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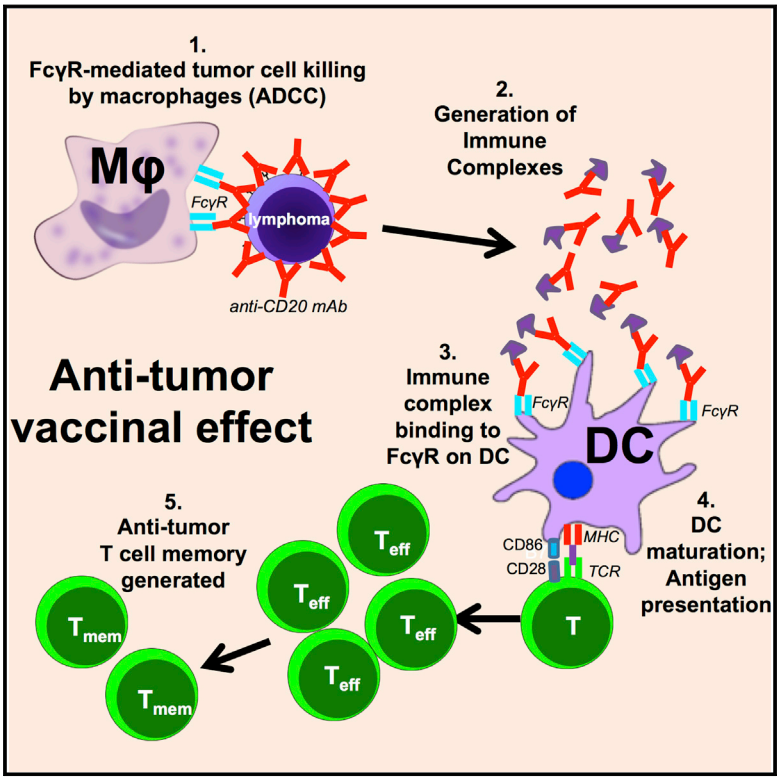
Differential Fc-Receptor Engagement Drives an Anti-tumor Vaccinal Effect

Graphical Abstract



Highlights

- Tumor-bearing mice given cytotoxic anti-tumor antibody develop T cell tumor immunity
- This anti-tumor vaccinal effect is differentially driven by two distinct human FcγRs
- ADCC-mediated tumor clearance requires engagement of hFcγRIIIA on macrophages
- Selective engagement of hFcγRIIA on DCs promotes long-term immunity against cancer

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In Brief

Cytotoxic antibodies targeting tumor cells can prime the cellular immune system for the generation of anti-tumor T cell memory. Tumor antigen immune complexes engage FcγRs expressed by CD11c^{+} antigen-presenting cells to stimulate this anti-tumor vaccinal effect.

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Differential Fc-Receptor Engagement Drives an Anti-tumor Vaccinal Effect

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SUMMARY

Passively administered anti-tumor monoclonal antibodies (mAbs) rapidly kill tumor targets via FcγR-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 FcγR expression by CD11c$^+$ antigen-presenting cells is required to generate anti-tumor T cell responses upon ADCC-mediated tumor clearance. Using FcγR-humanized mice, we demonstrate that anti-tumor human (h)IgG1 must engage hFcγRIIIA on macrophages to mediate ADCC, but also engage hFcγRIIA, the sole hFcγR expressed by human dendritic cells (DCs), to generate a potent vaccinal effect. Thus, while next-generation anti-tumor antibodies with enhanced binding to only hFcγRIIIA are now in clinical use, ideal anti-tumor antibodies must be optimized for both cytotoxic effects as well as hFcγRIIA 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 (FcγR)-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 FcγR-mediated mechanism of action, next-generation versions of anti-tumor mAbs that have been Fc-engineered for enhanced engagement of activating hFcγRIIIA 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-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-human (h)CD20 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-idiotype 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 (Abès 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 overexpressed 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 (mouse [m]FcγRI, 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, mIgG2a antibodies trigger cytotoxicity by virtue of this Fc having a 2-log greater affinity for the activating mFcγRIV receptor as compared to the inhibitory mFcγRIIb receptor, while mIgG1 preferentially engages the inhibitory mFcγRIIb receptor.

