed by human DCs) in order to stimulate a long-term anti-tumor cellular immune response. Thus, while next-generation anti-tumor antibodies with enhanced binding to only hFc γ RIIIA on innate effector cells are now in clinical use, our results indicate that anti-tumor antibodies with optimal long-term survival benefit must be optimized for both their short-acting cytotoxic effects through hFc γ RIIIA, as well as for engagement of hFc γ RIIA on DCs to stimulate long-term anti-tumor cellular immunity.

Results

Generation of an anti-tumor vaccinal effect by passive anti-tumor antibody treatment

To understand how passive anti-tumor mAb treatment can induce long-term anti-tumor cellular immune responses, we adapted a murine lymphoma model (Abes et al., 2010) of an

anti-neoantigen mAb-mediated vaccinal effect (Fig. 1A). Wild-type C57BL/6 mice were given syngenic EL4 lymphoma cells that express hCD20 as a tumor neoantigen (EL4-hCD20 cells), followed by mIgG2a isotype anti-hCD20 mAb. The mIgG2a isotype preferentially engages activating mFcγRs and is the most potent mouse subclass for triggering effector cells to result in cellular cytotoxicity, phagocytosis, and inflammatory responses (Nimmerjahn and Ravetch, 2005; Pincetic et al., 2014). Mice receiving lymphoma cells plus anti-hCD20 mAb clear the tumors and survive in an activating FcγR-dependent manner; wild-type mice survive the tumor challenge, while FcRα-null (Smith et al., 2012) mice (which lack all mFcγRs: mFcγRI, mFcγRIIB, mFcγRIII, and mFcγRIV) and *Fcer1g*^{-/-} mice (which lack all activating mFcγRs: mFcγRI, mFcγRIII, and mFcγRIV) do not (Fig. 1B and Supplementary Fig. 1A). The activating mFcγRIV is a major contributor during this clearance of tumor cells, since only 52% of *Fcgr4*^{-/-} mice survive tumor challenge after anti-hCD20 mAb treatment (p<0.001; Supplementary Fig. 1B). Non-FcγR binding DA265 mutant anti-hCD20 was also unable to clear tumors (Supplementary Fig. 1C). Thus, activating FcγRs mediate the initial ADCC clearance of tumor cells by anti-tumor mAb, as reported (Clynes et al., 2000; Uchida et al., 2004).

We next assessed whether an anti-tumor vaccinal effect was generated in mice that survived the initial EL4-hCD20 challenge via treatment with anti-hCD20 mAb. Ninety days after the initial challenge when anti-hCD20 mAb had been cleared (mIgG2a half-life = 7 days (Vieira and Rajewsky, 1988)), surviving tumor/mAb-primed mice were re-challenged with 5×10⁶ EL4-hCD20 tumor cells, a dose that is 10-fold greater than the initial challenge, without treatment with any additional anti-hCD20 mAb. These primed mice showed 100% survival during re-challenge with EL4-hCD20 cells (Fig. 1C). By contrast, surviving tumor/mAb-primed mice re-challenged with EL4-WT cells, which do not express hCD20, showed poor survival. Similar results were seen using a different anti-hCD20 mAb, clone 2B8, which is the parental hybridoma from which rituximab was generated (Supplementary Fig. 1D-E). Thus, mice primed with EL4-hCD20 and anti-hCD20 mAb generate a memory immune response and subsequently reject re-challenge with EL4-hCD20 cells, but not EL4 WT cells.

In this model of the vaccinal effect, both CD4⁺ and CD8⁺ T cells are required after antibody treatment in order to reject the tumor re-challenge (Abes et al., 2010). Specifically, T cell depletion studies demonstrated that CD4⁺ T cells are required during the initial phases of antibody therapy as well as during tumor re-challenge in order to reject tumors. Using CD8-deficient mice, it was also shown that CD8⁺ cells are required for tumor rejection during re-challenge. By contrast, mice do not mount any detectable antibody response against hCD20 or EL4-hCD20 cells, and adoptive transfer of serum from tumor/mAb-primed mice does not protect against tumor challenge (Abes et al., 2010). In figure 1C, we show that EL4-hCD20/anti-hCD20 mAb-primed mice only reject EL4-hCD20 cells, and not wild-type EL4 cells, suggesting that the vaccinal effect is directed against hCD20, with little detectable antigen spreading. To confirm that at least a portion of the vaccinal effect cellular immune response is directed at hCD20, mice primed with EL4-hCD20 cells and anti-hCD20 mAb were re-challenged with a distinct tumor cell line, B6BL lymphoma cells, that express either cell-surface hCD20 or an irrelevant antigen (mCD20). While 100% of mice re-challenged with B6BL-mCD20 died by day 31, 80% of mice re-challenged with B6BL-hCD20 cells survived

at least 90 days (Fig 1D; $p=0.0001$). Thus, only cells expressing hCD20 were capable of being rejected. Collectively, these experiments demonstrate that an anti-huCD20 immune response is generated after the initial Fc γ R-mediated clearance of tumor cells by ADCC.

Expression of mFc γ RIV on CD11c⁺ cells is required for the generation of an anti-tumor vaccinal effect

To understand the mechanistic basis for an anti-tumor vaccinal effect and to determine whether Fc γ R expression plays a role during this process, we utilized mice with a CD11c⁺ cell-specific deletion of mFc γ RIV. *Fcgr4^{fl/fl};cd11c-cre* mice (Nimmerjahn et al., 2010) show a complete absence of mFc γ RIV expression on spleen CD11c^{hi} DCs, but only a partial decrease in mFc γ RIV expression on spleen CD11b⁺Gr-1^{lo}SSC^{lo} monocytes (Fig. 2A). All CD11c^{hi} cells lose mFc γ RIV expression in *Fcgr4^{fl/fl};cd11c-cre* mice, while spleen CD11c^{int} cells show partial loss of mFc γ RIV and CD11c⁻ cells show only a modest decrease in mFc γ RIV (Supplementary Fig. 2A, Supplementary Table 1). The majority of CD11c^{hi}CD8⁺ DCs and a fraction of CD11c^{hi}CD4⁺ DCs express mFc γ RIV, both of which lose all mFc γ RIV expression in *Fcgr4^{fl/fl};cd11c-cre* mice (Supplementary Fig. 2E). While the majority of CD11c^{int/-}CD11b^{int} cells lose expression of mFc γ RIV, only a modest reduction in mFc γ RIV is seen in CD11c^{int/-}CD11b^{hi} cells in *Fcgr4^{fl/fl};cd11c-cre* mice (Supplementary Fig. 2B). Expression of mFc γ RIV is not affected in Ly6G⁺ neutrophils (Supplementary Fig. 2C). Most CD11b⁺CD11c^{int/-}F4/80^{hi} macrophages lose mFc γ RIV expression in *Fcgr4^{fl/fl};cd11c-cre* mice, as these cells express CD11c at intermediate levels (Supplementary Fig. 2D). Similar results demonstrating decreases in mFc γ RIV expression on CD11c⁺ cells and lesser decreases in CD11b⁺ or F4/80⁺ cells were seen in bone marrow, peritoneal cavity, and peripheral blood from *Fcgr4^{fl/fl};cd11c-cre* mice (Supplementary Figs. 3-5, Supplementary Table 1). Therefore, because mFc γ RIV expression was maintained to a sufficient degree on ADCC-mediating innate cells, both control *Fcgr4^{fl/fl}* and *Fcgr4^{fl/fl};cd11c-cre* mice were able to clear primary EL4-hCD20 lymphoma cell challenge after treatment with anti-hCD20 mAb (Fig. 2B), indicating that mFc γ RIV expression on CD11c⁺ cells is not required for anti-hCD20 mAb-mediated ADCC.

To assess the generation of the anti-hCD20 vaccinal effect in the context of CD11c⁺ cells lacking mFc γ RIV, surviving tumor/mAb-primed *Fcgr4^{fl/fl}* and *Fcgr4^{fl/fl};cd11c-cre* mice were re-challenged with EL4-hCD20 cells. Re-challenge of primed *Fcgr4^{fl/fl}* mice resulted in 100% survival (Fig. 2C). By contrast, only 57% of tumor/mAb-primed *Fcgr4^{fl/fl};cd11c-cre* mice survived re-challenge with EL4-hCD20 cells ($p=0.0069$). It is likely that activating mFc γ RIII, which is also expressed on murine DCs, compensates in the absence of mFc γ RIV, thereby explaining why a modest vaccinal effect remains in *Fcgr4^{fl/fl};cd11c-cre* mice. Thus, expression of the IgG2a-preferential activating mFc γ RIV on CD11c⁺ antigen-presenting cells is required for the generation of the anti-tumor vaccinal effect after mAb-mediated killing of tumor cells.

CD11c⁺ cell expression of mFc γ RIV is required to generate anti-tumor cellular immunity

To quantify anti-tumor cellular immunity in vivo, we performed adoptive transfer experiments (Fig. 3A). Mice were given EL4-hCD20 cells and mIgG2a anti-hCD20 mAb, with spleens harvested and total splenocytes or CD3⁺ T cells isolated and adoptively

transferred into naïve mice that were then challenged with EL4-hCD20 cells. Adoptive transfer of splenocytes or T cells from naïve mice was unable to protect against tumor growth, but transfer of splenocytes or T cells from tumor/mAb-primed mice resulted in 80% ($p=0.0044$) and 75% ($p=0.0067$) survival, respectively (Fig. 3B). Thus, tumor/mAb-primed mice generate a quantifiable anti-tumor T cell response.

