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14. ABSTRACT In general, a humoral immune response to breast cancer cells is not therapeutically effective, likely due, at least in part, to the overexpression of membrane bound complement inhibitory proteins on tumor cells. In these studies we proposed to investigate two novel fusion proteins aimed at overcoming complement inhibition of breast cancer cells. The recombinant fusion proteins have been successfully constructed, expressed and purified. The MUC1 vaccination study was completed, showing that C3d does function as a molecular adjuvant. Linking C3d to the tumor antigen MUC1 increased the humoral immune response and elicited a cellular immune response to the tumor. However, the T cell response was not MUC1 specific. CR2Fc has been characterized in vitro showing that it binds to C3 opsonized cells, increases complement activation and tumor cell lysis. CR2Fc has also preliminarily been characterized in vivo showing potential in a therapeutic study. Further experiments will be carried out to show the fusion proteins therapeutic efficacy and ability to modulate the immune response.					
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Table of Contents

	<u>Page</u>
Introduction.....	5
Body.....	5
Key Research Accomplishments.....	9
Reportable Outcomes.....	9
Conclusion.....	10
References.....	10
Appendices.....	10

Introduction

In general, a naturally occurring antibody response or therapeutically administered antibodies do not produce an effective response against breast cancer. Indeed, antibody immunotherapy of cancer has fallen short of clinical expectations. This is due, at least in part, to the expression of complement regulatory proteins, the expression of which is often upregulated on cancer cells, and the inhibition of complement mediated tumor cell destruction. Further, recent data has shown that complement inhibitors may also play a role in suppressing an anti-tumor cellular immune response [1, 2]. This project was based on the hypothesis that overcoming the complement resistance of tumor cells will enhance the outcome of a normally ineffective humoral immune response and potentially induce a protective cellular immune response to the tumor. To this end, we proposed to prepare and characterize two novel fusion proteins aimed at modulating complement to enhance the humoral immune response and potentially induce a cellular immune response to breast cancer cells.

One approach was to use the fusion protein MUC1-(C3d)₃ in a vaccination strategy designed to enhance the induction phase of the immune response. This fusion protein consists of MUC1, a tumor antigen, fused to three repeating units of mouse complement fragment C3d. Repeating units of C3d fused to antigen has been shown to enhance an antibody response by 10,000 fold [3]. Further, C3 has been shown to lower the antigen density threshold necessary to elicit an IgG response, is a regulator of T cell responses and has been shown to enhance avidity maturation [3-5]. Therefore, this approach is expected to induce a high titer IgG response to MUC1 and potentially a T cell response.

The second approach involved preparation and characterization of CR2Fc fusion protein, designed to enhance the effector phase of the immune response induced from monoclonal antibody therapy. The recombinant protein consists of a complement receptor 2 (CR2) fragment fused to the Fc region of mouse IgG2a. The CR2 portion of the fusion protein is the targeting domain. The C3 ligands (iC3b, C3d and C3dg) for CR2 are relatively long lived at sites of complement activation and would be present on tumor cells following binding of anti-cancer monoclonal antibodies and complement activation. The Fc portion is expected to further increase complement activation, further generating CR2 ligands and increasing complement dependent effector mechanisms, such as CDC (complement dependent cytotoxicity) and CDCC (complement dependent cellular cytotoxicity), as well as Fc dependent effector functions such as ADCC (antibody dependent cellular cytotoxicity).

Construct plasmids encoding MUC1 and MUC1-C3d (Task 1)

Protein constructs of five tandem repeats of MUC1 (MUC1/TR5) and five tandem repeats of MUC1 linked to three repeating units of C3d (MUC1/TR5-C3d) for vaccination studies were prepared (Figure 1).

Construct plasmid encoding CR2Fc (Task 1)

Due to a technical difficulty expressing and purifying mouse CR2Fc during year one it was necessary to construct a new plasmid encoding mouse CR2Fc.

During year two this problem was overcome and a stable clone successfully expressing CR2Fc was prepared.

Expression and Purification of recombinant proteins (Task 2)

The MUC1/TR5 and MUC1/TR5-C3d recombinant proteins were successfully expressed and purified. The proteins were purified from culture supernatant of stably transfected COS7 cells by DEAE FF Sepharose ion-exchange. SDS-PAGE and Western blot was used to confirm the identity of the purified protein. Figure 2 shows a Western blot revealing bands at the expected molecular weights of 20Kd (MUC1/TR5) and 120Kd (MUC1/TR5-C3d).