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

Here, using a murine lymphoma expressing a model tumor neoantigen (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 FcγRs required for macrophage-mediated ADCC, but activating FcγR 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 FcγR 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 FcγR-humanized mice. We show that anti-tumor mAb must engage hFcγRIIIA on clodronate liposome (CLOD)-sensitive macrophages to mediate immediate ADCC, as well as hFcγRIIA (the sole FcγR 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 hFcγRIIIA 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 hFcγRIIIA, as well as for engagement of hFcγRIIA 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 (Abès et al., 2010) of an anti-neoantigen mAb-mediated vaccinal effect (Figure 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 (that lack all mFcγRs: mFcγRI, mFcγRIIB, mFcγRIII, and mFcγRIV) and *Fcer1g*$^{-/-}$ mice (that lack all activating mFcγRs: mFcγRI, mFcγRIII, and mFcγRIV) do not (Figures 1B and S1A). 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 ($p < 0.001$; Figure S1B). Non-FcγR binding DA265 mutant anti-hCD20 was also unable to clear tumors (Figure S1C). 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 5×10^6 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 (Figure 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 (Figures S1D and S1E). 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.

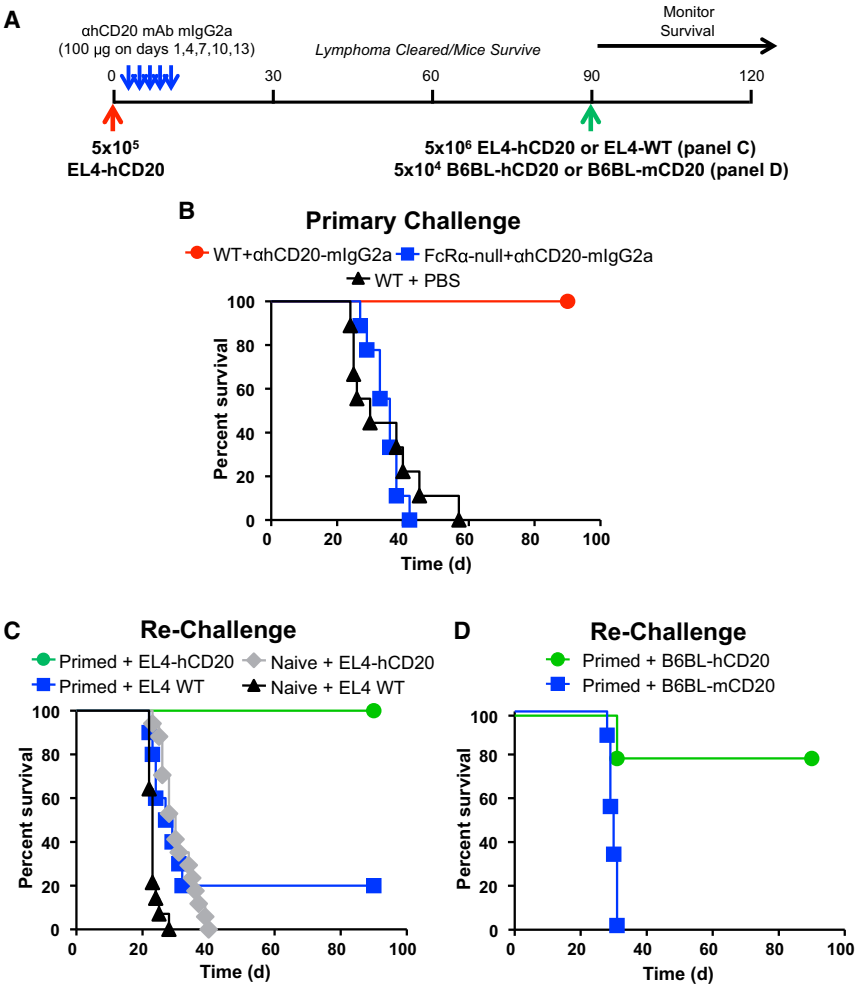


Figure 1. Fc-FcγR Interactions Are Required for the Clearance of Lymphoma by mAb and the Initiation of an Anti-tumor Vaccinal Effect

(A) Experimental protocol. Mice were injected i.v. with 5 × 10^5 EL4-hCD20 lymphoma cells on day 0 (red arrow) and received 100 μg 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) Wild-type (red circles) or FcRα null mice (blue squares) were injected with EL4-hCD20 cells and treated with mIgG2a 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, naive 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) and (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.

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 (Abès 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 (Abès 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 expressed 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 (Figure 1D; p = 0.0001). Thus, only cells expressing hCD20 were capable of being rejected. Collectively, these experiments demonstrate that an anti-hCD20 immune response is generated after the initial FcγR-mediated clearance of tumor cells by ADCC.

