

A targeted complement-dependent strategy to improve the outcome of mAb therapy, and characterization in a murine model of metastatic cancer

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Complement inhibitors expressed on tumor cells provide an evasion mechanism against mAb therapy and may modulate the development of an acquired anti-tumor immune response. Here we investigate a strategy to amplify mAb-targeted complement activation on a tumor cell, independent of a requirement to target and block complement inhibitor expression or function, which is difficult to achieve in vivo. We constructed a murine fusion protein, CR2Fc, and demonstrated that the protein

targets to C3 activation products deposited on a tumor cell by a specific mAb, and amplifies mAb-dependent complement activation and tumor cell lysis in vitro. In syngeneic models of metastatic lymphoma (EL4) and melanoma (B16), CR2Fc significantly enhanced the outcome of mAb therapy. Subsequent studies using the EL4 model with various genetically modified mice and macrophage-depleted mice revealed that CR2Fc enhanced the therapeutic effect of mAb therapy via

both macrophage-dependent Fc γ R-mediated antibody-dependent cellular cytotoxicity, and by direct complement-mediated lysis. Complement activation products can also modulate adaptive immunity, but we found no evidence that either mAb or CR2Fc treatment had any effect on an antitumor humoral or cellular immune response. CR2Fc represents a potential adjuvant treatment to increase the effectiveness of mAb therapy of cancer. (*Blood*. 2012;119(25):6043-6051)

Introduction

Complement plays important roles in the effector mechanisms of many anticancer antibodies,¹ whether the antibodies are induced or administered. Antibody-mediated activation of complement on a tumor cell leads to cleavage of C3, a central step in the complement pathway, and results in the opsonization of the tumor cell with C3 activation products. These C3 products are recognized by complement receptors on immune effector cells and can promote and enhance complement-dependent cellular cytotoxicity (CDCC) and antibody-dependent cellular cytotoxicity (ADCC).¹ This occurs via interaction of complement receptor 3 (CR3, CD11b/CD18) with the covalently bound C3 degradation products iC3b, C3d, and C3dg.² Other complement activation products that may be involved in an antitumor response include the anaphylatoxins C3a and C5a, which can recruit and activate immune cells and also modulate T-cell immunity,³⁻⁵ and the membrane attack complex (MAC), which can cause direct tumor cell lysis, often referred to as complement-dependent cytotoxicity (CDC).¹ Nevertheless, antibody-dependent complement activation is not, in general, an effective antitumor defense mechanism. This is thought to be the result, at least in part, of complement inhibitory mechanisms used by tumor cells.⁶⁻¹¹

Several studies have shown that interfering with complement inhibitor expression or function on tumor cells can enhance the effects of mAb immunotherapy in animal models.¹²⁻¹⁴ In addition, complement inhibitors have been shown to modulate the outcome of both humoral and cellular immune responses,^{3-5,15} and the down-regulation of a complement inhibitor on tumor cells has been

shown to result in a protective antitumor CD8⁺ T-cell response in a murine model.¹⁶ However, the down-regulation or blockade of a complement inhibitor on tumor cells in vivo is a technical challenge because of their widespread and abundant expression. One approach to overcome this problem, and one that has been applied in an animal model, is the use of a bispecific antibody against both a tumor antigen and a complement inhibitor (to block its function).¹³ However, this approach does not overcome problems of low tumor antigen density and conditions of limited antibody concentration, as well as potential off-target effects because of the engagement of complement inhibitors expressed on normal cells.

In this study, we investigated a novel strategy to amplify mAb-targeted complement activation on a tumor cell, independent of a requirement to target and block complement inhibitor expression or function. We prepared and characterized a construct consisting of a murine complement receptor 2 (CR2) targeting region linked to a murine IgG2a Fc complement activating region (supplemental Figure 1A, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). CR2 is naturally expressed predominantly on B cells and dendritic cells and recognizes C3 opsonins. When complement is activated on a cell surface, the initial covalently bound C3 activation product is C3b, which participates in amplifying further C3 cleavage and complement activation. However, C3b is quickly degraded to inactive iC3b, which is then more slowly degraded to C3d and C3dg. These relatively long-lived breakdown fragments of C3b are

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ligands for CR2 and would be expected to be present on tumor cells as a result of mAb-dependent complement activation. Thus, the CR2 region is predicted to target mAb-directed C3 activation products on a tumor cell, whereas the Fc region is predicted to amplify tumor-specific complement activation. In addition to amplifying complement activation, increased Fc deposition can potentially enhance Fc-dependent effector mechanisms, such as ADCC. In this regard, it has been shown that Fc receptors and ADCC can play important roles in mAb therapeutic mechanisms, both in the clinic and in experimental models.¹⁷ We previously demonstrated the feasibility of this approach using a human CR2Fc construct.¹⁸ Here we report on the characterization of a murine construct in a syngeneic model of cancer that allowed investigation of mAb therapy and complement-dependent effector and immune modulatory mechanisms in the context of a functional immune system.

Methods

Cell line, antibodies, and serum

The EL4 mouse lymphoma (ATCC) was grown at 37°C in 5% CO₂ in RPMI 1640 with 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. Anti-GD2 mAb 14G2a (IgG2a) was provided by Dr R. A. Reisfeld (Scripps Research Institute). The anti-mouse CR2 mAb 7G6¹⁹ and anti-gp75 mAb TA99 (ATCC) were purified from hybridoma supernatant by protein G affinity chromatography (GE Healthcare Bio-Sciences). Anti-mouse C3-FITC and anti-mouse Fc-HRP were purchased from MP Biomedical. HRP-conjugated anti-mouse C3a antibody, and rat anti-mouse IgM-FITC antibody was purchased from BD Biosciences PharMingen. All secondary-FITC-conjugated anti-mouse IgG and IgG isotype antibodies for flow cytometry were purchased from Santa Cruz Biotechnology. Normal mouse serum (NMS) was prepared from C57BL/6 male mice and all serum samples were stored in aliquots at -80°C.