To determine whether DC expression of mFc γ RIV is required for the generation of the anti-tumor cellular immune response after passive administration of anti-tumor mAb, tumor/mAb-primed splenocytes from *Fcgr4^{fl/fl}* and *Fcgr4^{fl/fl};cd11c-cre* mice were adoptively transferred into naïve mice before challenge with EL4-hCD20 cells. While 76% of mice receiving splenocytes from tumor/mAb-primed *Fcgr4^{fl/fl}* mice survived EL4-hCD20 challenge, only 34% of tumor/mAb-primed *Fcgr4^{fl/fl};cd11c-cre* mice survived the challenge ($p=0.0041$; Fig. 3C). Thus, expression of mFc γ RIV on CD11c⁺ antigen-presenting cells is required to mediate the generation of an anti-tumor cellular immune response after mAb-mediated clearance of tumor.

hFc γ RIIIA mediates ADCC of hIgG1 mAb-targeted tumor cells in Fc γ R-humanized mice

Fc receptors for mouse IgG are heterogeneous, differing in their binding affinities for IgG subclasses, their expression patterns on immune cells, and signaling properties (Nimmerjahn and Ravetch, 2006; Pincetic et al., 2014). For example, NK cells in the mouse express only mFc γ RIII, a low affinity activating Fc γ R. Macrophages and DCs express distinct combinations of both activating (mFc γ RI, mFc γ RIII, and mFc γ RIV) and inhibitory (mFc γ RIIB) receptors. Individual mouse subclasses show preferential mFc γ R binding affinities, with mIgG1 preferentially engaging the inhibitory mFc γ RIIB while mIgG2a engages the activating receptor mFc γ RIV with a 2 log higher affinity (Nimmerjahn and Ravetch, 2008, 2011). Thus, selecting an antibody for optimal Fc γ R engagement requires consideration of the receptors and cell types that are to be engaged.

Further complicating this situation are the inter-species differences between mice and humans. Human Fc γ R genes, expression patterns, and affinities for the various antibody isotypes differ significantly from mice. Importantly, humans express only the low-affinity activating Fc γ R – hFc γ RIIIA – on NK cells, the cells that are thought to primarily mediate cellular cytotoxicity in humans (Seidel et al., 2013), while antigen-presenting DCs express a single, distinct low-affinity activating Fc γ R, hFc γ RIIA (Boruchov et al., 2005; Nimmerjahn and Ravetch, 2008). Therefore, to engineer an antibody to optimize the generation of an anti-tumor vaccinal effect initiated by a hIgG1 antibody in the context of hFc γ Rs, we utilized Fc γ R-humanized mice, which express the full array of hFc γ Rs on a fully immunocompetent C57BL/6 background lacking all mFc γ Rs (Bournazos et al., 2014a; Smith et al., 2012). Fc γ R-humanized mice recapitulate hFc γ R expression patterns in mouse tissues; spleen CD11c^{hi} DCs express only hFc γ RIIA and hFc γ RIIB, but do not express hFc γ RIIIA, while spleen CD11b⁺Ly6G⁻Ly6C^{hi} and CD11b⁺Ly6G⁻Ly6C^{int/-} monocytes express hFc γ RIIIA, hFc γ RIIA and hFc γ RIIB in various combinations (Fig 4, Supplementary Fig. 6A-B).

To address the relative contributions of individual hFc γ Rs during mAb-mediated clearance of primary tumor challenge and during the generation of an anti-tumor vaccinal effect, we

generated anti-hCD20 mAb with a hIgG1 Fc backbone and introduced known point mutations (Bournazos et al., 2014b; Smith et al., 2012) that selectively enhance interactions with individual hFcγRs (Fig. 5A and Supplementary Table 2). Thus, the G236A (GA) mutant shows selectively enhanced binding to hFcγRIIA, the A330L/I332E (ALIE) mutant shows selectively enhanced binding to hFcγRIIIA, and the G236A/S239D/A330L/I332E (GASDALIE) mutant shows dramatically enhanced engagement to both hFcγRIIA and hFcγRIIIA.

To determine which hFcγRs are responsible for the initial ADCC-mediated clearance of tumor cells, FcγR-humanized mice were given EL4-hCD20 lymphoma cells, and were treated with the various Fc-engineered hIgG1 anti-hCD20 mAbs. The GA mutant anti-hCD20 mAb that selectively engages hFcγRIIA was unable to clear tumor, as 81% of mice receiving this antibody died after primary tumor challenge (Fig. 5B). By contrast, 82% and 85% of mice receiving ALIE mutant (selectively engaging hFcγRIIIA) and GASDALIE mutant (selectively enhance both hFcγRIIA and hFcγRIIIA) anti-hCD20 mAb survived the primary EL4-hCD20 tumor challenge, respectively. Further, mice expressing only hFcγRIIA (deficient for murine FcγRs) were unable to clear the primary tumor challenge after mAb treatment, while mice expressing only hFcγRIIIA and hFcγRIIIB (deficient for murine FcγRs) showed full survival when treated with GASDALIE mutant anti-hCD20 mAb (Fig. 5C). Notably, wild-type hIgG1 anti-hCD20 mAb does not protect FcγR-humanized mice or mice expressing either hFcγRIIA or hFcγRIIIA/B from EL4-hCD20 tumor challenge (Supplementary Fig. 6C-D), indicating that wild-type interactions between these hFcγRs and hIgG1 provide insufficient signaling to mediate effector functions at the doses used in this mouse model. Taken together, these results demonstrate that while hFcγRIIA is dispensable, hFcγRIIIA is both necessary and sufficient for mAb-mediated clearance of primary tumor challenge.

Clodronate liposome (CLOD)-sensitive macrophages mediate ADCC in the context of hIgG1 and the human FcγR system

In the context of the mouse FcγR system, CLOD-sensitive macrophages mediate ADCC of antibody-coated target cells (Uchida et al., 2004). NK cells are dispensable for ADCC in this context, presumably because they do not express mFcγRI or mFcγRIV, but only express the low affinity FcγR, FcγRIII, which interacts with mIgG2a antibodies with ~40-fold lower affinity than mFcγRIV (Otten et al., 2008). However, we now show that hFcγRIIIA mediates ADCC in vivo by hIgG1 antibody. Because hFcγRIIIA is expressed on both NK cells and monocytes/macrophages in FcγR-humanized mice (and humans), we determined whether CLOD-sensitive macrophages mediate ADCC of mAb-coated target cells in the context of the human FcγR system and human IgG1. CLOD decreased total numbers of splenic CD11b^{int}F4/80^{hi} red pulp macrophages by >90% (Supplementary Fig. 6E; $p < 0.0001$). With the exception of CD11c^{hi} DCs (33% depletion, $p = 0.01$), no other cellular populations analyzed were affected by CLOD treatment (Supplementary Fig. 6F).

We first confirmed that depletion of blood and spleen B220⁺ B cells in hCD20-Tg mice (these mice express hCD20 on mature B cells and also express the full array of murine FcγRs) by mIgG2a isotype anti-hCD20 was dependent on CLOD-sensitive macrophages.

Blood and spleen B cell numbers were decreased by 97.5%-98% and 63%-78% ($p < 0.0001$; Supplementary Fig. 7A), respectively, in mice receiving either PBS or control liposomes plus mIgG2a anti-hCD20 mAb. By contrast, no B cells were depleted in mice receiving CLOD plus mIgG2a anti-hCD20 mAb. Thus, as described (Uchida et al., 2004), ADCC of antibody-coated cells in vivo requires CLOD-sensitive macrophage populations.

We next tested the ability of hIgG1 (GASDALIE mutant) anti-hCD20 mAb to deplete hCD20⁺ B cells in the context of the human Fc γ R system in hCD20-Tg/Fc γ R-humanized mice (these mice express hCD20 on mature B cells and also express the full array of human Fc γ Rs, but lack all murine Fc γ Rs), as described above. Blood and spleen B cell numbers were decreased by 86%-90% and 81%-82% ($p < 0.001$), respectively, in hCD20-Tg/Fc γ R-humanized mice receiving either PBS or control liposomes plus hIgG1 (GASDALIE) anti-hCD20 mAb. By contrast, no B cells were depleted in mice receiving CLOD plus GASDALIE anti-hCD20 mAb (Supplementary Fig. 7B). Similar results were seen in Fc γ R-humanized mice treated with CLOD and a depleting hIgG1 (GASDALIE) anti-mCD4 mAb (Supplementary Fig. 7C). Therefore, CLOD-sensitive macrophages are required for ADCC mediated by hIgG1 antibody in the context of the human Fc γ R system.

hFc γ RIIA engagement by anti-tumor mAb mediates the generation of the anti-tumor vaccinal effect in Fc γ R-humanized mice

Finally, we assessed to what extent the anti-tumor vaccinal effect was generated in Fc γ R-humanized mice (Fig. 6A) treated with anti-hCD20 mAb mutants that selectively engage only hFc γ RIIA (ALIE mutant) or both hFc γ RIIA and hFc γ RIIA (GASDALIE mutant). Only 20% of Fc γ R-humanized mice receiving ALIE mutant anti-hCD20 mAb survived re-challenge with EL4-hCD20 cells, while 77% of mice receiving GASDALIE mutant anti-hCD20 mAb survived re-challenge ($p < 0.0001$; Fig. 6B). Further, only 36% ($p = 0.01$) of hFc γ RIIA/B-Tg mice (which lack all murine Fc γ Rs and express only hFc γ RIIA and hFc γ RIIB) survive EL4-hCD20 re-challenge, again demonstrating that expression of hFc γ RIIA is required for optimal induction of an anti-tumor vaccinal effect. Thus, engagement of hFc γ RIIA, which is the only activating hFc γ R expressed by human DCs, is required for the generation of an anti-tumor vaccinal effect by passively-administered anti-tumor mAb.

Discussion

It has long been hypothesized that passive administration of anti-tumor antibodies may generate immune complexes that, upon uptake by antigen-presenting cells, stimulate anti-tumor cellular immunity. Taken together, our results now mechanistically demonstrate how passively-administered anti-tumor antibody achieves such an effect using a lymphoma cell line that expresses a model tumor neoantigen. Anti-tumor mAb opsonizes tumor cells and targets them for killing by Fc γ R-mediated ADCC, a process that generates antibody:tumor antigen immune complexes. These immune complexes engage activating Fc γ Rs expressed by CD11c⁺ antigen-presenting cells, which results in stimulation of DC maturation and presentation of tumor antigens to T cells, thereby leading to long-term anti-tumor cellular memory formation (Fig. 7) (Boruchov et al., 2005; Dhodapkar et al., 2005; Kalergis and

Ravetch, 2002; Nimmerjahn and Ravetch, 2008). In the human Fc γ R system, the vaccinal effect requires interactions with hFc γ R1IA, the sole activating Fc γ R expressed by DCs. Thus, these results now mechanistically explain how passively-administered anti-tumor mAb stimulates anti-tumor cellular immune responses in vivo and suggest novel methods for augmenting such an effect.