Expression of mFcγRIV 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 FcγR expression plays a role during this process, we utilized mice with a $CD11c^+$ cell-specific deletion of mFcγRIV. *Fcgr4*$^{fl/fl;cd11c\text{-}cre}$ mice (Nimmerjahn et al., 2010) show a complete absence of mFcγRIV expression on spleen $CD11c^{hi}$ DCs, but only a partial decrease in mFcγRIV expression on spleen $CD11b^+Gr\text{-}1^{lo}SSC^{lo}$ monocytes (Figure 2A). All $CD11c^{hi}$ cells lose mFcγRIV expression in *Fcgr4*$^{fl/fl;cd11c\text{-}cre}$ mice, while spleen $CD11c^{int}$ cells show partial loss of mFcγRIV and $CD11c^-$ cells show only a modest decrease in mFcγRIV (Figure S2A; Table S1). The majority of $CD11c^{hi}CD8^+$ DCs and a fraction of $CD11c^{hi}CD4^+$ DCs express mFcγRIV, both of which lose all mFcγRIV expression in *Fcgr4*$^{fl/fl;cd11c\text{-}cre}$ mice (Figure S2E). While the majority of $CD11c^{int/-}CD11b^{int}$ cells lose expression of mFcγRIV, only a modest reduction in mFcγRIV is seen in $CD11c^{int/-}CD11b^{hi}$ cells in *Fcgr4*$^{fl/fl;cd11c\text{-}cre}$ mice (Figure S2B). Expression of mFcγRIV is not affected in

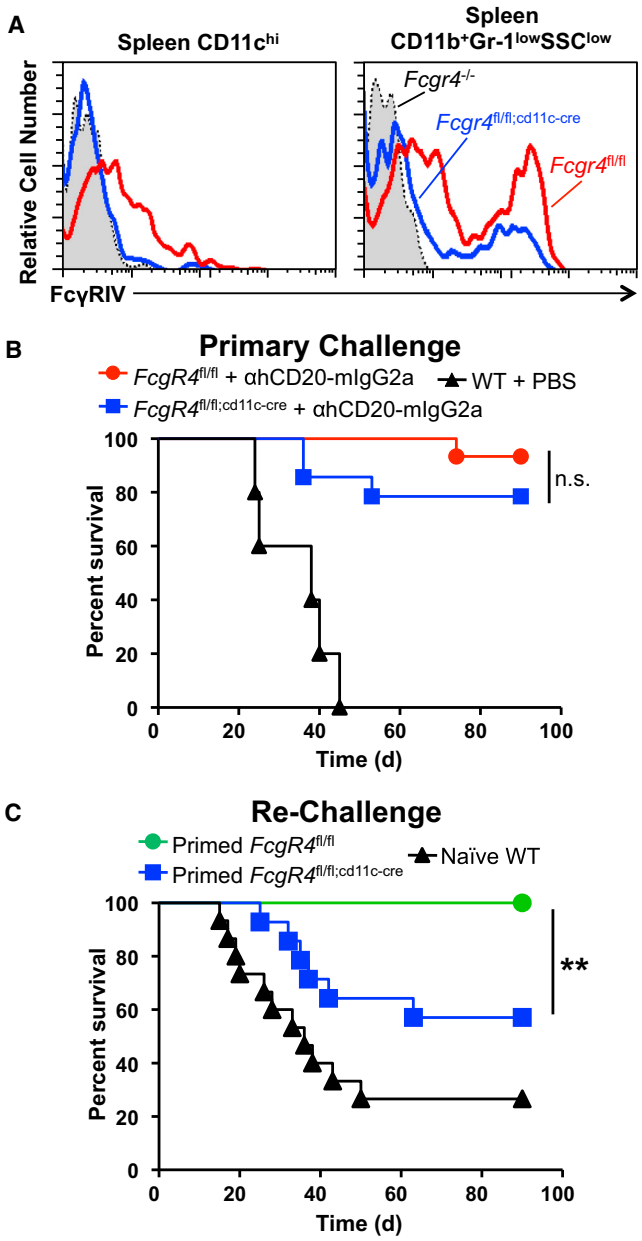


Figure 2. Expression of mFcγRIV on CD11c$^+$ Cells Is Required for the Generation of an Anti-tumor Vaccinal Effect

(A) mFcγRIV expression levels on spleen innate cell subsets. Spleen lymphocytes were harvested from *Fcgr4*$^{fl/fl}$ (red line), *Fcgr4*$^{fl/fl;cd11c\text{-}cre}$ (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*$^{fl/fl;cd11c\text{-}cre}$ (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*$^{fl/fl;cd11c\text{-}cre}$ (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.

See also Table S1.