Preparation of mouse CR2Fc fusion protein

For construction of plasmids for expression of CR2Fc fusion protein, the sequence encoding the 4 NH₂-terminal short consensus repeats of mouse CR2 (residues 1-257 of mature protein, GenBank accession number M35684) was linked to the sequence encoding the complement activating Fc region of mouse IgG2a (residues 237-469 of mature protein, GenBank accession number S37483). The construct was prepared by standard PCR methods as previously described.²⁰ For expression, plasmids were transfected into CHO cells with Fugene-HD transfection reagent according to the manufacturer's protocol (Roche Applied Science), and stably transfected clones selected by limiting dilution. Protein expression was confirmed by SDS-PAGE and Western blot under reducing and nonreducing conditions using anti-mouse CR2 and anti-mouse Fc antibodies (supplemental Figure 1B). CR2Fc was purified from cell culture supernatants by protein A affinity chromatography, and more than 95% purity was verified by SDS-PAGE.

In vitro assays

For analysis of CR2Fc binding to complement-opsonized cells, 1×10^6 EL4 cells were resuspended in 100 µL of RPMI with 5 µg/mL anti-GD2 and incubated for 30 minutes at 4°C. After washing, cells were resuspended in 100 µL RPMI 1640 with 5% NMS and incubated for 30 minutes at 37°C. Cells were washed and incubated with 20 µg/mL AlexaFluor-488-conjugated CR2Fc diluted in RPMI 1640 with or without 5% NMS for 1 hour at 37°C. Finally, cells were washed and resuspended in 500 µL PBS and analyzed by flow cytometry. Analysis of complement activation and complement-dependent lysis (CDC) was performed by incubation of 1×10^6 EL4 or B16 cells in 100 µL RPMI 1640 with either 5 µg/mL

anti-GD2 or anti-gp75, respectively, for 30 minutes at 4°C. Cells were washed and resuspended in 100 µL of 5% or 50% NMS for analysis of C3 activation, or normal rat serum for analysis of cell lysis, with or without 50 µg/mL CR2Fc. Rat serum was used for lysis assays because of the low lytic capacity of mouse serum in a homologous system. After 1 hour at 37°C, 20 µL of 20mM EDTA was added to the cells to prevent further complement activation. The supernatant was collected and analyzed for complement-mediated lysis by lactate dehydrogenase assay (Bioassay Systems) or complement activation by C3a Western blot.

Mice

Wild-type C57BL/6 mice were purchased from The Jackson Laboratory and Harlan Laboratory. Fcγ receptor γ chain-deficient mice (backcrossed 12 generations to C57BL/6 background) were purchased from Taconic Farms. SCID beige mice were provided by Dr M. Nishimura (Loyola University, Chicago, IL). Mice with a natural deficiency of C6 were backcrossed onto the C57BL/6 background.²¹ All animal procedures were approved by the Medical University of South Carolina Animal Care and Use Committee.

Metastatic lymphoma and melanoma models, and mAb/CR2Fc treatment

Mice were injected intravenously via the tail vein with 1×10^5 EL4 cells suspended in 0.1 mL sterile PBS. Two days after tumor challenge, mice were injected intraperitoneally with 25 µg of the anti-GD2 mAb 14G2a or PBS, followed 6 hours later by injection with 50 µg CR2Fc or PBS. The mAb and CR2Fc doses used here were found to be optimal in our previous study using the EL4 model in immune-deficient mice.¹⁸ For the B16 model, inoculum and treatment schedule was the same, except a 50-µg dose of anti-gp75 mAb was used, which was previously shown to provide suboptimal protection in a B16 metastatic model.²² For survival studies, the endpoint was death or more than 15% weight loss. For the rechallenge experiment, surviving mice were rechallenged with 5×10^4 EL4 cells at day 35. For analysis of immune response, animals were killed at day 21 unless otherwise indicated.

Depletion of macrophages

Macrophages and monocytes were depleted *in vivo* with clodronate liposomes and depletion confirmed by immunohistochemical staining of liver sections for F4/80 as previously described.²³

Analysis of antitumor antibody response

Serum was collected from mice before tumor challenge and at death on day 19 after tumor challenge. Cultured EL4 cells were incubated with 1:5 diluted serum samples for 1 hour at 4°C. After washing, cells were resuspended in 50 µL of 1:100 diluted anti-mouse IgM or IgG FITC-labeled antibody for 1 hour at 4°C. Cells were then washed, resuspended in PBS/propidium iodide, and analyzed by flow cytometry. A similar protocol was used to determine anti-EL4 IgG isotypes.

Analysis of antitumor T-cell response

Mice were killed at day 21 after tumor cell challenge and their spleens removed. Splenocytes were isolated and resuspended in complete medium at 1×10^7 cells/mL. Cultured EL4 or MB49 (control) target cells were harvested and resuspended at 1×10^6 cells/mL in complete medium. Splenocytes and tumor cells were plated at a 10:1 ratio, respectively, and incubated at 37°C for 48 hours. The supernatant was then collected and assayed for IFN-γ and IL-10 by ELISA (BD Biosciences) according to the manufacturer's protocol. For T cell specific analysis, splenocytes were isolated and CD4⁺ and CD8⁺ cells separated using MACS CD8a⁺ or CD4⁺ T cell isolation kit II (Miltenyi Biotec) according to the manufacturer's protocol. Isolated CD4⁺ or CD8a⁺ cells were plated at 2:1 effector-to-target ratio and incubated for 48 hours. The supernatant was collected and assayed for IFN-γ and IL-10 by ELISA.