It is clear that activating Fc γ R_s expressed by antigen-presenting cells, especially DCs, are capable of capturing antibody/tumor antigen immune complexes. Upon immune complex binding, DCs undergo maturation, upregulate MHC-II and co-stimulatory molecules, and stimulate CD4 and CD8 T cells responses through traditional antigen presentation and cross-presentation (Nimmerjahn and Ravetch, 2008; Rafiq et al., 2002). DCs loaded with antibody/tumor antigen immune complexes stimulate potent T cell responses that are capable of eradicating tumors (Kalergis and Ravetch, 2002). In vitro studies have demonstrated that anti-CD20 mAb treatment of lymphoma cells stimulates DC maturation and CD8 T cell activation (Selenko et al., 2002), and a synergistic effect between vaccination with hCD20⁺ tumor cells and anti-hCD20 mAb treatment has been demonstrated in mice (Gadri et al., 2009). CD8⁺ DCs are considered to be excellent cross-presenters of cell-associated antigens (Mayer et al., 2014), and soluble immune complexes stimulate cross-presentation by DCs more potently than antigen alone (Berlyn et al., 2001; Rafiq et al., 2002). Correspondingly, a much larger fraction of CD8⁺ spleen CD11c^{hi} cells expresses mFc γ RIV compared to CD8⁻CD11c^{hi} cells (60% vs. 28%, Supplementary Fig. 2E), suggesting that CD8⁺ DCs may play a significant role during the induction of the mAb-mediated anti-tumor vaccinal effect. Further, clinical trials have shown that combining anti-CD20 mAb treatment with administration of immunomodulatory cytokines that promote the activation of DCs or T cell responses, such as IFN- α (Kimby et al., 2008) or GM-CSF (Cartron et al., 2008), synergistically increased anti-CD20 mAb efficacy, suggesting that augmenting antigen presentation and T cell responses in the context of anti-tumor mAb therapy may augment an anti-tumor vaccinal effect. Murine studies have also demonstrated a heightened vaccinal effect when administration of the pleiotropic cytokine IL-2, which activates both innate cells and T cells, is combined with anti-hCD20 mAb treatment (Abes et al., 2010). Thus, our studies now mechanistically explain how the anti-tumor cellular immune responses generated by passive antibody treatment are generated in vivo.

While healthy individuals are normally tolerized to self-antigens such as CD20 and would not develop memory T cells reactive with self-antigens, cancer-bearing patients often break tolerance and autoimmune disorders are common in these patients (Abu-Shakra et al., 2001). Thus, while tolerance to over-expressed antigens, oncoproteins, tumor suppressor proteins, differentiation antigens, or neoantigens engaged by antibodies may be broken and lead to the generation of anti-tumor memory T cells, these cells are often anergized or exhausted and unable to mount an effective cytotoxic T cell response to the tumor. Activating these T cells to become effector cells and target tumor cells is thus a goal of current immunotherapy approaches, most recently achieved by blocking inhibitory signals such as CTLA-4 (Hodi et al., 2010) and PD-1 (Brahmer et al., 2012; Topalian et al., 2012). Our data support an alternative approach in which combining anti-tumor cytotoxic antibody therapeutics with various immunotherapies that boost cellular immune responses (i.e., agonistic anti-CD40 mAb (Li and Ravetch, 2011), or antagonistic anti-CTLA-4 or anti-PD-1 mAbs) may

synergistically combine with an anti-tumor mAb vaccinal effect to boost cellular memory formation. Thus, our results suggest a general mechanism by which anti-tumor antibodies can stimulate anti-tumor cellular immune responses against a variety of tumor antigens.

Significant efforts were put towards identifying the antigen-specific T cells that mediate the vaccinal effect in this study, but *ex vivo* re-stimulation (ELISPOT and intracellular cytokine staining) studies with tumor cell lysates, peptides, or irradiated tumor cells were not sensitive enough to detect rare tumor-specific T cells. Regardless, conclusions regarding the specificity of the anti-tumor vaccinal effect T cell response can be made. Mice primed with mAb and hCD20⁺ EL4 lymphoma cells rejected lymphoma re-challenge with hCD20⁺ EL4 cells but not wild-type EL4 cells (Fig. 1C). This result suggests that the vaccinal effect T cell response is directed at the hCD20 neoantigen and that no detectable epitope-spreading occurs in this model. We have confirmed that the T cell response is, at least in part, directed at hCD20 because mice primed with mAb and hCD20⁺ EL4 lymphoma rejected re-challenge with a distinct tumor cell line expressing hCD20, but not the same cell line expressing a control antigen (Fig. 1D). Whether all vaccinal effect anti-tumor T cell responses are solely directed at the mAb-targeted antigen, as in the current EL4 tumor model, or whether different tumor models or different tumor microenvironments (i.e., lymphoid vs. solid tumors) will result in different mAb-induced anti-tumor T cell responses and epitope spreading remains unclear. Whether combining passive anti-tumor mAb with checkpoint inhibitor blockade or adjuvants will result in synergistically enhanced epitope spreading and anti-neoantigen T cell responses is also unknown.

Our studies in Fc γ R-humanized mice with hIgG1 anti-hCD20 mAb-mediated ADCC of hCD20⁺ tumor cells clearly demonstrate that hFc γ RIIIA is both necessary and sufficient for ADCC-mediated clearance of antibody-coated tumor cells; hFc γ RIIA plays no role in this process (Fig. 5). These results correspond to findings in humans that *FCGR3A* polymorphisms correlate with response rates in lymphoma patients treated with anti-CD20 mAb (Cartron et al., 2002) or breast cancer patients treated with anti-Her2 mAb (Musolino et al., 2008). Importantly, Fc γ R signaling is required for ADCC *in vivo*, rather than simple cross-linking of antigen (de Haij et al., 2010). Because they solely express hFc γ RIIIA, dogma has dictated that NK cells are the main mediators of ADCC in humans (Seidel et al., 2013). Further promoting the belief that NK cells are the major mediators of ADCC in humans, NK cells from human peripheral blood are routinely used during *in vitro* ADCC assays, which inadequately attempt to artificially re-create a complex *in vivo* process. Nonetheless, it has been demonstrated that CLOD-sensitive macrophages ((Uchida et al., 2004), Supplementary Fig. 7A), but not NK cells, are required for ADCC by mIgG in the context of murine Fc γ Rs. This was partially thought to be due to the lone expression of Fc γ RIII on murine NK cells (Otten et al., 2008), which weakly interacts with mouse antibody Fc compared to Fc γ RIV. Therefore, we have now clearly determined the cellular requirements for hIgG1-triggered ADCC mediated by human Fc γ Rs, and demonstrate that CLOD-sensitive macrophages mediate ADCC of antibody-coated target cells *in vivo* in the context of hIgG1 antibody and the human Fc γ R system (Supplementary Fig. 7B-C). This result is significant, because new therapies aimed at augmenting human NK cell activity *in vivo* to enhance ADCC of mAb-targeted tumor cells are currently under investigation.

Further studies determining any functional differences between murine and human NK cells will shed more light on this important matter.

The results reported here highlight the importance of properly engineering antibody therapeutics to engage the appropriate Fc γ Rs to mediate appropriate effector functions. Current efforts to augment anti-tumor antibodies have only focused on enhancing their cytotoxic effects by modulating hIgG1 Fc interactions with hFc γ RIIIA to augment ADCC by innate cells, as exemplified by the next-generation glyco-engineered anti-hCD20 mAb, obinutuzumab (Goede et al., 2014). Obinutuzumab is afucosylated for augmented affinity to only hFc γ RIIIA, and accordingly, extends survival by approximately one year in CLL patients when directly compared to an unmodified anti-CD20 antibody (Rituximab). However, afucosylation of obinutuzumab does not affect Fc engagement of hFc γ RIIA, which is the sole activating hFc γ R expressed on human antigen-presenting DCs for engagement of immune complexes and stimulation of T cell responses. Thus, our current results argue that an ideal anti-tumor therapeutic should not only optimally engage hFc γ RIIIA for cytotoxic effector function, but also hFc γ RIIA on DCs in order to induce long-term cellular anti-tumor immunity.

Experimental Procedures

Cell lines and mice

EL4-WT and 293T cells were obtained from ATCC (Manassas, VA) and maintained in Dulbecco's minimum essential medium (DMEM; Life Technologies, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies, Inc), 100 units/ml of penicillin, and 100 g/ml of streptomycin (Life Technologies, Inc). EL4-hCD20 cells were obtained from Oliver Press (Fred Hutchinson Cancer Research Center, Seattle, WA) with permission from Josée Golay (Ospedali Riuniti di Bergamo, Bergamo, Italy) and maintained in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 g/ml of streptomycin. B6BL cells, a spontaneous B cell lymphoma line isolated from p53^{fl/fl}CD19-Cre⁺ mice on pure B6 genetic background, have been previously described (Robbiani et al., 2009), and were retrovirally transduced with constructs encoding either hCD20 or mCD20 (pVPack Vectors, Agilent Technologies, La Jolla, CA), selected to make stable cell lines, and sorted for CD20⁺ cells, as described previously (Li and Ravetch, 2011). C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). *Fcer1g*^{-/-} (Takai et al., 1994), *Fcgr4*^{-/-} (Nimmerjahn et al., 2010), FcR α -null (Smith et al., 2012), *FCGR3A/B*-Tg (Li et al., 1996) (crossed to FcR α -null mice), and *FCGR2A*-Tg (McKenzie et al., 1999) (crossed to *Fcer1g*^{-/-}*Fcgr2b*^{-/-} mice) mice on the C57BL/6 genetic background have been previously described. Fc γ R-humanized mice, which express all hFc γ Rs on the FcR α -null C57BL/6 genetic background, have been described (Smith et al., 2012). *Fcgr4*^{fl^{ox}} mice (Nimmerjahn et al., 2010) were crossed with mice expressing cre recombinase under the control of CD11c promoter/enhancer regions (B6.Cg-Tg(Itgax-cre)1-1Reiz/J mice, Jackson Laboratories). hCD20-Tg mice were kindly provided by Dr. Andrew Chan (Genentech, San Francisco, CA), and crossed onto the Fc γ R-humanized background. All mice were maintained in a specific pathogen-free facility at the

Rockefeller University, and all studies were approved by the Rockefeller University Institutional Animal Care and Use Committee.