Ly6G$^+$ neutrophils (Figure S2C). Most CD11b$^+$CD11c$^{int/-}$F4/80^{hi} macrophages lose mFcγRIV expression in *Fcgr4*$^{fl/fl;cd11c\text{-}cre}$ mice, as these cells express CD11c at intermediate levels (Figure S2D). Similar results demonstrating decreases in mFcγRIV 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*$^{fl/fl;cd11c\text{-}cre}$ mice (Figures S3, S4, and S5; Table S1). Therefore, because mFcγRIV expression was maintained to a sufficient degree on ADCC-mediating innate cells, both control *Fcgr4*$^{fl/fl}$ and *Fcgr4*$^{fl/fl;cd11c\text{-}cre}$ mice were able to clear primary EL4-hCD20 lymphoma cell challenge after treatment with anti-hCD20 mAb (Figure 2B), indicating that mFcγRIV 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 mFcγRIV, surviving tumor/mAb-primed *Fcgr4*$^{fl/fl}$ and *Fcgr4*$^{fl/fl;cd11c\text{-}cre}$ mice were re-challenged with EL4-hCD20 cells. Re-challenge of primed *Fcgr4*$^{fl/fl}$ mice resulted in 100% survival (Figure 2C). By contrast, only 57% of tumor/mAb-primed *Fcgr4*$^{fl/fl;cd11c\text{-}cre}$ mice survived re-challenge with EL4-hCD20 cells (p = 0.0069). It is likely that activating mFcγRIII, which is also expressed on murine DCs, compensates in the absence of mFcγRIV, thereby explaining why a modest vaccinal effect remains in *Fcgr4*$^{fl/fl;cd11c\text{-}cre}$ mice. Thus, expression of the IgG2a-preferential activating mFcγRIV 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 mFcγRIV Is Required to Generate Anti-tumor Cellular Immunity

To quantify anti-tumor cellular immunity in vivo, we performed adoptive transfer experiments (Figure 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 naive mice that were then challenged with EL4-hCD20 cells. Adoptive transfer of splenocytes or T cells from naive 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 (Figure 3B). Thus, tumor/mAb-primed mice generate a quantifiable anti-tumor T cell response.

To determine whether DC expression of mFcγRIV 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*$^{fl/fl;cd11c\text{-}cre}$ mice were adoptively transferred into naive 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*$^{fl/fl;cd11c\text{-}cre}$ mice survived the challenge (p = 0.0041; Figure 3C). Thus, expression of mFcγRIV on CD11c$^+$ antigen-presenting cells is required to mediate the generation of an anti-tumor cellular immune response after mAb-mediated clearance of tumor.

hFcγRIIIA Mediates ADCC of hIgG1 mAb-Targeted Tumor Cells in FcγR-Humanized Mice

Fc receptors for mouse IgG are heterogeneous, differing in their binding affinities for IgG subclasses, their expression patterns on

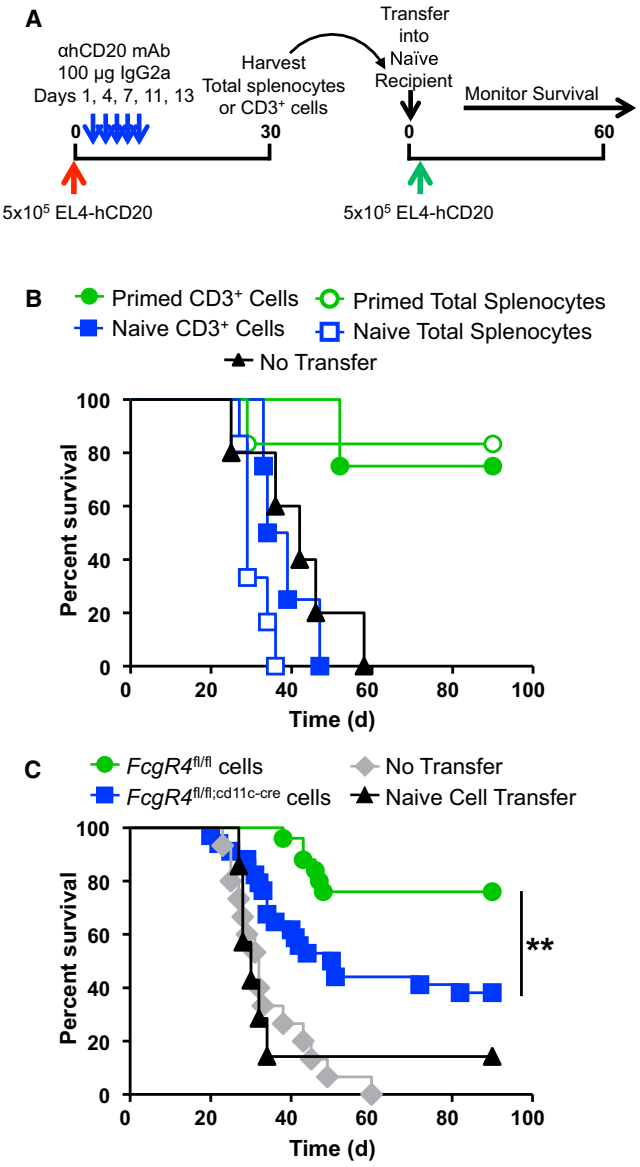


Figure 3. CD11c$^+$ Cell-Specific Expression of mFcγRIV Is Required for the Generation of Anti-tumor Cellular Immunity

(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 naive mice one day before i.v. challenge with EL4-hCD20 lymphoma cells (green arrow).