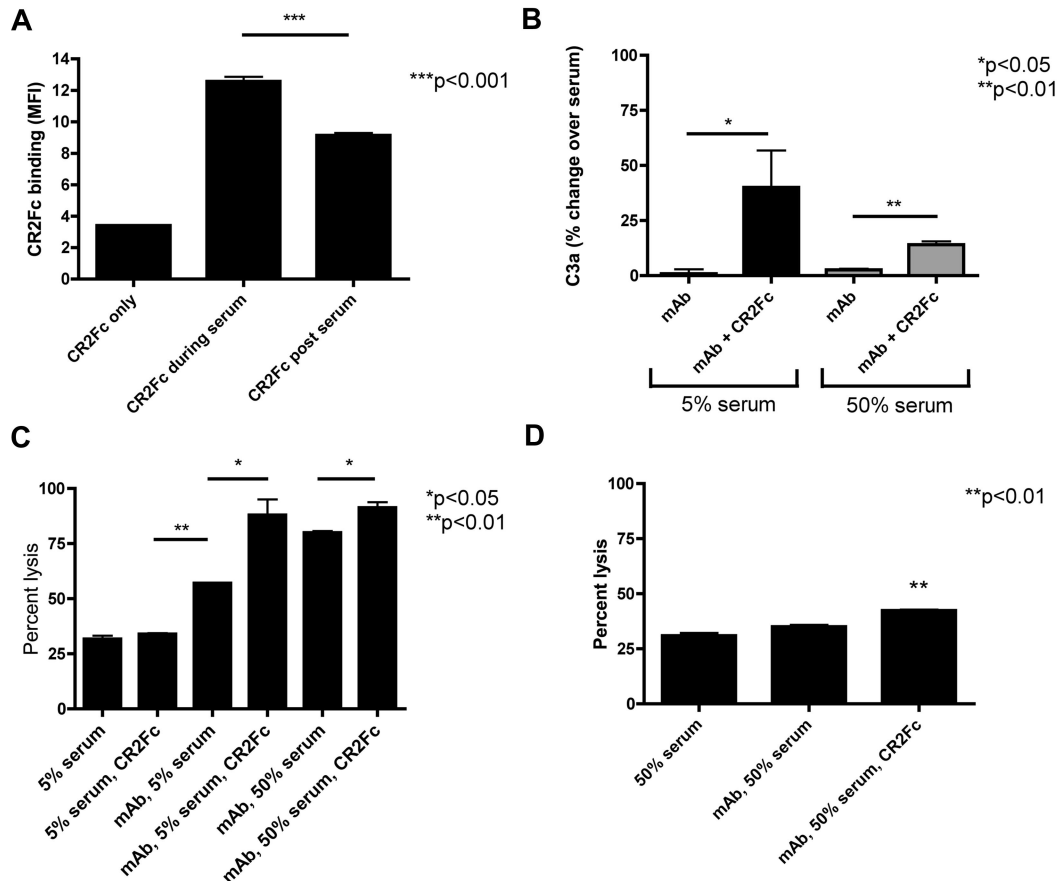


Figure 1. In vitro characterization of CR2Fc. CR2Fc binds to C3-opsonized cells and increases Fc opsonization by amplifying its own ligand. (A) CR2Fc binding: EL4 cells sensitized with anti-GD2 14G2a mAbs were incubated with NMS and 20 μ g/mL AlexaFluor-488–labeled CR2Fc added during serum incubation or after serum incubation (after washing). CR2Fc binding in the absence of mAb sensitization (CR2Fc only) was also determined. (B) CR2Fc-enhanced complement activation: EL4 cells were incubated in 5% or 50% NMS, with 14G2a and CR2Fc as indicated, and C3a levels in supernatant determined by Western blot and densitometry. (C) CR2Fc-enhanced complement-dependent EL4 lysis: EL4 cells were incubated in 5% or 50% normal rat serum, and with 14G2a and CR2Fc as indicated. (D) CR2Fc-enhanced complement-dependent B16 lysis: B16 cells were incubated in 50% normal rat serum, and with anti-gp75 TA99 mAb and CR2Fc as indicated. Cell lysis determined by measuring lactate dehydrogenase release. Data are mean \pm SEM; n = 3.

Statistical analysis

GraphPad Prism Version 4 statistical software was used for analysis. Student *t* tests were used to determine statistical differences. The log-rank test was applied to determine differences in survival curves. Significance was accepted at the *P* less than .05 level.

Results

In vitro characterization of CR2Fc

The CR2Fc fusion protein was first characterized in vitro with respect to its ability to bind C3-opsonized cells and to amplify mAb-mediated complement activation and cell lysis. C3 binding function of CR2Fc was demonstrated by flow cytometric analysis using C3-opsonized EL4 cells (Figure 1A). CR2Fc binding to antibody-sensitized EL4 cells was increased when it was present during serum incubation compared with when CR2Fc was added after C3 opsonization (cells washed after serum incubation), indicating that CR2Fc increases Fc opsonization by generating its own ligand. To determine the effect of CR2Fc on mAb-mediated complement activation, EL4 cells were incubated with anti-GD2 mAb and NMS, either with or without CR2Fc. Complement activation was assayed by measuring C3a in the supernatant. There was a significant increase in C3a levels when EL4 cells were

incubated with both mAb and CR2Fc compared with mAb alone (Figure 1B). We also determined whether increased complement activation and C3 cleavage correlated to increased CDC. CR2Fc significantly increased anti-GD2 mAb-mediated lysis of EL4 at both low and high serum concentration, as determined by lactate dehydrogenase release (Figure 1C). Although EL4 cells express high levels of Crry, they are CD59 negative/low. We therefore also tested whether CR2Fc enhanced mAb-mediated lysis of a CD59-positive tumor cell line. The B16 melanoma cell expresses both Crry and CD59 (supplemental Figure 2), and CR2Fc also enhanced mAb-mediated complement-dependent lysis of this cell line (Figure 1D).