Mouse CR2Fc was successfully expressed and purified. Two different CR2Fc constructs have been produced, one with the CR2 linked to the Fc region without a linker and one with the CR2Fc connected with a (SG₄)₂ linker. The proteins were purified from culture supernatant of stably transfected CHO cells using a Protein A column. A high expressing CHO cell clone was isolated by limiting dilution, and the average yield was 13 mg/L of supernatant. The purity of the recombinant protein was confirmed by SDS-Page (data not shown). The identity of the purified proteins was confirmed by SDS-Page and Western blot. The Western blot was stained with 7G6 (an anti-mouse CR2 mAb), revealing a band at the expected molecular weight of 54 Kd (Figure 3).

***In vitro* characterization of CR2Fc (Task 3)**

CR2Fc has been characterized *in vitro* for binding to complement component C3 opsonized cells, its ability to increase complement activation and tumor cell lysis.

Using a flow cytometric binding assay, it was shown that both CR2Fc constructs (with and without linker) bind to C3 deposited on CHO cells (Figure 4). We next investigated the ability of CR2Fc to increase complement activation. This was investigated by several different methods. First, we investigated the ability of CR2Fc to increase C3 deposition on the cell surface by flow cytometry. Surprisingly, we saw a slight decrease in C3 deposition in the presence of CR2Fc (Figure 5; mean fluorescence 71.15 vs 93.72). However, this is likely caused by the binding site for the detection antibody being covered by the CR2 portion of the fusion protein. Therefore, to investigate if CR2Fc is increasing complement activation we undertook a different approach and measured the release of a soluble complement activation fragment. Following antibody-mediated complement activation in the presence or absence of CR2Fc, supernatants were analyzed by SDS-Page and Western blot for C3a. We found that in the presence of CR2Fc, there was an over 2-fold increase in C3a in the supernatant compared to the sample containing antibody and serum alone (Figure 6; band density 2019.5 vs 924.46).

The ability of CR2Fc to increase tumor cell lysis was next evaluated by a LDH assay and trypan blue exclusion method. CR2Fc was shown to increase tumor cell lysis as detected by both methods. Cancer cells were incubated with complement activating antibody, followed by incubation with serum. The cells were then stained with trypan blue or the supernatant was collected and analyzed for LDH activity. In the trypan blue experiment, an increase in lysis from 17% cytotoxicity in the absence of CR2Fc to 30% cytotoxicity in the presence of CR2Fc was seen (Figure 7).

We then evaluated CR2Fc's ability to increase tumor cell lysis using an LDH assay. In this experiment, in the presence of CR2Fc there was 95% lysis of the tumor cells, whereas in the absence of CR2Fc lysis decreased to 59% (Figure 8).

Collectively, this data demonstrates that *in vitro* CR2Fc binds to C3 deposited on the cell surface, increases complement activation and complement dependent tumor cell lysis.

MUC1 vaccination study (Task 4)

The MUC1/TR5 and MUC1/TR5-C3d proteins were characterized in a vaccination study. MUC1 transgenic mice on a C57BL/6 background were immunized intramuscularly with MUC1/TR5 or MUC1/TR5-C3d and boosted on day 28 and day 69. The complete immunization schedule is depicted (Figure 9). At day 35, serum was taken to analyze the antibody response by ELISA (Figure 10). Seven days following the second boost (day 78) serum and spleen were taken to again analyze the antibody response, as well as the T cell response by ELISPOT (Figure 11). MUC1 transgenic mice were used to analyze responses in the context of tolerance and autoimmunity.

MUC1 transgenic (MUC1-Tg) mice vaccinated with either protein construct had anti-MUC1 IgM and IgG antibodies present in their sera at day 28. However, the IgM response was similar in both groups. The IgG response, however, at days 28, 35 and 78, was increased in mice inoculated with MUC1/TR5-C3d compared to mice inoculated with MUC1/TR5 (Figure 10). The IgG levels in mice inoculated with MUC1/TR5 or MUC1/TR5-C3d was further investigated by titrating the IgG levels from sera taken on days 35 and 78. Disappointingly, the mice immunized with MUC1/TR5-C3d had a relative IgG titer that was only four times higher at day 35 and eight times higher at day 78 compared to mice immunized with MUC1/TR5 (Figure 10). This very modest increase in anti-MUC1 IgG titer compares to over a 1000-fold increase seen using a different antigen, although not in the context of tolerance (3). Furthermore, sera isolated from these mice did not have increased tumor lysis activity compared to unimmunized mice. Finally, we also prepared constructs for DNA vaccination and followed a similar immunization protocol as outlined for protein immunization. However, we failed to elicit any increase in immune response by this method (data not shown).