(B) Survival was measured in naive 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 naive mice. Another group of naive mice received no adoptive transfer (black triangles). n = 4–6 mice per group.

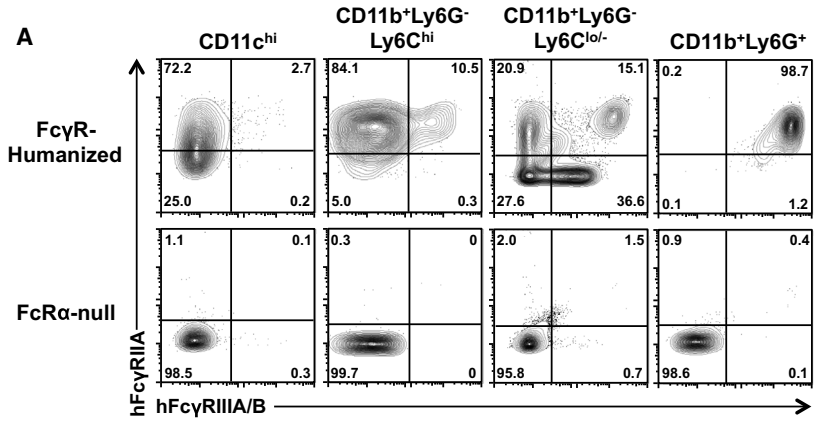
(C) Survival in naive mice receiving total splenocytes from tumor and mAb-primed *Fcgr4*$^{fl/fl}$ (green circles; n = 25) or *Fcgr4*$^{fl/fl;cd11c\text{-}cre}$ (blue squares, n = 34) mice before EL4-hCD20 cell challenge. Other groups of naive mice received splenocytes from naive mice (black triangles; n = 7) or no adoptive transfer (gray diamonds; n = 15). Significant differences between groups are indicated: **p = 0.0041.

immune cells, and signaling properties (Nimmerjahn and Ravetch, 2006; Pincetic et al., 2014). For example, NK cells in the mouse express only mFcγRIII, a low-affinity activating FcγR. Macrophages and DCs express distinct combinations of both activating (mFcγRI, mFcγRIII, and mFcγRIV) and inhibitory (mFcγRIIB) receptors. Individual mouse subclasses show preferential mFcγR binding affinities, with mIgG1 preferentially engaging the inhibitory mFcγRIIB while mIgG2a engages the activating receptor mFcγRIV with a 2 log higher affinity (Nimmerjahn and Ravetch, 2008, 2011). Thus, selecting an antibody for optimal FcγR 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 FcγR genes, expression patterns, and affinities for the various antibody isotypes differ significantly from mice. Importantly, humans express only the low-affinity activating FcγR—FcγRIIIA—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 FcγR, hFcγRIIA (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 hFcγRs, we utilized FcγR-humanized mice, which express the full array of hFcγRs on a fully immunocompetent C57BL/6 background lacking all mFcγRs (Bournazos et al., 2014a; Smith et al., 2012). FcγR-humanized mice recapitulate hFcγR expression patterns in mouse tissues; spleen CD11c^{hi} DCs express only hFcγRIIA and hFcγRIIB, but do not express hFcγRIIIA, while spleen CD11b$^+$Ly6G$^-$Ly6C^{hi} and CD11b$^+$Ly6G$^-$Ly6C$^{int/-}$ monocytes express hFcγRIIIA, hFcγRIIA and hFcγRIIB in various combinations (Figures 4, S6A, and S6B).

To address the relative contributions of individual hFcγRs 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; DiLillo et al., 2014; Smith et al., 2012) that selectively enhance interactions with individual hFcγRs (Figure 5A; Table S2). 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 (Figure 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