CR2Fc enhances the therapeutic outcome of anti-GD2 immunotherapy

The effect of CR2Fc on mAb therapy was investigated in the EL4 metastatic mouse lymphoma model. This model is well characterized with respect to tumor metastasis and growth, as well as anti-GD2 mAb therapy.^{24,25} Forty-eight hours after intravenous challenge with EL4 cells, mice were treated with either PBS or a suboptimal dose of anti-GD2 mAb (25 μ g, 14G2a). Mice from each group were then treated with either PBS or CR2Fc (50 μ g). Outcome was measured in terms of survival and the number of liver micrometastases. CR2Fc significantly enhanced the outcome

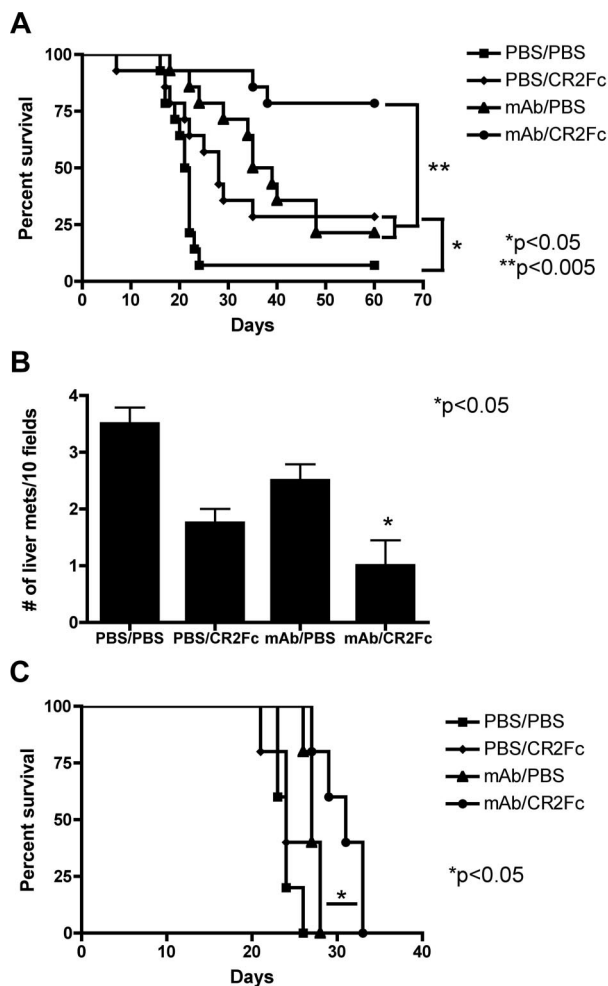


Figure 2. CR2Fc enhances the therapeutic outcome of mAb immunotherapy. Survival (A) and number of liver micrometastases at day 19 (B) of mice challenged with EL4 cells and treated with 14G2a mAb alone or in combination with CR2Fc. Long-term survival is significantly improved with mAb and CR2Fc treatment over mAb immunotherapy alone. Survival data are combined from 2 independent experiments ($n = 14$), each of which yielded similar results. (B) Data are mean \pm SEM; $n = 4$ or 5. (C) Survival of mice challenged with B16 cells and treated with TA99 mAb alone or in combination with CR2Fc; $n = 5$.

of anti-GD2 mAb therapy by both measures (Figure 2A-B). Long-term survival was increased from 20% (mAb alone) to 80% (mAb + CR2Fc; $P < .005$), and all surviving mice were tumor free at death on day 60. At day 19 after tumor challenge, liver metastases were reduced from 2.5 metastases/10 high power fields (mAb alone) to less than 1 metastasis/10 high power fields (mAb + CR2Fc; $P < .05$). To broaden the significance of these findings, we investigated the ability of CR2Fc to enhance mAb therapy in an additional tumor model. CR2Fc significantly extended survival time in the metastatic B16 melanoma model after therapy with the anti-gp75 mAb, TA99 (Figure 2C). Although combined treatment did not result in increased long-term survival, as it did in the EL4 model, we investigated only a single-treatment regimen that was in line with the EL4 model. Indeed, it has been shown previously that an optimal treatment protocol with TA99 mAb alone is multiple 150- μ g injections of the mAb over a 2-week period after B16 inoculation.²² Notably, here we were able to significantly extend survival of mice challenged with B16 cells using a single injection of 50 μ g TA99 and 50 μ g CR2Fc.

Of note, in the EL4 model, treatment of mice with CR2Fc alone resulted in a similar therapeutic outcome as treatment with mAb alone; and although the enhanced survival was significantly lower than for combined treatment, there was a significant improvement compared with PBS only treated controls. This may be the result of activation of complement by EL4 cells in vivo and the subsequent targeting of CR2Fc, and we show in “Effect of CR2Fc treatment on antitumor antibody and T-cell responses” that naive C57BL/6 mice contain low levels of EL4 reactive IgM.

Role of innate and acquired immunity in CR2Fc mechanism of action

In addition to enhancing innate complement-dependent effector mechanisms, increased complement activation has been shown to enhance B- and T-cell immune responses. We therefore determined the effect of CR2Fc on anti-GD2 mAb therapy using the EL4 model in SCID/beige mice that lack functional T, B, and NK cells. In this model, combined mAb and CR2Fc treatment significantly improved long-term survival compared with treatment with either mAb or CR2Fc alone (50% vs 0%, $P = .005$), indicating that an adaptive immune response is not required for CR2Fc-mediated enhancement of mAb therapy (Figure 3). We note that combined mAb and CR2Fc treatment in this model did not appear to be as effective as in the wt C57BL/6 model; and unlike wt mice, there was also no survival of SCID/beige mice treated with either mAb or CR2Fc alone. Although these differences between wt and SCID/beige were not statistically significant, it is possible that there may be a contribution of NK cells to ADCC in wt mice, which may be more prominent at lower complement and Fc densities. In subsequent experiments (see next 2 sections), we therefore investigated specific complement-dependent effector mechanisms, as well as antitumor B- and T-cell responses. We also cannot rule out the possibility that differences in complement levels between wt and SCID/beige mice may contribute to the small differences seen in the effect of mAb and CR2Fc, although with a few exceptions, complement activity in most inbred mouse strains is very similar.^{26,27}