In conclusion, these studies indicate that C3d does function as a molecular adjuvant when linked to a cancer associated antigen. Although promising, the IgG response elicited by MUC1/TR5-C3d was not strong. In order to achieve protective immunity the response to the vaccine will need to be increased. To this end, we have begun to construct adenoviral vectors expressing MUC1/TR5 and MUC1/TR5-C3d. Since adenoviral vectors are known to be highly immunogenic, it is expected that this approach may boost the immune response to the vaccine and potentially elicit protective immunity. However, these studies will extend beyond the current scope of this project.

Determine outcome of MUC1 vaccination with CR2Fc and beta-glucan treatment (Task 5)

Due to the poor outcome of the MUC1 vaccination study, this task was not completed.

Determine outcome of anti-MUC1 immunotherapy with CR2Fc and beta-glucan treatment (Task 6)

To investigate the ability of CR2Fc to enhance mAb immunotherapy we used an already established metastatic model with EL4 cell line due to a technical problem with continued stable expression of MUC1 on the EO771 breast cancer cell line in vivo. EL4 cells endogenously express the tumor antigen GD2, and our lab has the hybridoma to produce anti-GD2 (14G2a) for mAb immunotherapy. In a preliminary experiment, performed principally to determine that mice were susceptible to cancer at cell dose used, and that mAb is protective, mice were inoculated intravenously with tumor cells and 48 hours later treated with mAb (14G2a) or PBS. Six hours after mAb therapy the mice were treated with CR2Fc or PBS and survival was followed to day 80. As expected, in the absence of mAb therapy, mice survived between 30 and 36 days. In the presence of mAb therapy alone survival was 50% at day 80. With combined mAb and CR2Fc treatment the survival rate at day 80 was 75% (Figure 12). However, while promising, due to the small group size, we cannot draw conclusions on the efficacy of CR2Fc from this experiment. A second experiment with increased group size is underway.

Determine the effect of MUC1 vaccination and complement modulation by CR2Fc on the outcome of a T cell response (Task 8)

Spleens were harvested from the mice vaccinated with MUC1/TR5 or MUC1/TR5-C3d at day 78. To evaluate a T cell response the isolated splenocytes were analyzed by an interferon gamma (IFN- γ) ELISPOT. EO771 cells transfected with MUC1 or vector were gamma irradiated and used as targets. Splenocytes isolated from mice immunized with MUC1/TR5 did not elicit a T cell response against vector or MUC1 transfected EO771 cells. However, a strong response was seen with splenocytes isolated from mice immunized with MUC1/TR5-C3d against both MUC1 and vector transfected EO771 cells (Figure 11). A T cell response was seen in response to MUC1/TR5-C3d and not MUC1/TR5. However, since the splenocytes produced IFN- γ in response to both MUC1 and vector (control) transfected target cells (EO771), we conclude that the induced T cell response is not MUC1 specific.

Breast cancer related training tasks and accomplishments

During the three-year award period I have grown as a breast cancer researcher. I have completed several courses, participated in scientific meetings and retreats and attended numerous seminars. My knowledge in the field of breast cancer research has grown along with my scientific communication skills.

I have completed the 12 required credit hours past the core curriculum. I took courses in Tumor Immunology and Immunotherapy, Histology, Mechanisms of Apoptosis and Microbiology-Immunity. These courses furthered my knowledge of cancer biology. I also completed courses in Biostatistics and biomedical and

scientific ethics, which have helped prepare me for my career as a breast cancer researcher.

I participated in several seminar series around campus. My department, Microbiology and Immunology, has weekly seminars that I attended and presented at once a year. I also participated in biweekly meetings with a Complement research group. These meetings are smaller and more interactive than the departmental seminars and allow for discussion of an individuals data. I have presented and discussed my data at these meetings several times a year. Finally, I attended seminars by invited speakers at the Hollings Cancer Center several times a month. These experiences helped me develop my presentation and communication skills for a career as a research scientist.

I have attended meetings that have furthered my scientific development. The university hosts a Student Research Day annually that I have participated in during the three years of my award. The Hollings Cancer Center hosted a weekend retreat for members of the Cancer Immunology and Immunotherapy group that I participated in. Finally, I presented at the DOD Era of Hope meeting in Baltimore, MD in June of 2008. This experience allowed for interaction with many of the most respected breast cancer researchers and also taught me more about research from the patients' perspective.

My mentor has also helped me develop my research skills in the last three years. We have biweekly meetings to discuss my progress and his door is always open if I have any questions or problems. Overall, in the last three years I have grown immensely as a breast cancer researcher and feel well prepared for a career in the field.