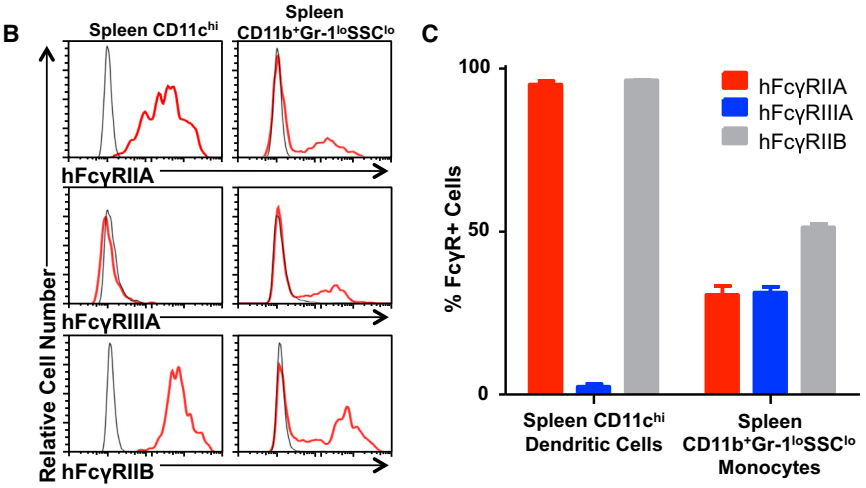


Figure 4. Human FcγR Expression on Innate Cells in FcγR-Humanized Mice

(A) Representative flow cytometry dot plots show hFcγRIIA versus hFcγRIIIA/B expression on spleen cells from FcγR-humanized or FcRα null mice. Numbers represent the frequency of cells in the indicated gate.

(B) DCs and monocytes from FcγR-humanized mouse spleens (red lines) were stained for hFcγRIIA, hFcγRIIIA/B, or hFcγRIIB. Background staining by hFcγR$^-$ cells is shown (gray lines).

(C) Frequencies (±SEM) of hFcγR$^+$ cells among spleen DCs and monocytes (n = 3 per group), with frequencies generated by background staining subtracted.

treatment, while mice expressing only hFcγRIIIA and hFcγRIIIB (deficient for murine FcγRs) showed full survival when treated with GASDALIE mutant anti-hCD20 mAb (Figure 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 (Figures S6C and S6D), 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-Sensitive Macrophages Mediate ADCC in the Context of hIgG1 and the Human FcγR System

In the context of the mouse FcγR system, clodronate liposome (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 msIgG2a 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% (Figure S6E; $p < 0.0001$). With the exception of CD11c^{hi} DCs (33% depletion, $p = 0.01$), no other cellular populations analyzed were affected by CLOD treatment (Figure S6F).

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% ($p < 0.0001$; Figure S7A), 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 FcγR system in hCD20-Tg/FcγR-humanized mice (these mice express hCD20 on mature B cells and also express the full array of human FcγRs, but lack all murine FcγRs), as described above. Blood and spleen B cell numbers were decreased by 86%–90% and 81%–82% ($p < 0.001$), respectively, in hCD20-Tg/FcγR-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 (Figure S7B). Similar results were seen in FcγR-humanized mice treated with CLOD and a depleting hIgG1 (GASDALIE) anti-mCD4 mAb (Figure S7C). Therefore, CLOD-sensitive macrophages are required for ADCC mediated by hIgG1 antibody in the context of the human FcγR system.

A

Mutant	FcγRIIA	FcγRIIB	FcγRIIIA
huIgG1 (wild-type)	+	+	+
GA	++	+	+
ALIE	+	+	++
GASDALIE	+++	+	+++

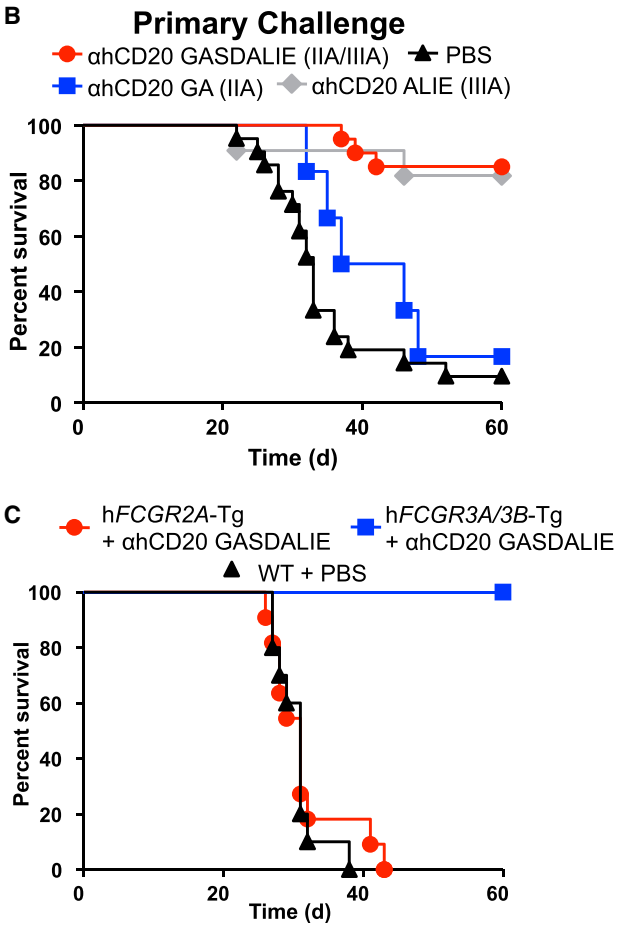


Figure 5. Differential hFcγR Engagement Mediates Tumor Cytotoxicity

(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 (Table S2).