Antibodies, flow cytometry, and other reagents

To generate CAT-13.6E12 and 2B8 mAb constructs, total RNA was obtained from hybridoma cells (DSMZ, Braunschweig, Germany and ATCC, Manassas, VA, respectively), and cDNA was generated by using SuperscriptIII reverse transcriptase (Life Technologies) and immunoglobulin gene-specific primers. The V_H and V_K genes were amplified by PCR and cloned in frame into mammalian expression vectors with mouse IgG2a, mouse IgG1, mouse DA265 mutant, mouse Kappa, huIgG1, or huKappa Fc backbones. The human G236A, A330L/I332E, and GASDALIE Fc mutants were generated by site-directed mutagenesis with PCR amplification of the entire vector, using complementary primers containing the desired point mutations, as described (Li and Ravetch, 2011). Antibodies were produced by transient transfection of 293T cells and subsequent protein G purification from culture supernatants, as described (Nimmerjahn et al., 2005). Anti-mCD4 antibody (clone GK1.5) with a hIgG1 backbone and containing GASDALIE point mutations was generated previously (Smith et al., 2012). Fluorescently-conjugated antibodies and staining procedures are listed in the Supplemental Experimental Procedures. To deplete macrophages in vivo, mice received 200 μ l of clodronate liposomes or PBS liposomes i.v. through lateral tail veins (Clophosome-A, Formumax, Palo Alto, CA).

Tumor model

For the primary tumor challenge, mice were injected intravenously through lateral tail veins with 5×10^5 EL4-hCD20 cells in 200 μ l PBS on day 0. Mice then received intraperitoneal injections of 100 μ g of antibody in 200 μ l of PBS on days 1, 4, 7, 10, and 13. Survival was assessed daily. In some experiments, 90 days after primary tumor challenge, surviving mice were re-challenged intravenously with 5×10^6 EL4-hCD20 or EL4-WT cells, with survival assessed daily. In experiments in which hIgG1 antibodies or mutants were administered, Fc γ R-humanized mice were given 5×10^5 EL4-hCD20 cells in 200 μ l PBS on day 0, with 250 μ g of antibody in 200 μ l of PBS given i.p. on days 1 and 2. Surviving mice were re-challenged with 5×10^6 EL4-hCD20 cells on day 60. In some experiments, mice were re-challenged i.v. with 5×10^4 B6BL cells expressing either hCD20 or mCD20.

In adoptive transfer experiments, splenocytes from mice 30 days after primary tumor challenge were harvested and red blood cells were lysed. In some cases, CD3⁺ cells were negatively selected using magnetic beads (Miltenyi Biotec, San Diego, CA). Then, 50×10^6 total splenocytes or 15×10^6 CD3⁺ cells were adoptively transferred into naïve C57BL/6 mice. One day later, the mice were challenged with 5×10^5 EL4-hCD20 cells in 200 μ l PBS, with survival assessed daily.

Statistics

Statistical differences between survival rates were analyzed by comparing Kaplan-Meier curves using the log-rank test and GraphPad Prism Software. All other statistical differences were compared using the student's T test analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Patrick Smith and Stylianos Bournazos for assistance with these studies. We are grateful to Oliver Press (Fred Hutchinson Cancer Research Center, Seattle, WA) and Josée Golay (Ospedali Riuniti di Bergamo, Bergamo, Italy) for providing EL4-hCD20 cells, and to Andrew Chan (Genentech, San Francisco, CA) for providing hCD20-Tg mice. Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under Award Number R01CA080757 to JVR. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This work was also supported by funding from the New York Community Trust Grants for Blood Disorder Research, Francis Florio Fund (to DJD). DJD received a postdoctoral career development fellowship from the Leukemia and Lymphoma Society of America.

References

- Abes R, Gelize E, Fridman WH, Teillaud JL. Long-lasting antitumor protection by anti-CD20 antibody through cellular immune response. *Blood*. 2010; 116:926–934. [PubMed: 20439625]
- Abu-Shakra M, Buskila D, Ehrenfeld M, Conrad K, Shoenfeld Y. Cancer and autoimmunity: autoimmune and rheumatic features in patients with malignancies. *Annals of the rheumatic diseases*. 2001; 60:433–441. [PubMed: 11302861]
- Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Borresen-Dale AL, et al. Signatures of mutational processes in human cancer. *Nature*. 2013; 500:415–421. [PubMed: 23945592]
- Berlyn KA, Schultes B, Leveugle B, Noujaim AA, Alexander RB, Mann DL. Generation of CD4(+) and CD8(+) T lymphocyte responses by dendritic cells armed with PSA/anti-PSA (antigen/antibody) complexes. *Clinical immunology*. 2001; 101:276–283. [PubMed: 11726219]
- Boruchov AM, Heller G, Veri MC, Bonvini E, Ravetch JV, Young JW. Activating and inhibitory IgG Fc receptors on human DCs mediate opposing functions. *The Journal of clinical investigation*. 2005; 115:2914–2923. [PubMed: 16167082]
- Bournazos S, DiLillo DJ, Ravetch JV. humanized mice to study FcγRIIIb function. *Current topics in microbiology and immunology*. 2014a; 382:237–248. [PubMed: 25116103]
- Bournazos S, Klein F, Pietzsch J, Seaman MS, Nussenzweig MC, Ravetch JV. Broadly Neutralizing Anti-HIV-1 Antibodies Require Fc Effector Functions for In Vivo Activity. *Cell*. 2014b; 158:1243–1253. [PubMed: 25215485]
- Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *The New England journal of medicine*. 2012; 366:2455–2465. [PubMed: 22658128]
- Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, Watier H. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcγRIIIa gene. *Blood*. 2002; 99:754–758. [PubMed: 11806974]
- Cartron G, Watier H, Golay J, Solal-Celigny P. From the bench to the bedside: ways to improve rituximab efficacy. *Blood*. 2004; 104:2635–2642. [PubMed: 15226177]
- Cartron G, Zhao-Yang L, Baudard M, Kanouni T, Rouille V, Quittet P, Klein B, Rossi JF. Granulocyte-macrophage colony-stimulating factor potentiates rituximab in patients with relapsed follicular lymphoma: results of a phase II study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2008; 26:2725–2731. [PubMed: 18427151]
- Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nature medicine*. 2000; 6:443–446.
- de Bono JS, Rha SY, Stephenson J, Schultes BC, Monroe P, Eckhardt GS, Hammond LA, Whiteside TL, Nicodemus CF, Cermak JM, et al. Phase I trial of a murine antibody to MUC1 in patients with metastatic cancer: evidence for the activation of humoral and cellular antitumor immunity. *Annals*