Role of Fc γ receptors and direct complement-mediated lysis in CR2Fc activity

Complement is known to enhance ADCC, and CR2Fc enhances complement activation in addition to directly increasing target cell Fc concentration. We therefore determined the effect of mAb and CR2Fc treatment in Fc γ R γ chain-deficient mice challenged with EL4. Fc γ R deficiency completely abrogated the protective effect of mAb treatment or of combined mAb + CR2Fc treatment (Figure

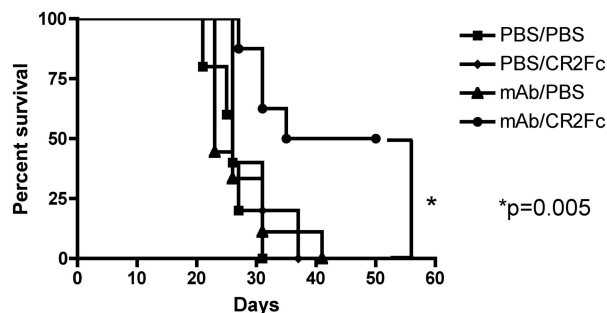


Figure 3. CR2Fc enhances mAb therapy in SCID beige mice. Survival of SCID beige mice challenged with EL4 cells and treated with 14G2a alone or in combination with CR2Fc. Long-term survival of mice treated with mAb and CR2Fc is significantly extended compared with mice treated with mAb therapy alone; $n = 8$ or 9.

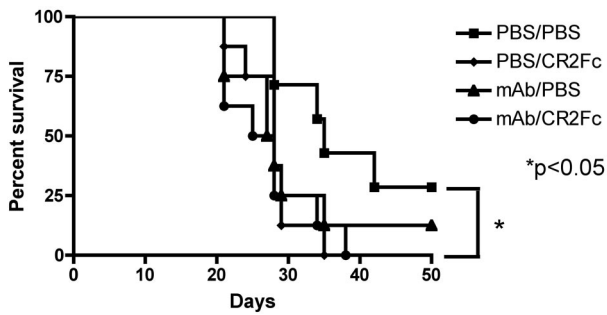


Figure 4. mAb and CR2Fc treatment is not protective in FcγR-deficient mice. Survival of FcγR-deficient mice challenged with EL4 and treated with 14G2a alone or in combination with CR2Fc. In the absence of Fcγ-activating receptors, the protective effect of mAb and CR2Fc treatment is ablated in EL4-challenged mice; n = 7 or 8.

4). These data indicate an essential role for ADCC and/or FcγR-mediated phagocytosis in both mAb therapy of EL4 and the enhancing effect of CR2Fc. Furthermore, the data reported in the previous section using NK cell-deficient SCID/beige mice, indicate that enhanced ADCC and/or phagocytosis are mediated predominantly by either macrophages or granulocytes.

Notably, untreated FcγR-deficient mice (PBS/PBS treatment group) had a significant increased survival (25%) compared with all treatment groups. One explanation for this result is a shift in the balance of activating and inhibitory signals in FcγR-deficient mice. In wt mice, mAb and CR2Fc are able to interact with FcγRs on immune cells leading to a protective, inflammatory antitumor effect. Although CR2Fc will also interact with the inhibitory FcγR in wt mice, the increased Fc levels on tumor cells (because of CR2Fc binding) may shift the balance toward an inflammatory phenotype. Increased Fc opsonization in the absence of activating FcγRs may lead to increased stimulation of the inhibitory FcγR, leading to a more protumor, anti-inflammatory phenotype.

Because the data indicate that either macrophages or granulocytes represent the primary effector cells for enhanced ADCC and/or phagocytosis in our therapeutic paradigm, we investigated the effect of macrophage/monocyte depletion. Depletion of macrophages and monocytes ablated the therapeutic effect of both mAb treatment and combined mAb and CR2Fc treatment (Figure 5). Together, these data indicate that the therapeutic effect of mAb and CR2Fc treatment is mediated primarily via a macrophage-mediated FcγR-dependent mechanism, although the data do not allow us to distinguish between macrophage-mediated ADCC and enhanced phagocytosis. Mice not depleted of macrophages were included as a control group; and similar to data shown in Figure 2A, undepleted wt mice were protected from EL4 challenge by mAb and CR2Fc treatment.

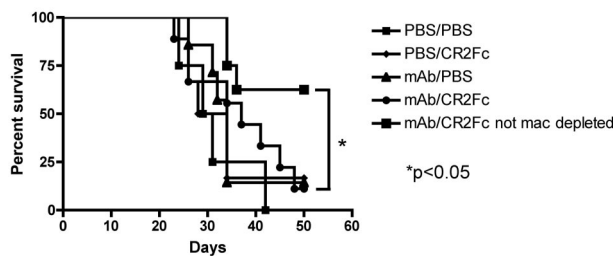


Figure 5. Macrophage depletion ablates mAb and CR2Fc-mediated protection. Survival of macrophage/monocyte-depleted mice challenged with EL4 and treated with 14G2a alone or in combination with CR2Fc. There was no difference in survival between any of the treatment groups in macrophage-depleted mice. Mice not depleted of macrophages (control) had significantly improved long-term survival, consistent with data shown in Figure 2A; n = 6 to 9.