(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.

hFcγRIIA Engagement by Anti-tumor mAb Mediates the Generation of the Anti-tumor Vaccinal Effect in FcγR-Humanized Mice

Finally, we assessed to what extent the anti-tumor vaccinal effect was generated in FcγR-humanized mice (Figure 6A) treated with anti-hCD20 mAb mutants that selectively engage only hFcγRIIIA (ALIE mutant) or both hFcγRIIA and hFcγRIIIA (GASDALIE mutant). Only 20% of FcγR-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 ($p < 0.0001$; Figure 6B). Further, only 36% ($p = 0.01$) of hFcγRIIIA/B-Tg mice (that lack all murine FcγRs and express only hFcγRIIIA and hFcγRIIIB) survive EL4-hCD20 re-challenge, again demonstrating that expression of hFcγRIIA is required for optimal induction of an anti-tumor vaccinal effect. Thus, engagement of hFcγRIIA, which is the only activating hFcγR 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 FcγR-mediated ADCC, a process that generates antibody:tumor antigen immune complexes. These immune complexes engage activating FcγRs 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 (Figure 7) (Boruchov et al., 2005; Dhodapkar et al., 2005; Kalergis and Ravetch, 2002; Nimmerjahn and Ravetch, 2008). In the human FcγR system, the vaccinal effect requires interactions with hFcγRIIA, the sole activating FcγR 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 FcγRs 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

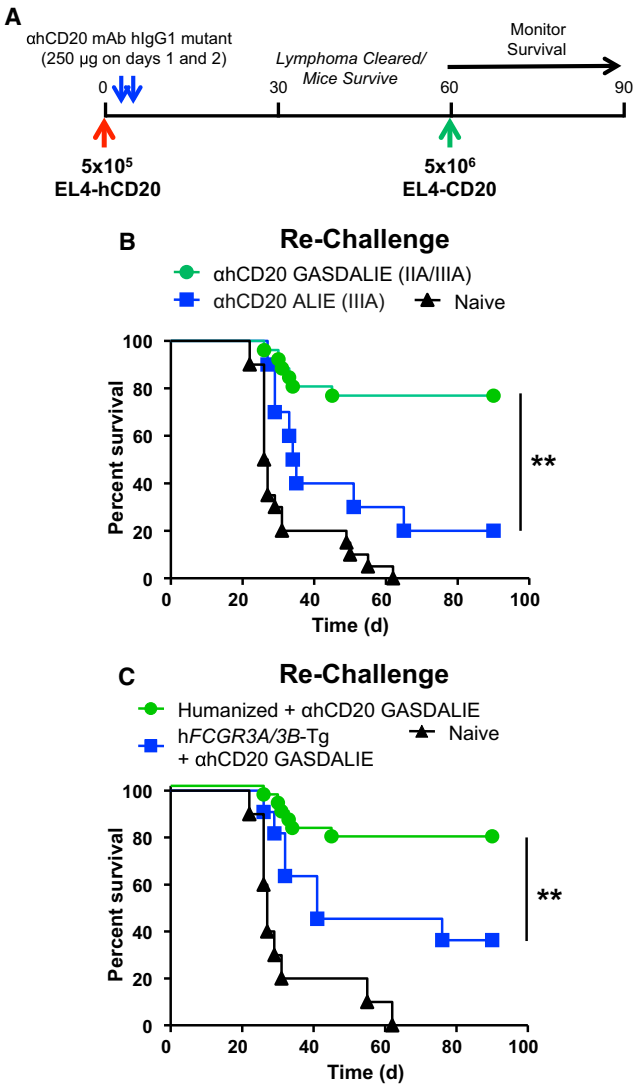


Figure 6. Selective Engagement of hFcγRIIA Mediates an Anti-tumor Vaccinal Effect

(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 Figure 5B, or naive mice (black triangles; n = 15) were re-challenged with EL4-hCD20 cells.