Key Research Accomplishments:

- Construction, expression and purification of MUC1/TR5 and MUC1/TR5-C3d
- Construction, expression and purification of two CR2Fc constructs (with and without linker)
- Characterization of CR2Fc *in vitro*; showing binding, increased complement activation and complement dependent lysis
- Preliminary *in vivo* studies with CR2Fc, showing feasibility
- Completion of the MUC1 vaccination study
- Determined that C3d does function as a molecular adjuvant, but in the context of tolerance (ie. vaccinating against human MUC1 in a human MUC1 transgenic mouse), the induced Ab response is low and not protective.
- Determined that while an anti-tumor T cell response is elicited by MUC1/TR5-C3d, it is not specific for the immunizing antigen (MUC1).

Reportable outcomes:

- Rapisardo M, Ohta R and Tomlinson S. Enhancement of the immune response following immunization with a MUC1-C3d fusion protein. DOD Era of Hope BCRP meeting. Poster #P52-3. Baltimore, MD. June 25-28, 2008.

Conclusions:

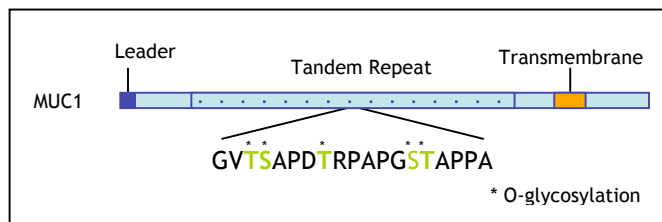
From the vaccination study, we conclude that C3d does have the potential to function as a molecular adjuvant when linked to a tumor associated antigen. Vaccination with C3d linked to antigen increased the immune response by eliciting an enhanced IgG response, as well as a T cell response. However, Ab response was of low titer, and the T cell response was not MUC1 (antigen) specific. Future studies will need to be done to increase the immune response further to produce protective anti-tumor immunity.

We have produced a CR2Fc fusion protein that functions as hypothesized. The fusion protein has been characterized *in vitro* and shown to bind to C3 opsonized cells, increase complement activation and enhance antibody-mediated lysis of tumor cells. Preliminary *in vivo* therapeutic studies are promising, although inconclusive at present. Ongoing studies will determine the therapeutic efficacy of CR2Fc, as well as if and how it modulates the immune response due to increased complement activation.

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1. Liu, J., et al., *The complement inhibitory protein DAF (CD55) suppresses T cell immunity in vivo*. J Exp Med, 2005. 201(4): p. 567-77.
2. Heeger, P.S., et al., *Decay-accelerating factor modulates induction of T cell immunity*. J Exp Med, 2005. 201(10): p. 1523-30.
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5. Test, S.T., et al., *Increased immunogenicity and induction of class switching by conjugation of complement C3d to pneumococcal serotype 14 capsular polysaccharide*. Infect Immun, 2001. 69(5): p. 3031-40.

Appendices:



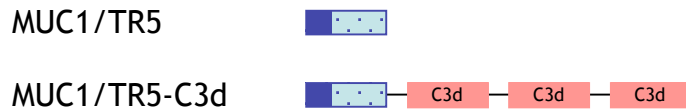


Figure 1. MUC1/TR5 and MUC1/TR5-C3d constructs. Both constructs contain five tandem repeating units of MUC1. MUC1/TR5-C3d is fused to three repeating units of C3d.

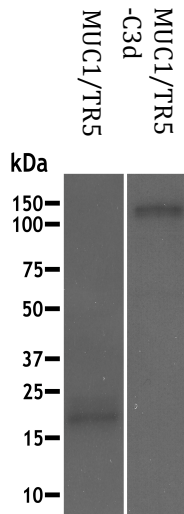


Figure 2. Purification of MUC1/TR5 and MUC1/TR5-C3d recombinant proteins. Recombinant proteins were purified from culture supernatant of COS7 transfected cells by DEAE FF Sepharose ion-exchange. The purified protein was confirmed by SDS-PAGE and Western blot, revealing proteins with in the expected molecular weight range.

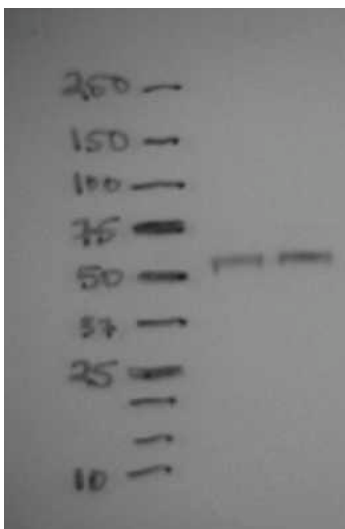


Figure 3. Western blot confirming identity of purified CR2Fc. Blot was stained with 7G6 (anti-CR2) antibody and shows a band at the expected molecular weight (54 Kd).