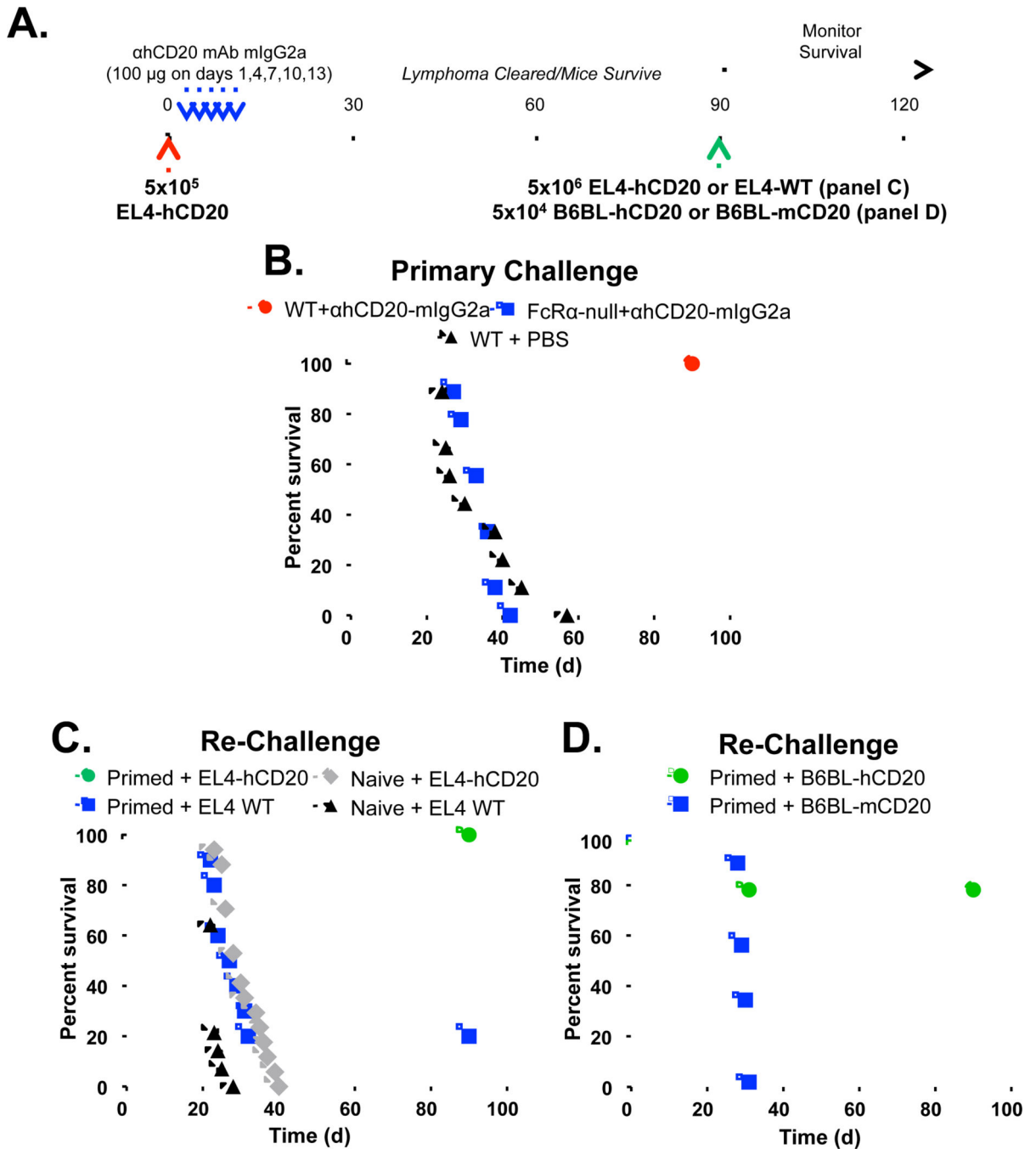
- of oncology : official journal of the European Society for Medical Oncology / ESMO. 2004; 15:1825–1833. [PubMed: 15550589]
- de Haij S, Jansen JH, Boross P, Beurskens FJ, Bakema JE, Bos DL, Martens A, Verbeek JS, Parren PW, van de Winkel JG, et al. In vivo cytotoxicity of type I CD20 antibodies critically depends on Fc receptor ITAM signaling. *Cancer research*. 2010; 70:3209–3217. [PubMed: 20354182]
- Dhodapkar KM, Kaufman JL, Ehlers M, Banerjee DK, Bonvini E, Koenig S, Steinman RM, Ravetch JV, Dhodapkar MV. Selective blockade of inhibitory Fcγ receptor enables human dendritic cell maturation with IL-12p70 production and immunity to antibody-coated tumor cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:2910–2915. [PubMed: 15703291]
- Fritsch EF, Hacohen N, Wu CJ. Personal neoantigen cancer vaccines: The momentum builds. *Oncoimmunology*. 2014; 3:e29311. [PubMed: 25101225]
- Gadri Z, Kukulansky T, Bar-Or E, Haimovich J, Hollander N. Synergistic effect of dendritic cell vaccination and anti-CD20 antibody treatment in the therapy of murine lymphoma. *Journal of immunotherapy*. 2009; 32:333–340. [PubMed: 19342972]
- Goede V, Fischer K, Busch R, Engelke A, Eichhorst B, Wendtner CM, Chagorova T, de la Serna J, Dilhuydy MS, Illmer T, et al. Obinutuzumab plus chlorambucil in patients with CLL and coexisting conditions. *The New England journal of medicine*. 2014; 370:1101–1110. [PubMed: 24401022]
- Gros A, Robbins PF, Yao X, Li YF, Turcotte S, Tran E, Wunderlich JR, Mixon A, Farid S, Dudley ME, et al. PD-1 identifies the patient-specific CD8(+) tumor-reactive repertoire infiltrating human tumors. *The Journal of clinical investigation*. 2014; 124:2246–2259. [PubMed: 24667641]
- Hilchey SP, Hyrien O, Mosmann TR, Livingstone AM, Friedberg JW, Young F, Fisher RI, Kelleher RJ Jr, Bankert RB, Bernstein SH. Rituximab immunotherapy results in the induction of a lymphoma idiotype-specific T-cell response in patients with follicular lymphoma: support for a “vaccinal effect” of rituximab. *Blood*. 2009; 113:3809–3812. [PubMed: 19196657]
- Hodi FS, O’Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *The New England journal of medicine*. 2010; 363:711–723. [PubMed: 20525992]
- Kalergis AM, Ravetch JV. Inducing tumor immunity through the selective engagement of activating Fcγ receptors on dendritic cells. *The Journal of experimental medicine*. 2002; 195:1653–1659. [PubMed: 12070293]
- Kimby E, Jurlander J, Geisler C, Hagberg H, Holte H, Lehtinen T, Ostenstad B, Hansen M, Osterborg A, Linden O, et al. Long-term molecular remissions in patients with indolent lymphoma treated with rituximab as a single agent or in combination with interferon alpha-2a: a randomized phase II study from the Nordic Lymphoma Group. *Leukemia & lymphoma*. 2008; 49:102–112. [PubMed: 18203019]
- Li F, Ravetch JV. Inhibitory Fcγ receptor engagement drives adjuvant and anti-tumor activities of agonistic CD40 antibodies. *Science*. 2011; 333:1030–1034. [PubMed: 21852502]
- Li M, Wirthmueller U, Ravetch JV. Reconstitution of human Fc gamma RIII cell type specificity in transgenic mice. *The Journal of experimental medicine*. 1996; 183:1259–1263. [PubMed: 8642269]
- Mayer CT, Ghorbani P, Nandan A, Dudek M, Arnold-Schrauf C, Hesse C, Berod L, Stuve P, Puttner F, Merad M, et al. Selective and efficient generation of functional Batf3-dependent CD103+ dendritic cells from mouse bone marrow. *Blood*. 2014; 124:3081–3091. [PubMed: 25100743]
- McKenzie SE, Taylor SM, Malladi P, Yuhan H, Cassel DL, Chien P, Schwartz E, Schreiber AD, Surrey S, Reilly MP. The role of the human Fc receptor Fc gamma RIIA in the immune clearance of platelets: a transgenic mouse model. *Journal of immunology*. 1999; 162:4311–4318.
- Musolino A, Naldi N, Bortesi B, Pezzuolo D, Capelletti M, Missale G, Laccabue D, Zerbini A, Camisa R, Bisagni G, et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2008; 26:1789–1796. [PubMed: 18347005]

- Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV. Fcγ₄RIV: a novel FcR with distinct IgG subclass specificity. *Immunity*. 2005; 23:41–51. [PubMed: 16039578]
- Nimmerjahn F, Lux A, Albert H, Woigk M, Lehmann C, Dudziak D, Smith P, Ravetch JV. Fcγ₄RIV deletion reveals its central role for IgG_{2a} and IgG_{2b} activity in vivo. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:19396–19401. [PubMed: 20974962]
- Nimmerjahn F, Ravetch JV. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science*. 2005; 310:1510–1512. [PubMed: 16322460]
- Nimmerjahn F, Ravetch JV. Fcγ receptors: old friends and new family members. *Immunity*. 2006; 24:19–28. [PubMed: 16413920]
- Nimmerjahn F, Ravetch JV. Fc-receptors as regulators of immunity. *Adv Immunol*. 2007; 96:179–204. [PubMed: 17981207]
- Nimmerjahn F, Ravetch JV. Fcγ receptors as regulators of immune responses. *Nature reviews Immunology*. 2008; 8:34–47.
- Nimmerjahn F, Ravetch JV. Fcγ₄R in health and disease. *Current topics in microbiology and immunology*. 2011; 350:105–125. [PubMed: 20680807]
- Otten MA, van der Bij GJ, Verbeek SJ, Nimmerjahn F, Ravetch JV, Beelen RH, van de Winkel JG, van Egmond M. Experimental antibody therapy of liver metastases reveals functional redundancy between Fc γ₁R and Fc γ₄RIV. *Journal of immunology*. 2008; 181:6829–6836.
- Park S, Jiang Z, Mortenson ED, Deng L, Radkevich-Brown O, Yang X, Sattar H, Wang Y, Brown NK, Greene M, et al. The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. *Cancer cell*. 2010; 18:160–170. [PubMed: 20708157]
- Pincetic A, Bournazos S, DiLillo DJ, Maamary J, Wang TT, Dahan R, Fiebiger BM, Ravetch JV. Type I and type II Fc receptors regulate innate and adaptive immunity. *Nature immunology*. 2014; 15:707–716. [PubMed: 25045879]
- Rafiq K, Bergtold A, Clynes R. Immune complex-mediated antigen presentation induces tumor immunity. *The Journal of clinical investigation*. 2002; 110:71–79. [PubMed: 12093890]
- Robbiani DF, Bunting S, Feldhahn N, Bothmer A, Camps J, Deroubaix S, McBride KM, Klein IA, Stone G, Eisenreich TR, et al. AID produces DNA double-strand breaks in non-Ig genes and mature B cell lymphomas with reciprocal chromosome translocations. *Molecular cell*. 2009; 36:631–641. [PubMed: 19941823]
- Seidel UJ, Schlegel P, Lang P. Natural killer cell mediated antibody-dependent cellular cytotoxicity in tumor immunotherapy with therapeutic antibodies. *Frontiers in immunology*. 2013; 4:76. [PubMed: 23543707]
- Selenko N, Majdic O, Jager U, Sillaber C, Stockl J, Knapp W. Cross-priming of cytotoxic T cells promoted by apoptosis-inducing tumor cell reactive antibodies? *Journal of clinical immunology*. 2002; 22:124–130. [PubMed: 12078853]
- Smith P, DiLillo DJ, Bournazos S, Li F, Ravetch JV. Mouse model recapitulating human Fcγ receptor structural and functional diversity. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109:6181–6186. [PubMed: 22474370]
- Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV. FcR γ chain deletion results in pleiotrophic effector cell defects. *Cell*. 1994; 76:519–529. [PubMed: 8313472]
- Taylor C, Hershman D, Shah N, Suci-Foca N, Petrylak DP, Taub R, Vahdat L, Cheng B, Pegram M, Knutson KL, et al. Augmented HER-2 specific immunity during treatment with trastuzumab and chemotherapy. *Clin Cancer Res*. 2007; 13:5133–5143. [PubMed: 17785568]
- Taylor RP, Lindorfer MA. Immunotherapeutic mechanisms of anti-CD20 monoclonal antibodies. *Current opinion in immunology*. 2008; 20:444–449. [PubMed: 18585457]
- Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *The New England journal of medicine*. 2012; 366:2443–2454. [PubMed: 22658127]
- Tran E, Turcotte S, Gros A, Robbins PF, Lu YC, Dudley ME, Wunderlich JR, Somerville RP, Hogan K, Hinrichs CS, et al. Cancer immunotherapy based on mutation-specific CD4⁺ T cells in a patient with epithelial cancer. *Science*. 2014; 344:641–645. [PubMed: 24812403]