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

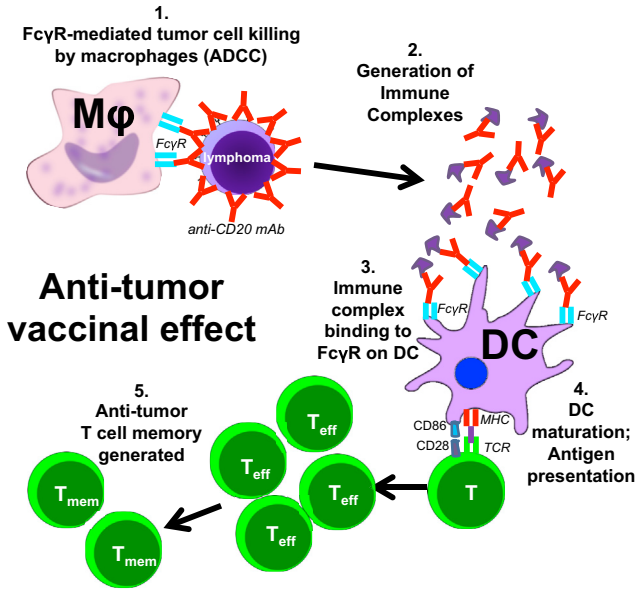


Figure 7. Model for the Generation of an Anti-tumor Vaccinal Effect

Anti-tumor mAb opsonizes tumor cells and targets them for killing by FcγR-mediated ADCC, a process that generates antibody:tumor antigen immune complexes. These immune complexes engage activating FcγRs 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.

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 mFcγRIV compared to CD8$^-$CD11c^{hi} cells (60% versus 28%, Figure S2E), 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 (Abès 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 overexpressed 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 toward 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 (Figure 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 (Figure 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 versus 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 FcγR-humanized mice with hIgG1 anti-hCD20 mAb-mediated ADCC of hCD20$^+$ tumor cells clearly demonstrate that hFcγRIIIA is both necessary and sufficient for ADCC-mediated clearance of antibody-coated tumor cells; hFcγRIIA plays no role in this process (Figure 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, FcγR signaling is required for ADCC in vivo, rather than simple cross-linking of antigen (de Haij et al., 2010). Because they solely express hFcγRIIIA, 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) (Figure S7A), but not NK cells, are required for ADCC by mIgG in the context of murine FcγRs. This was partially thought to be due to the lone expression of FcγRIII on murine NK cells (Otten et al., 2008), which weakly interacts with mouse antibody Fc compared to FcγRIV. Therefore, we have now clearly determined the cellular requirements for hIgG1-triggered ADCC mediated by human FcγRs and demonstrate that CLOD-sensitive macrophages mediate ADCC of antibody-coated target cells in vivo in the context of hIgG1 antibody and the human FcγR system (Figures S7B and S7C). 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 FcγRs 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 hFcγRIIIA 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 hFcγRIIIA, and accordingly, extends survival by ~1 year in CLL patients when directly compared to an unmodified anti-CD20 antibody (Rituximab). However, afucosylation of obinutuzumab does not affect Fc engagement of hFcγRIIA, which is the sole activating hFcγR 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 hFcγRIIIA for cytotoxic effector function, but also hFcγRIIA 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 and maintained in Dulbecco's minimum essential medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Life Technologies). 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 U/ml of penicillin, and 100 μg/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), 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. *Fcer1g*$^{-/-}$ (Takai et al., 1994), *Fcgr4*$^{-/-}$ (Nimmerjahn et al., 2010), FcRα null (Smith et al., 2012), *FCGR3A/B*-Tg (Li et al., 1996) (crossed to FcRα 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. FcγR-humanized mice, which express all hFcγRs on the FcRα null C57BL/6 genetic background, have been described (Smith et al., 2012). *Fcgr4*^{flox} 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) and crossed onto the FcγR-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 and ATCC, 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, hIgG1, 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 μl of clodronate liposomes or PBS liposomes intravenously (i.v.) through lateral tail veins (Clophosome-A, Formumax).

Tumor Model

For the primary tumor challenge, mice were injected i.v. through lateral tail veins with 5×10^5 EL4-hCD20 cells in 200 μl PBS on day 0. Mice then received intraperitoneal (i.p.) injections of 100 μg of antibody in 200 μl 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 i.v. with 5×10^6 EL4-hCD20 or EL4-WT cells, with survival assessed daily. In experiments in which hIgG1 antibodies or mutants were administered, FcγR-humanized mice were given 5×10^5 EL4-hCD20 cells in 200 μl PBS on day 0, with 250 μg of antibody in 200 μl 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). Then, 50×10^6 total splenocytes or 15×10^6 $CD3^+$ cells were adoptively transferred into naive C57BL/6 mice. One day later, the mice were challenged with 5×10^5 EL4-hCD20 cells in 200 μl 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.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.04.016.

AUTHOR CONTRIBUTIONS

D.J.D. and J.V.R. designed experiments, analyzed the data, and wrote the manuscript. D.J.D. performed the experiments.

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