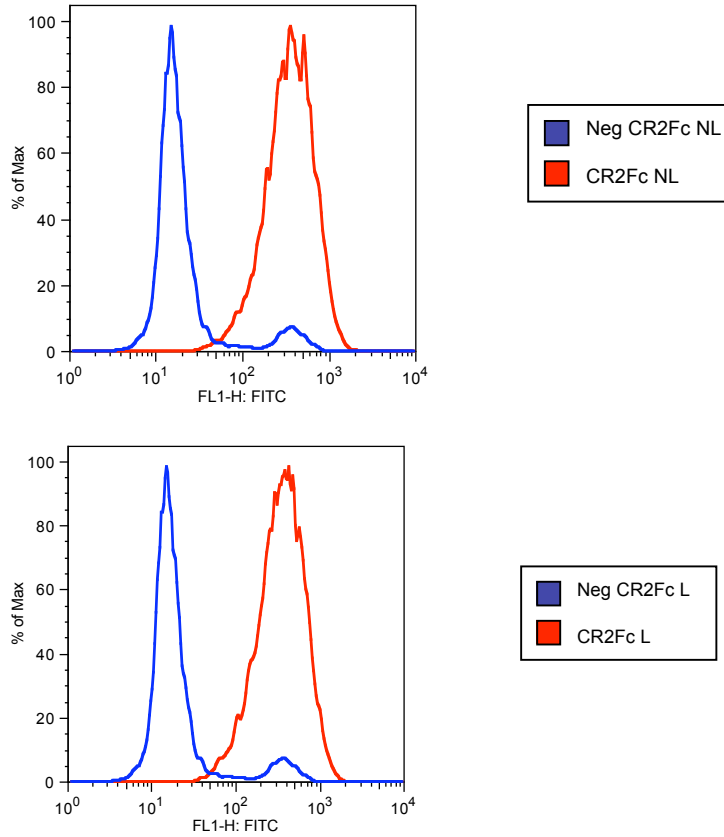


Figure 4. Binding of CR2Fc to CHO cells. The binding of both CR2Fc linker and no linker proteins to C3 opsonized CHO cells were shown by flow cytometry. Representative result.

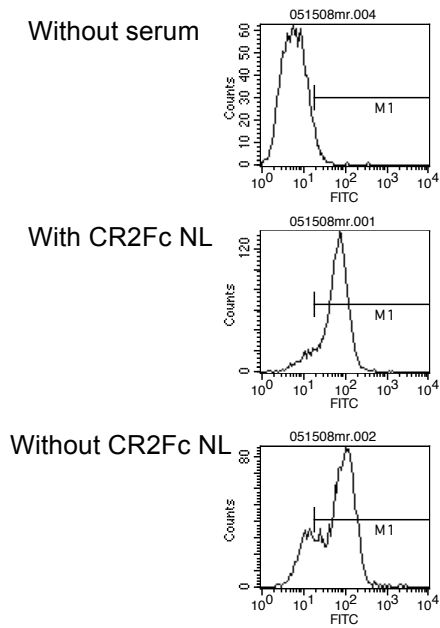


Figure 5. . Complement deposition following incubation with CR2Fc. The ability of CR2Fc to increase C3 deposition on CHO cells was evaluated by flow cytometry. Mean fluorescence; with CR2Fc NL 71.15 vs without CR2Fc NL 93.72. Representative result.

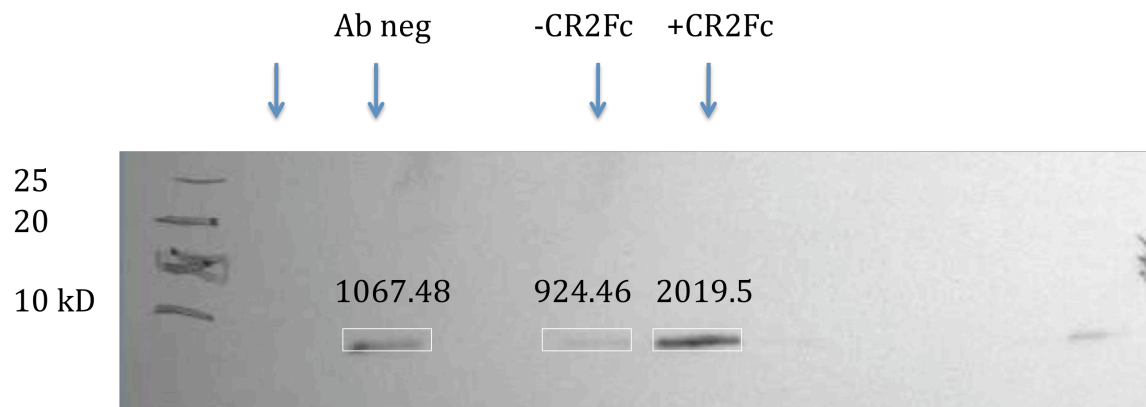


Figure 6. C3a Western Blot. The complement activation product ,C3a, is increased in the presence of CR2Fc, compared to sample containing antibody and serum alone; band density 2019.5 vs 924.46. Representative of three separate experiments.