- Uchida J, Hamaguchi Y, Oliver JA, Ravetch JV, Poe JC, Haas KM, Tedder TF. The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *The Journal of experimental medicine*. 2004; 199:1659–1669. [PubMed: 15210744]
- van Rooij N, van Buuren MM, Philips D, Velds A, Toebes M, Heemskerk B, van Dijk LJ, Behjati S, Hilkmann H, El Atmioui D, et al. Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2013; 31:e439–442. [PubMed: 24043743]
- Vieira P, Rajewsky K. The half-lives of serum immunoglobulins in adult mice. *European journal of immunology*. 1988; 18:313–316. [PubMed: 3350037]

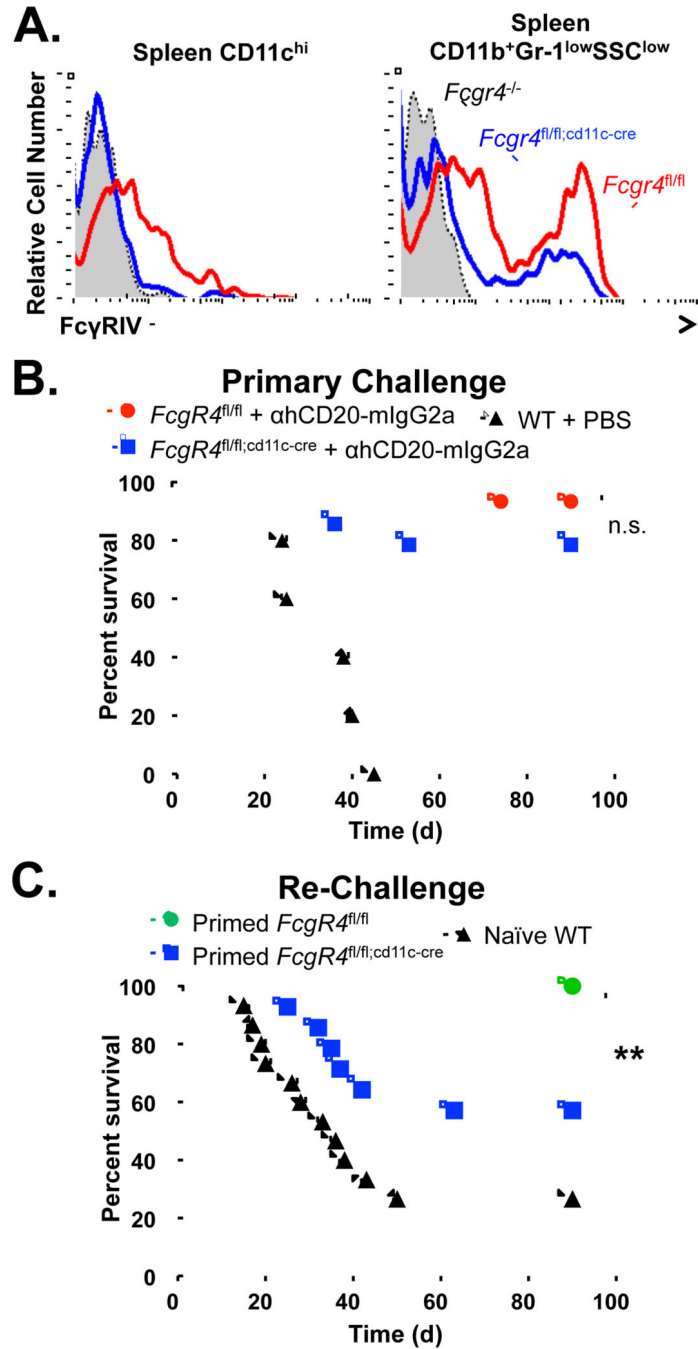
Highlights

- Tumor-bearing mice given cytotoxic anti-tumor antibody develop T cell tumor immunity.
- ADCC-mediated tumor clearance requires engagement of hFc γ RIIIA on macrophages
- Selective engagement of hFc[.gamma]RIIA in DCs promotes long-term immunity against cancer

**Figure 1.**

Fc-Fc γ R interactions are required for the clearance of lymphoma by mAb and the initiation of an anti-tumor vaccinal effect. **A**, Experimental protocol: Mice were injected i.v. with 5×10^5 EL4-hCD20 lymphoma cells on day 0 (red arrow), and received 100µg of mIgG2a isotype anti-hCD20 mAb (clone CAT13.6E12) on days 1, 4, 7, 10, and 13 (blue arrows). On day 90, surviving mice were re-challenged i.v. with 5×10^6 EL4-hCD20 lymphoma cells (green arrow) or EL4-WT cells, a 10-fold greater dose of tumor compared to the primary lymphoma challenge, and survival was monitored daily. Alternatively, surviving mice were

re-challenged with 5×10^4 B6BL-hCD20 or B6BL-mCD20 cells i.v. **B**, Wildtype (red circles) or FcR α -null mice (blue squares) were injected with EL4-hCD20 cells and treated with IgG2a isotype anti-hCD20 mAb, with survival assessed daily (n=9-11 mice per group). **C**, After 90 days, surviving (primed) mice treated with mIgG2a anti-hCD20 mAb from (b) were re-challenged with EL4-hCD20 cells (green circles) or EL4-WT cells (blue squares) with survival assessed daily. For comparison, naïve mice were also challenged with EL4-hCD20 cells (gray diamonds) or EL4-WT cells (filled triangles). n=10-13 mice per group. **D**, Mice were primed with EL4-hCD20 lymphoma cells and mAb as in (A-B) before re-challenge on day 90 with B6BL tumor cells expressing either hCD20 (green circles) or mCD20 (blue squares). n=10 mice per group.

**Figure 2.**

Expression of mFcγRIV on CD11c⁺ cells is required for the generation of an anti-tumor vaccinal effect. **A**, mFcγRIV expression levels on spleen innate cell subsets. Spleen lymphocytes were harvested from *Fcgr4^{fl/fl}* (red line), *Fcgr4^{fl/fl};cd11c-cre* (blue line), or *Fcgr4^{-/-}* (shaded line) mice and mFcγRIV expression levels on CD11c⁺ dendritic cells and CD11b⁺Gr-1^{low}SSC^{low} resident monocytes was assessed. Representative flow cytometry histograms from three independent experiments are shown. **B**, *Fcgr4^{fl/fl}* (red circles; n=15) or *Fcgr4^{fl/fl};cd11c-cre* (blue squares; n=14) mice were given EL4-hCD20 cells and treated with

mIgG2a anti-hCD20 mAb, with survival monitored daily. **C**, After 90 days, surviving *Fcgr4^{fl/fl}* (green circles) or *Fcgr4^{fl/fl;cd1c-cre}* (blue squares) mice treated with mIgG2a isotype anti-hCD20 mAb from (B) were re-challenged with EL4-hCD20 cells, with survival assessed daily (n=14-16 mice per group). Significant differences between groups are indicated: **, p=0.0065; n.s., not significant

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

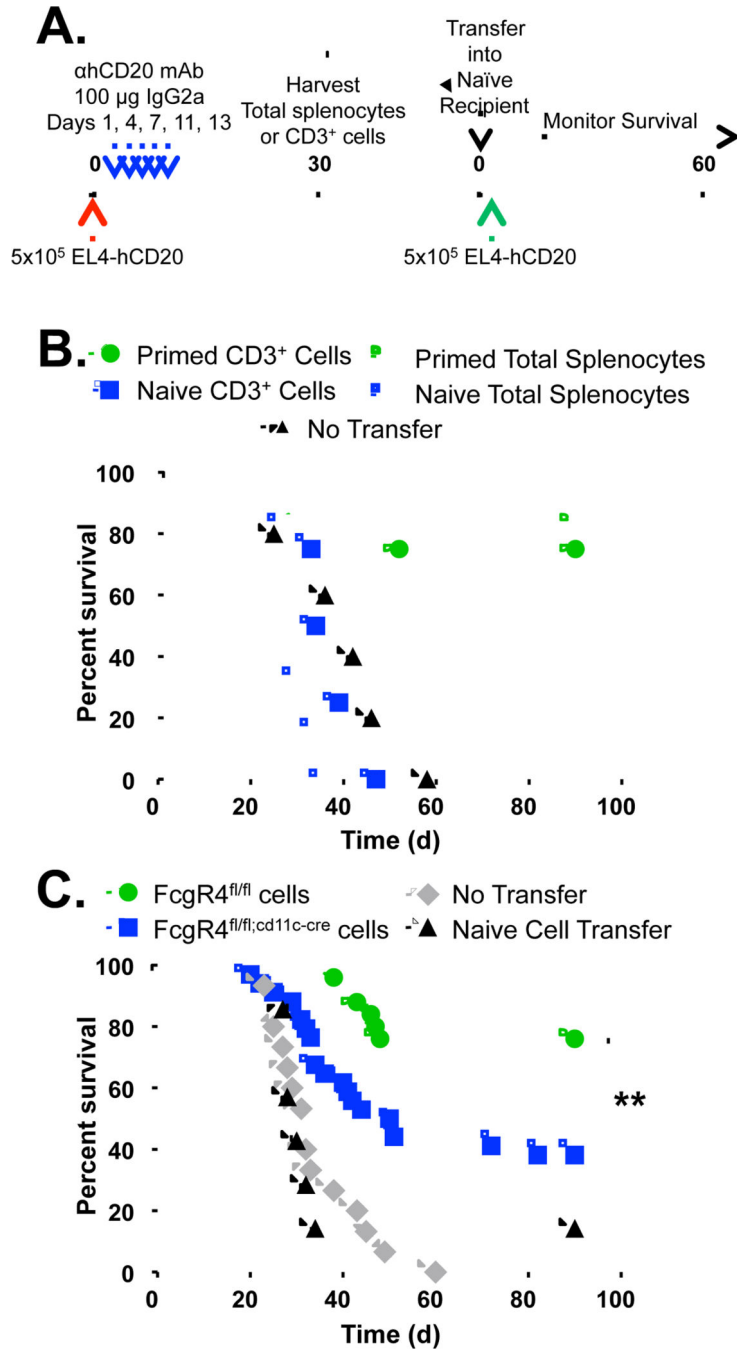


Figure 3. CD11c⁺ cell-specific expression of mFcγRIV is required for the generation of anti-tumor cellular immunity. **A**, Experimental protocol: Mice were injected i.v. with EL4-hCD20 lymphoma cells on day 0 (red arrow), and received mIgG2a anti-hCD20 mAb (blue arrows). On day 30, spleens were harvested and total splenocytes were isolated or CD3⁺ cells were purified. Then, 50×10^6 total splenocytes or 15×10^6 CD3⁺ cells were adoptively transferred into naïve mice one day before i.v. challenge with EL4-hCD20 lymphoma cells (green arrow). **B**, Survival was measured in naïve mice receiving CD3⁺ cells (green filled circles)