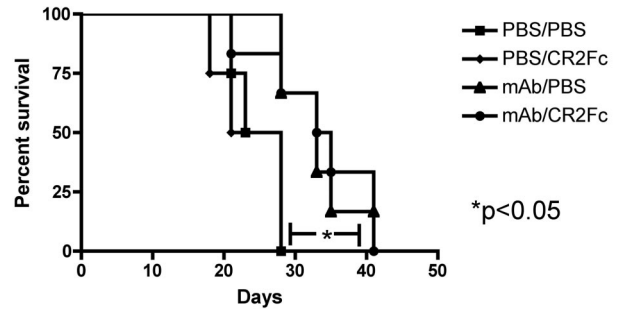


Figure 6. mAb and CR2Fc treatment does not increase overall survival in C6-deficient mice. Survival of C6-deficient mice challenged with EL4 and treated with 14G2a alone or in combination with CR2Fc. mAb therapy with or without CR2Fc did not provide long-term protection in the absence of C6, although it did significantly ($P < .05$) prolong survival time. CR2Fc did not enhance the effect of mAb therapy in C6-deficient mice; n = 4 to 6.

Finally, to determine whether the terminal pathway of complement and CDC plays any role in mAb and CR2Fc mechanism of action, the therapeutic study was repeated in C6-deficient mice. Monoclonal antibody alone and combined mAb + CR2Fc treatment extended median survival time from 25 to 33.5 days, but neither treatment provided any overall survival benefit (Figure 6). These data indicate that CDC contributes to therapeutic efficacy and the enhancing effect of CR2Fc on mAb therapy.

Effect of CR2Fc treatment on antitumor antibody and T-cell responses

The data in the previous 2 sections do not exclude the possibility that enhanced complement activation is also modulating antibody or T-cell immunity, for which there is precedent. We therefore additionally investigated whether an adaptive immune response was being induced secondary to initial FcγR-dependent/CDC mediated protection. Serum levels of anti-EL4 IgM and IgG were measured in naive mice and in mice 19 days after tumor challenge. Naive mice were found to have low levels of naturally occurring anti-EL4 IgM, but these levels did not change significantly by day 19 in any group (Figure 7A). Anti-EL4 IgG was not detected in naive mice, and analysis of IgG isotypes 19 days after tumor challenge and treatment revealed that only IgG2a was elevated and to similar levels in mAb and mAb + CR2Fc treated mice (Figure 7B). However, IgG2a is the isotype of the treatment mAb; and in a second experiment, we detected similar increased levels of IgG2a in mAb and CR2Fc-treated mice that were not challenged with EL4 (Figure 7C; data for IgG1, IgG2b, and IgG3 are shown in supplemental Figure 3). Thus, we conclude that anti-EL4 IgG2a detected after 19 days is the treatment antibody, and there is no induced antibody response to EL4.

Because complement can modulate T-cell responses, we also investigated whether an antitumor T-cell response was promoted by anti-EL4 mAb and CR2Fc treatment. Isolated splenic CD4⁺ or CD8a⁺ T cells were plated with either EL4 or MB49 (control) target cells, and secreted IFN-γ and IL-10 levels assayed by ELISA. Neither CD4⁺ nor CD8⁺ T cells from any treatment group produced detectable levels of IFN-γ after incubation with EL4 cells (or MB49 cells). There was also no detectable production of IL-10 from CD8⁺ T cells incubated with EL4 (supplemental Figure 4). CD4⁺ T cells plated with EL4 targets produced IL-10, but at similar levels from each treatment group (supplemental Figure 4). These data indicate that neither anti-EL4 mAb treatment nor mAb + CR2Fc treatment has any effect on anti-EL4 T-cell immunity.