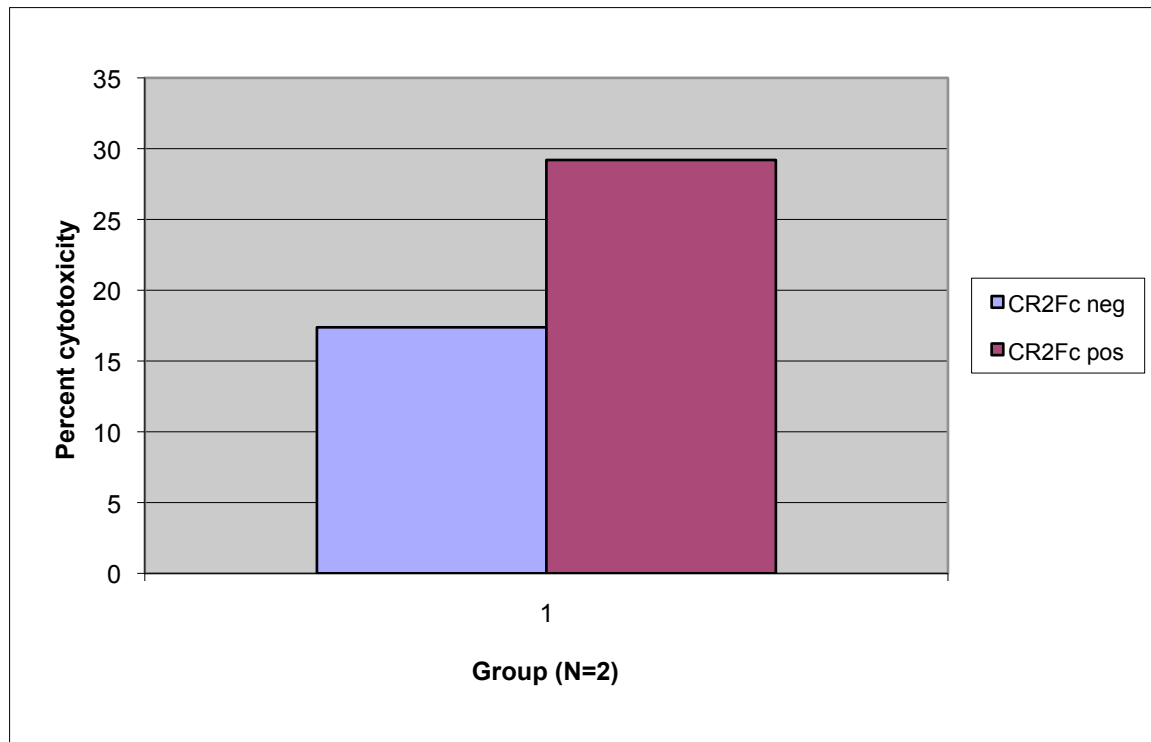


Figure 7. Lysis assay for CR2Fc dependent increase in tumor cell lysis. Cells were incubated with antibody and serum in the presence or absence of CR2Fc for 20 minutes. Following incubation cells were stained with trypan blue to detect cell viability. Percent cytotoxicity in the absence of CR2Fc was 17.38% and in the presence of CR2Fc percent cytotoxicity increased to 29.2%. N=2