or total splenocytes (green open circles) from tumor and mAb-primed wild-type mice, or CD3⁺ cells (blue filled squares) or total splenocytes (blue open squares) from naïve mice. Another group of naïve mice received no adoptive transfer (black triangles). n=4-6 mice per group. C, Survival in naïve mice receiving total splenocytes from tumor and mAb-primed *Fcgr4^{fl/fl}* (green circles; n=25) or *Fcgr4^{fl/fl;cd1c-cre}* (blue squares, n=34) mice before EL4-hCD20 cell challenge. Other groups of naïve mice received splenocytes from naïve mice (black triangles; n=7) or no adoptive transfer (gray diamonds; n=15). Significant differences between groups are indicated: **, p=0.0041.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

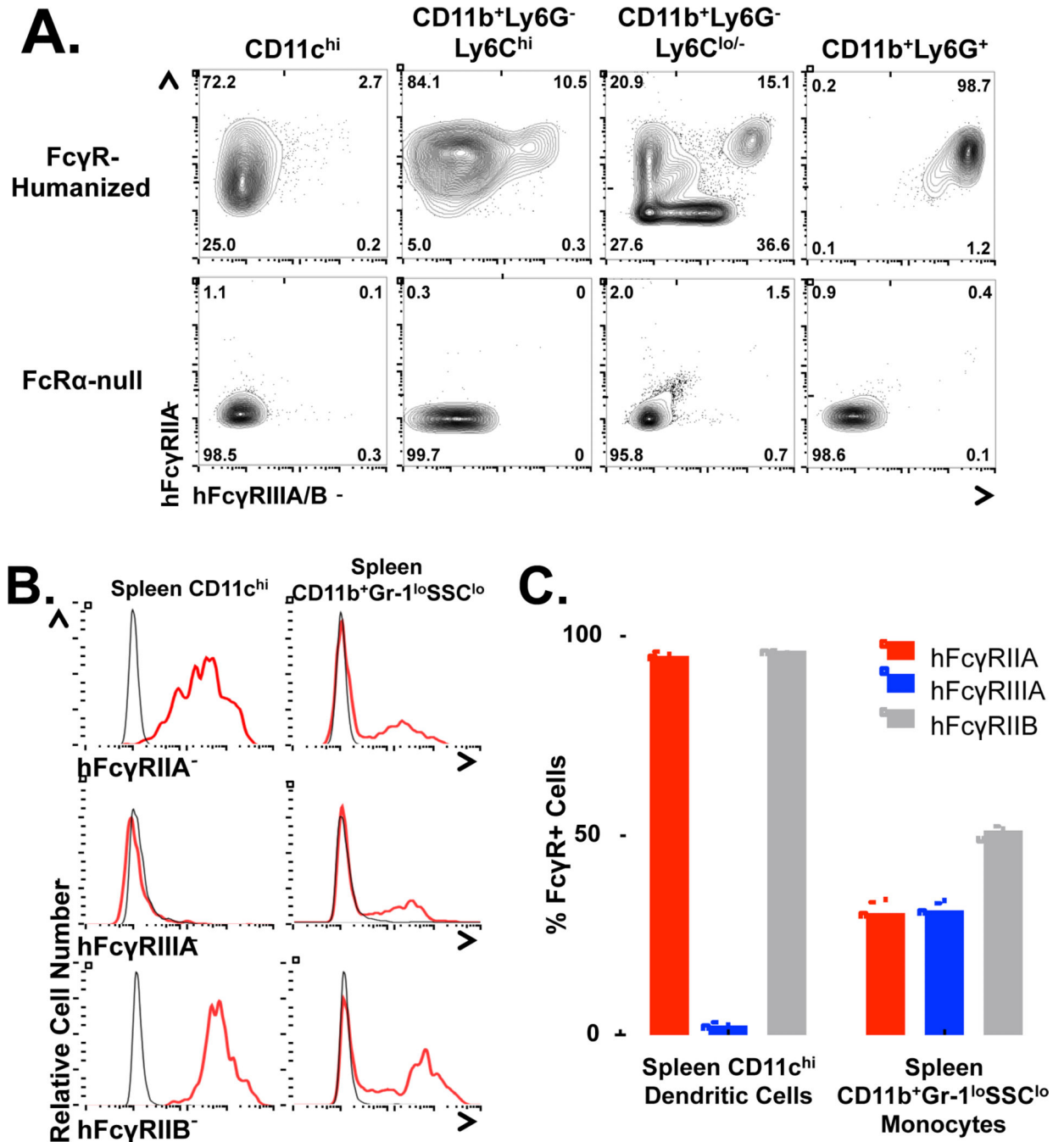
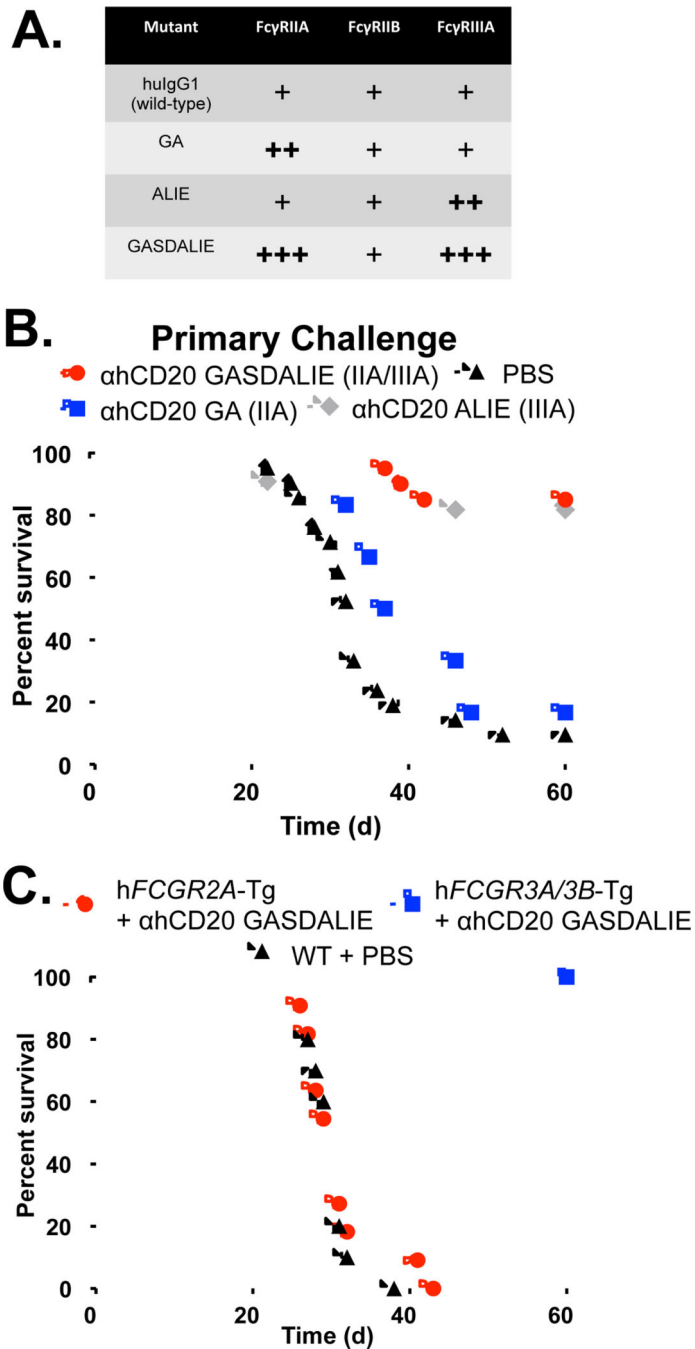


Figure 4.

Human Fc γ R expression on innate cells in Fc γ R-humanized mice. **A**, Representative flow cytometry dot plots show hFc γ RIIA vs. hFc γ RIIIA/B expression on spleen cells from Fc γ R-humanized or FcR α -null mice. Numbers represent the frequency of cells in the indicated gate. **B**, DCs and monocytes from Fc γ R-humanized mouse spleens (red lines) were stained for hFc γ RIIA, hFc γ RIIIA/B, or hFc γ RIIB. Background staining by hFc γ R⁻ cells is shown (gray lines). **C**, Frequencies of hFc γ R⁺ cells among spleen DCs and monocytes (n=3 per group), with frequencies generated by background staining subtracted.