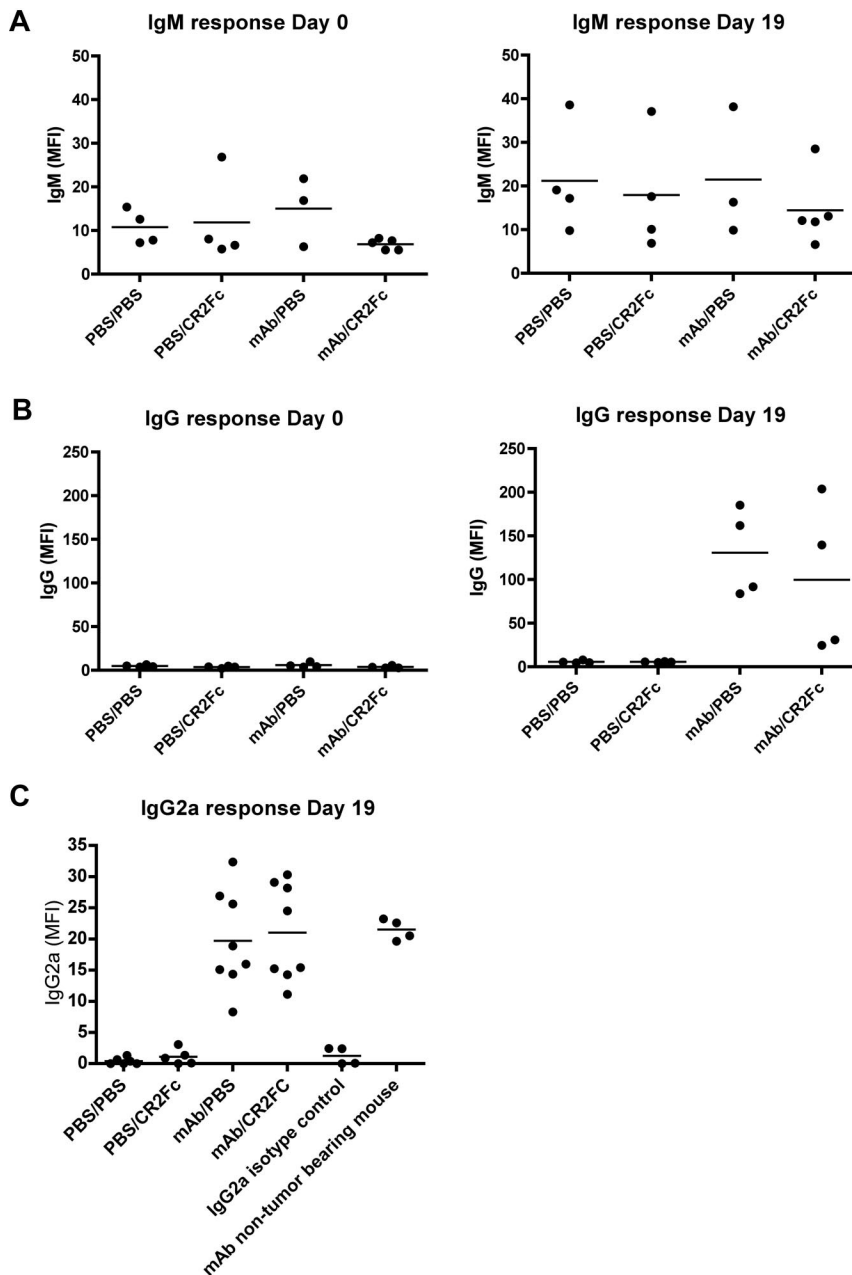


Figure 7. CR2Fc treatment does not induce an anti-EL4 IgM or IgG response. IgM (A), IgG (B), or IgG2a (C) was measured in the serum at day 0 and on day 19 after tumor challenge by a flow cytometry-based method. No significant differences were detected in IgM response between groups (A). Increased anti-EL4 IgG was seen in mAb and mAb + CR2Fc-treated mice (B), but only IgG2a isotype was elevated (C), which is the isotype of the treatment mAb.

Finally, we investigated whether there was induction of a memory response in EL4-challenged mice that had been treated with either mAb or mAb + CR2Fc. Surviving mice from each group were rechallenged with EL4, but there was no significant difference in survival between the 2 groups, and all mice died by day 50 after rechallenge, indicating no long-term memory response (supplemental Figure 5). This is consistent with the CD4⁺ and CD8⁺ T cell data shown in supplemental Figure 4.

Discussion

Complement inhibitors can be a hindrance to the effectiveness of mAb immunotherapy, and many types of tumors up-regulate their expression,^{8-10,28} indicating that they play a role in tumor immune evasion. However, the *in vivo* application of approaches to block or

down-regulate complement inhibitors on tumor cells is a considerable technical challenge because of the widespread and abundant expression of complement inhibitors on host cells, which prevents effective targeting. Here we investigate a strategy that directs and amplifies complement activation on a tumor cell using the targeting specificity of an antitumor mAb. Specifically, the current data show that an antitumor mAb activates complement and deposits C3d on a tumor cell, generating the ligand for CR2Fc. The Fc domain of the targeted CR2Fc molecule then further activates complement and generates more of its own ligand. The resulting increase in Fc opsonization and complement activation on the tumor cell enhances the outcome of mAb therapy via complement-dependent and Fc-dependent mechanisms.

In the current study, we focused on a metastatic lymphoma model and an mAb therapy protocol in the context of a clinically relevant tumor antigen, GD2. The anti-GD2 mAb alone had some

therapeutic benefit, but the coadministration of CR2Fc significantly improved the outcome of mAb therapy. We broadened the significance of these findings by demonstrating that CR2Fc also enhanced the outcome of anti-gp75 mAb therapy in a B16 metastatic melanoma model. In the EL4 model, an initial and unexpected observation was that CR2Fc alone also had some therapeutic benefit. However, the detection of low levels of antitumor IgM in naive mice offers a likely explanation for this finding in that IgM can activate complement and provide the C3d ligand for CR2Fc binding to tumor cells. Of note, antitumor IgM has been demonstrated in other mouse models of cancer,^{12,16} as well as in patients with different types of cancer.^{10,29,30} Furthermore, tumor-specific deposition of IgM was previously shown in studies investigating the therapeutic potential of an mAb specific for iC3b,²⁹ a ligand also recognized by CR2Fc. In this earlier study, the authors showed IgM deposition on prostate tumor cells after incubation with human serum, and immunohistochemical analysis of clinical samples demonstrated that iC3b was specifically deposited on malignant prostate tissue, supporting the use of C3 activation products as tumor targeting ligands. Subsequent studies by the same group demonstrated that an anti-iC3b mAb enhanced C3b deposition on lymphoma cell lines treated with rituximab (anti-CD20) and enhanced rituximab-mediated tumor cell lysis *in vitro*.³¹ Further, using primary cells isolated from non-Hodgkin lymphoma and chronic lymphocytic leukemia patients, it was shown that a mouse-human chimeric mAb specific for C3b and iC3b enhanced rituximab-mediated CDC *ex vivo*.³² In contrast to the current study using CR2Fc, however, there are no reports of anti-iC3b enhancing the therapeutic efficacy or outcome of mAb therapy in terms of tumor burden or survival in an *in vivo* model. It is also significant that CR2Fc recognizes the C3 activation products iC3b, C3dg, and C3d, whereas analysis of the anti-iC3b mAb used in the aforementioned studies revealed specificity for C3b and iC3b.¹⁸ This is an important distinction because deposited C3b and iC3b are fairly rapidly degraded to the longer-lived degradation fragments C3dg and C3d.³³

The data presented indicate that the primary protective mechanism of CR2Fc is dependent on innate complement effector mechanisms. To investigate the role of an FcγR-dependent mechanism and CDC, we determined the therapeutic outcome of mAb and CR2Fc treatment in FcγR γ chain-deficient mice and in C6-deficient mice. The data show a dependence on FcγR γ chain for the protective effect of CR2Fc, indicating that ADCC and/or enhanced phagocytosis represents the primary mechanism of action for CR2Fc-mediated enhancement of mAb therapy. Unexpectedly, however, we saw a significant increase in survival of PBS-treated (control) versus mAb + CR2Fc-treated FcγR γ chain-deficient mice. This may be because of a shift in the balance of FcγR activating and inhibitory signals, as explained in "Role of Fcγ receptors and direct complement-mediated lysis in CR2Fc activity." However, it has been shown that complement activation can promote tumor growth via C5a-mediated recruitment of myeloid-derived suppressor cells, although only in a single solid tumor model.³⁴ Thus, an additional possibility is that, in the absence of activating FcγRs and phagocyte activation, CR2Fc-enhanced complement activation may promote an immune suppressive environment in liver EL4 micrometastases.