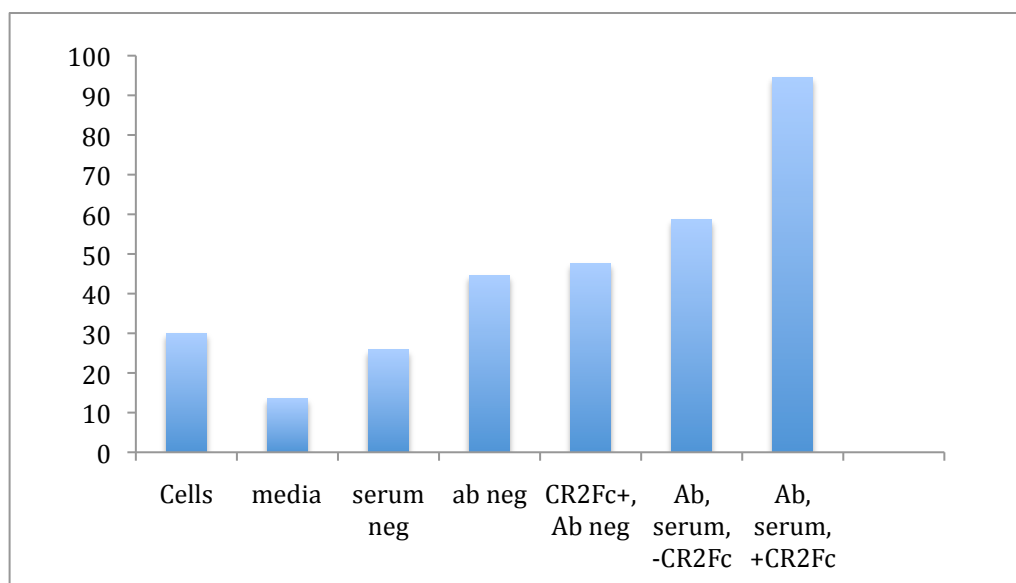


Figure 8. LDH assay for CR2Fc dependent increase in tumor cell lysis. Cells were incubated with antibody and serum in the presence or absence of CR2Fc for 1 hour

and 15 min. Following incubation supernatants were collected and assayed for LDH activity. In the presence of CR2Fc percent lysis was 94.5%, whereas in the absence of CR2Fc percent lysis decreased to 58.6%. Representative of three separate experiments.

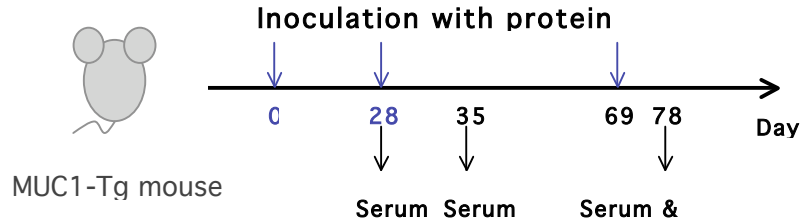


Figure 9. Vaccination strategy for MUC1 vaccination study.