**Figure 5.**

Differential hFcγR engagement mediates tumor cytotoxicity. **A**, Anti-hCD20 hIgG1 mAb Fc mutants for selectively-enhanced engagement of hFcγRs. Relative binding capabilities to the indicated hFcγRs are shown, based on binding affinities from biacore experiments (Supplementary Table 2). **B**, hFcγRIIIA engagement mediates cytotoxic clearance of tumor cells by mAb. FcγR-humanized mice were given EL4-hCD20 cells and treated with hIgG1 mutant versions of anti-hCD20 mAb: GASDALIE mutant (enhanced engagement of hFcγRIIA and hFcγRIIIA; red circles; n=20), GA mutant (preferential hFcγRIIA

engagement; blue squares; n=6), ALIE mutant (preferential hFcγRIIIA engagement; gray diamonds; n=12), or PBS (black triangles; n=17), with survival monitored daily. **C.** hFcγRIIIA is necessary and sufficient to mediate the immediate cytotoxic clearance of EL4-hCD20 lymphoma cells. EL4-hCD20 cells were injected into hFCGR2A-Tg mice that were given GASDALIE mutant anti-hCD20 mAb (filled red circles; n=11), hFCGR3A/B-Tg mice given GASDALIE mutant anti-hCD20 mAb (filled blue squares; n=11), or wild-type mice given PBS (filled triangles; n=10) with survival monitored daily.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

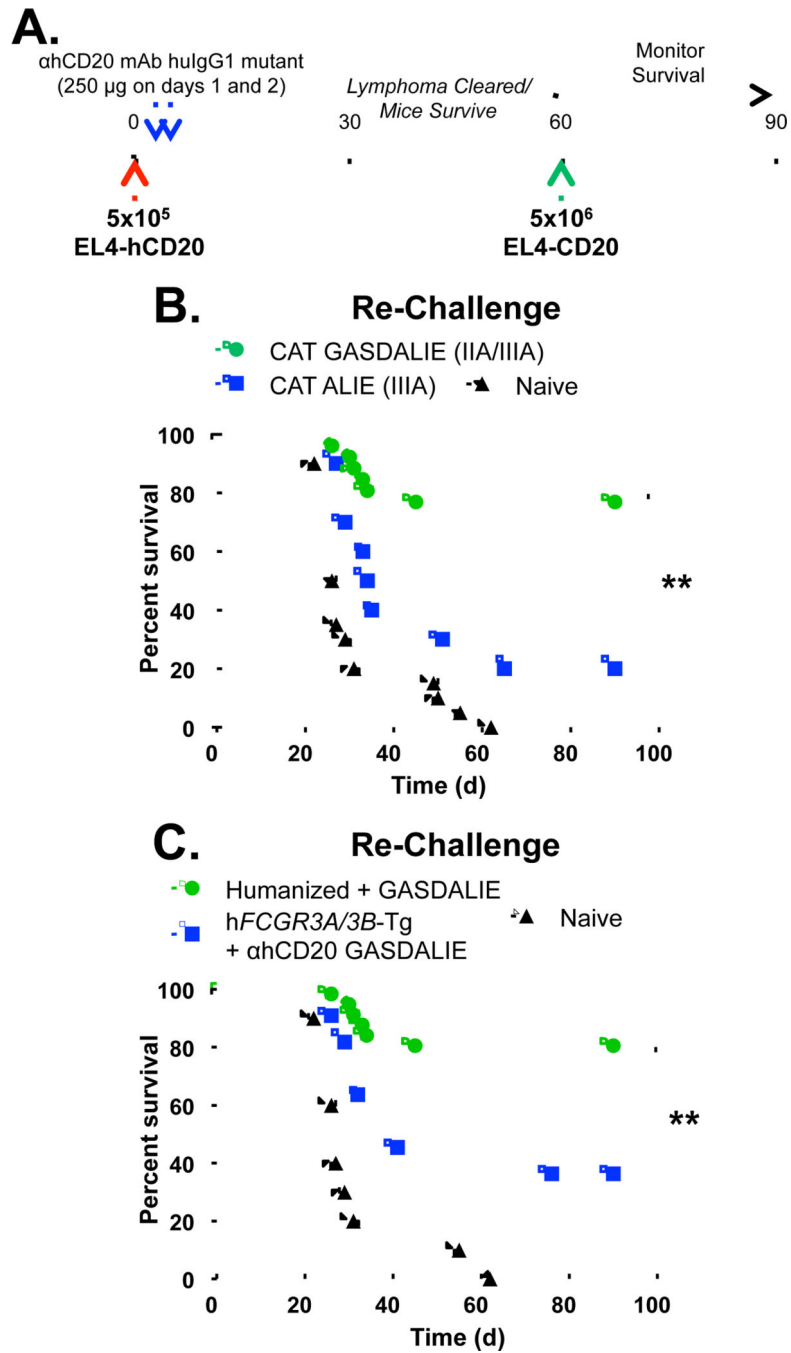


Figure 6.

Selective engagement of hFcγRIIA mediates an anti-tumor vaccinal effect. **A**, Experimental protocol: FcγR-humanized mice were injected i.v. with 5×10^5 EL4-hCD20 lymphoma cells on day 0 (red arrow), and received 250 µg of hIgG1 mutant anti-hCD20 mAb on days 1 and 2 (blue arrows). On day 60, surviving mice were re-challenged i.v. with 5×10^6 EL4-hCD20 lymphoma cells (green arrow), and survival was monitored daily. **B**, Surviving tumor-primed mice that received GASDALIE hIgG1 anti-hCD20 mAb (green circles; n=28) or ALIE hIgG1 anti-hCD20 mAb (blue squares; n=10) from Fig. 5B, or naïve mice (black

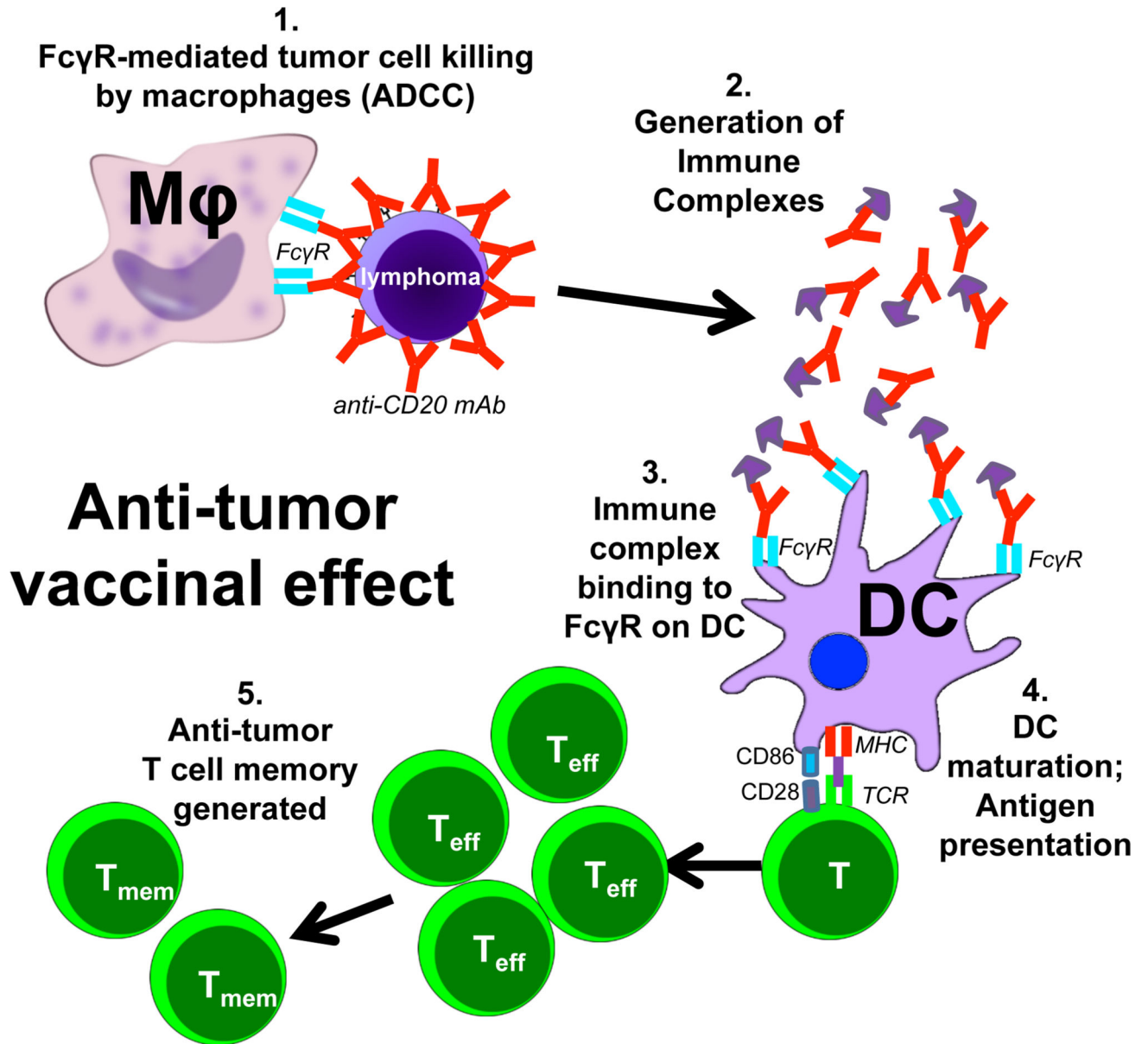
triangles; n=15) were re-challenged with EL4-hCD20 cells. **C**, Surviving tumor-primed Fc γ R-humanized (green circles; n=28) or hFc γ R11A/B-Tg mice (blue squares, n=11) that received GASDALIE hIgG1 anti-hCD20 mAb from Fig. 5C, or naïve mice (black triangles; n=10) were re-challenged with EL4-hCD20 cells. Significant differences between groups are indicated: **, p<0.01.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

**Figure 7.**

Model for the generation of an anti-tumor vaccinal effect. Anti-tumor mAb opsonizes tumor cells and targets them for killing by Fc γ R-mediated ADCC, a process that generates antibody:tumor antigen immune complexes. These immune complexes engage activating Fc γ Rs expressed by mouse or human CD11c⁺ cells, which results in stimulation of DC maturation and presentation of tumor antigens to T cells, thereby leading to long-term anti-tumor cellular memory formation