Our data using NK-deficient SCID/beige mice and macrophage-depleted mice indicate that macrophages are necessary for tumor rejection after mAb and CR2Fc treatment. Of significance, our data indicating a key role for macrophage-dependent ADCC and/or phagocytosis in the EL4 model is also in accord with data from

previous mAb therapy studies using the B16 melanoma model of cancer, in which it was shown that Fc receptors and ADCC are required for effective therapy with anti-gp75 mAb treatment.³⁵ In this previous study, it was also shown that macrophages require the FcγR γ-chain for efficient lysis of IgG2a-coated tumor cells *in vitro*,³⁵ and in an earlier study it was shown that anti-gp75 therapy remains intact in SCID/beige mice,³⁶ together indicating an important role for macrophage-mediated FcγR-dependent mechanisms in the B16/anti-gp75 model. Although the focus of our studies here is the EL4 model, we also demonstrate that CR2Fc enhances the outcome of anti-gp75 mAb therapy in the B16 model. Of note, FcγRs on fixed reticuloendothelial cells have been shown to play an important role in the clearance of circulating IgG-opsonized cells and particles.³⁷⁻³⁹ However, this is an unlikely mechanism of macrophage-dependent clearance in the current study because we have shown that injected EL4 cells are no longer present in the circulation when mAb and CR2Fc are injected 2 days after tumor challenge (M.E., unpublished observation, February 2011).

Direct complement-dependent (MAC-mediated) lysis or CDC has been shown to contribute to the therapeutic efficacy of FDA-approved mAbs for the treatment of hematologic cancers, namely, the anti-CD20 mAbs rituximab and ofatumumab, and the anti-CD52 mAb alemtuzumab.⁴⁰ Indeed, new anti-CD20 mAbs have been designed to more effectively activate complement and enhance CDC,⁴¹ and one such anti-CD20 mAb, ofatumumab, has been shown to have significantly improved clinical efficacy compared with rituximab for the treatment of relapsed chronic lymphocytic leukemia.⁴² To address the issue of whether CDC is contributing to the therapeutic effect of anti-GD2 mAb therapy in our murine lymphoma model, we used mice deficient in the terminal complement pathway protein C6, which are unable to assemble the MAC. Although in C6-deficient mice we found a significantly extended survival time of mAb and mAb + CR2Fc-treated mice compared with PBS-treated controls, it did not improve overall long-term survival, and CR2Fc did not enhance the effect of mAb therapy. These data demonstrate that CDC contributes to the efficacy of mAb therapy and the enhancing effect of CR2Fc on mAb therapy.

One potential concern with CR2Fc therapy is toxicity because CR2 ligands are being constantly formed at very low levels in the body, and toxicity would need to be formally investigated before any clinical development of this reagent. However, we did examine kidney sections from mice treated with mAb + CR2Fc and CR2Fc alone and could detect no evidence of glomerular inflammation or injury (not shown). This was despite the fact that we have previously shown in a biodistribution study that human CR2Fc (which has a similar affinity for mouse and human C3d) localizes in the kidney, possibly representing a site of clearance.¹⁸

Finally, although it has been shown that complement activation products can modulate humoral and T cell-mediated immunity, we found no evidence that anti-GD2 mAb therapy, either with or without CR2Fc treatment, had any effect on an acquired anti-EL4 tumor immune response. Interestingly, however, it was previously shown that mAb therapy in a human CD20⁺ EL4 model induces a cell-mediated antitumor response.⁴³ In this earlier study, it was shown that treatment with an anti-CD20 mAb induces a cellular immune response that requires CD4⁺ T cells for protection from initial tumor challenge, and a CD4⁺ and CD8⁺ T cells for protection from a subsequent challenge. Similar to our data, anti-CD20 therapy did not induce an antitumor IgM or IgG response. Both studies use IgG2a treatment antibodies; and other than the fact that the 2 studies targeted different tumor antigens, it is

also possible that the treatment regimen may account for the differences observed in T-cell immunity. We used a single injection of mAb after tumor cell inoculation, whereas Abes et al treated mice over a prolonged period (2 weeks) with significantly higher mAb doses.⁴³ Continued immune activation by the antitumor mAb may facilitate development of an adaptive immune response. It is also noteworthy that when mAb and CR2Fc therapy was initiated 7 days after EL4 inoculation when liver micrometastases are well established, there was no protective effect (data not shown), suggesting the principal benefit of mAb and CR2Fc may be in an adjuvant setting.

In conclusion, we describe a reagent and strategy to direct and amplify complement activation on a tumor cell that uses the targeting specificity of an antitumor mAb. We show that the reagent, CR2Fc, enhances the therapeutic effect of mAb therapy in mouse models of metastatic lymphoma and melanoma. We show that, in the lymphoma model, therapeutic effect occurs via macrophage-mediated ADCC and direct complement mediated lysis. In a clinical context, it has also been reported that the *in vivo* mechanism of clinically approved anti-CD20 antibodies is complement-dependent at low tumor loads, and at higher tumor burdens are dependent on a combination of complement and Fc γ R-mediated mechanisms.⁴⁴ The results presented here indicate that CR2Fc may be a beneficial adjuvant to mAb immunotherapy of cancer, particularly in a metastatic setting.

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Authorship

Contribution: S.T. and M.E. designed research and wrote the paper; M.E., Y.H., F.Q., and C.A. performed research and collected data; S.T., M.E., and C.A. analyzed and interpreted data; N.v.R. contributed vital new reagent; and B.P.M. contributed vital new reagents and reviewed the manuscript.

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