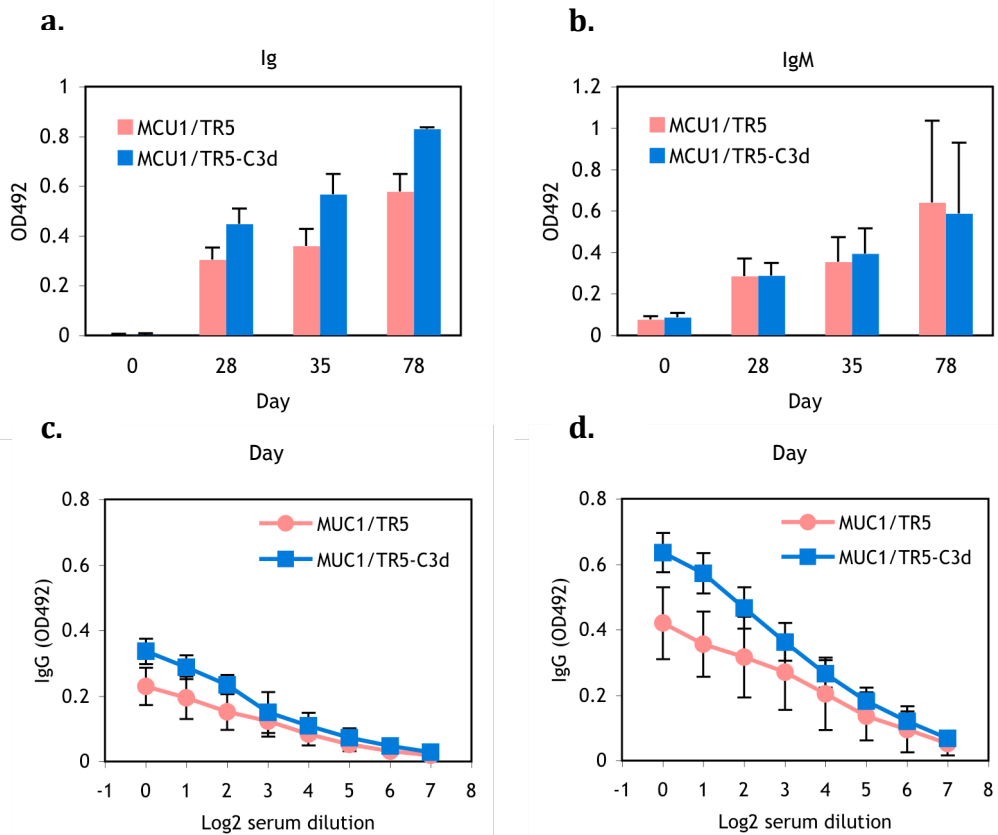


Figure 10. . Humoral immune response to MUC1/TR5 and MUC1/TR5-C3d in MUC1-transgenic mice. MUC1 transgenic mice on a C57BL/6 background were immunized i.m. with 500 pmol recombinant protein and boosted on days 28 and 69. Panels a and b). The IgG (a) and IgM (b) titers from the serum were analyzed on days 28, 35 and 78 by ELISA. Panels c and d). IgG levels from sera on days 35 (c) and 78 (d) were titrated to investigate the difference between MUC1/TR5 and MUC1/TR5-C3d immunized mice

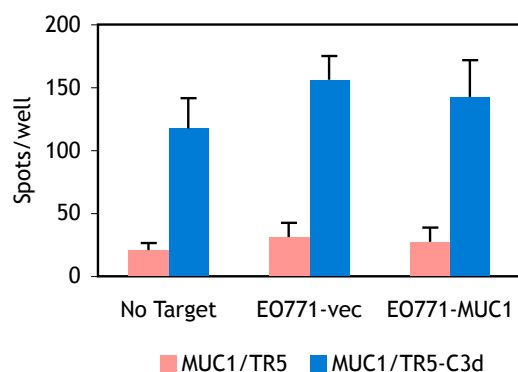


Figure 11. T cell response to MUC1/TR5 and MUC1/TR5-C3d in MUC1-transgenic mice. T cell response was analyzed on day 78 by interferon gamma ELISPOT. Gamma irradiated vector or MUC1 transfected EO771 cells were used as targets. Results are representative of two independent experiments.

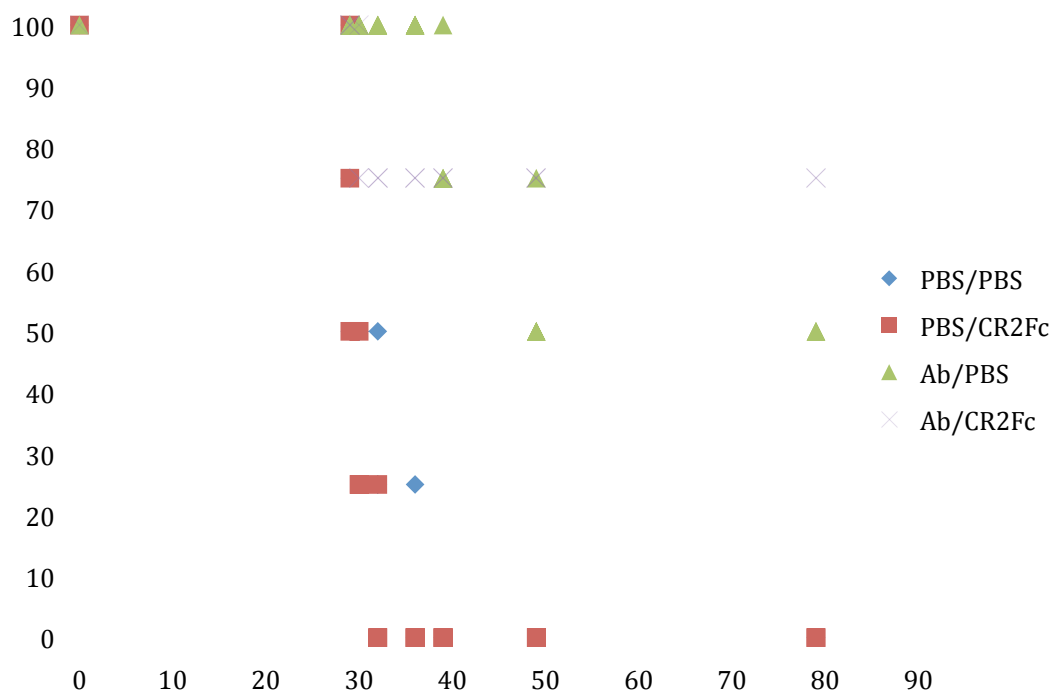


Figure 12. Therapeutic study to investigate the outcome of mAb therapy with CR2Fc treatment. Mice were inoculated with tumor cells and 48 hours later treated with mAb or PBS. Six hours following mAb therapy mice were treated with CR2Fc or PBS. Animal survival was followed to day 80.