Liposomal Fc Domain Conjugated to a Cancer Vaccine Enhances Both Humoral and Cellular Immunity

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Supporting Information

ABSTRACT: Targeted delivery of antigens to antigen-presenting cells (APCs) by utilizing natural antiacarbohydrate antibodies is a promising approach for selective uptake and enhanced antigen presentation. Previously, we reported that in the presence of a natural antibody, anti-rhamnose antibody (anti-Rha), the bacterial sugar rhamnose conjugated with liposomal cancer antigen MUC1-Tn enhances antigen presentation by APCs such as dendritic cells by targeting Fc gamma receptors. The idea was to utilize the natural human anti-Rha antibodies present in human serum for targeted delivery of cancer-specific antigens. Recently, we found that the IgG3 antibody isotype was the most prevalent anti-Rha antibody generated in mice immunized with rhamnose-Ficoll (Rha-Ficoll) antigen. In this manuscript, we have conjugated the murine IgG3-Fc with a MUC1-containing cancer vaccine and compared the humoral and cellular immune response to this vaccine with one targeted via the human anti-Rha antibody and to the MUC1 vaccine alone. This Fc approach enhanced antibody production and T-cell proliferation almost to the same level as using the anti-Rha antibody. These results suggest that targeting Fc directly to dendritic cells can be an alternative approach to human anti-Rha for generating effective antigen-primed T-cells.

INTRODUCTION

The target of vaccination, whether preventative or therapeutic, is to prevent the spread of disease. Preventative vaccines are designed mainly to produce specific antibodies and memory B cells, whereas therapeutic vaccines are prepared to stimulate the immune system to additionally produce antigen-specific cytotoxic T-cells (CTLs). A successful therapeutic vaccine induces both humoral and cellular immunity and effectively prevents the spread of a disease. Dendritic cells (DCs) participate between innate and adaptive immunity by capturing antigen and presenting it to T-cells after processing.¹² This is very important to generate effective antigen-specific immunity. After capturing antigen and receiving an activation signal, DCs migrate to secondary lymphoid organs from the peripheral tissue and present peptide from the processed antigen to T-cells through major histocompatibility complex (MHC) molecules.³ Depending on the absence or presence of inflammatory signals, DCs can be tolerogenic or immunogenic.⁴ DCs possess receptors for inflammatory signals or microbial products and can become mature cells, which are essential for adaptive immunity.⁵,⁶

Different receptors on DCs have been identified for effective capture and presentation of antigens.⁷⁻⁹ Fc receptors (FcRs) are very common for antibody-mediated antigen uptake.¹⁰⁻¹¹ Utilizing a natural antiacarbohydrate antibody to enhance the antigen presentation of a vaccine by forming a complex between the antibody and corresponding vaccine antigen has been found effective. α-gal antibodies are among the most common natural antiacarbohydrate antibodies present in human serum. They have been targeted to form complexes with the α-gal epitope in vivo and were found to augment immunogenicity by the interaction between FcRs of DCs and the Fc portion of the antigen antibody.¹²,¹³ Similarly, anti-Rha is another antiacarbohydrate antibody found in human serum in large amounts.¹⁴ Targeting the anti-Rha antibody for effective uptake and presentation of a cancer vaccine conjugated with rhamnose has been found to be very promising in terms of the production of both antibody and cancer-killing CTLs (CD8 T-cells).¹⁵⁻¹⁷ However, the success of this approach entirely depends on the presence of specific natural antibodies in human serum which limits its usability.

Direct conjugation of the Fc portion of an antibody isotype with a liposomal cancer vaccine is another approach that does not depend on the presence of natural antibodies. This helps in targeting the cancer vaccine directly to DCs through FcRs without the need of anti-Rha antibodies.

In this article, we have compared both approaches and showed that the Fc approach can be equally effective as a natural antibody-targeting (human anti-Rha antibody) approach in generating both cancer antigen-specific antibody production and inducing cellular immunity.
As a liposomal cancer vaccine, we used one of the most common tumor-associated antigens, mucin 1 (MUC1). It is a glycoprotein usually overexpressed on the apical surface of tumor cells. In normal cells, it is highly glycosylated with elongated O-glycans that shield the protein backbone from the immune system. However, on cancer cells, MUC1 is usually hypoglycosylated with fewer truncated O-glycans. Some tumor-associated carbohydrate antigens (TACAs) are found on MUC1, notably Tn, TF, and STn. However, because they are not highly immunogenic, TACAs have been conjugated with different carrier proteins by different research groups. The problem with carrier proteins, however, is that they are immunogenic and might produce immune responses, which can impede the desired immune response. In our vaccine design, we used a TLR-2 agonist (Pam3-Cys-SK4) covalently linked to a MUC1-Tn peptide, which helps in DC maturation and antigen presentation to T-cells.

## RESULTS AND DISCUSSION

Targeting whole IgG Fc domains to Fc gamma receptors (FcγR) of DCs for antigen loading can be critical considering FcγR can be both activating and inhibitory. Under normal conditions, this targeting seemed insufficient for DC maturation because of the presence of inhibitory FcγRIIB. Earlier studies revealed that knocking out the FcγRIIB receptor from mice or blocking of FcγRIIB through mAb resulted in the up-regulation of MHC II molecules. These studies motivated us to use the Fc domain from an IgG isotype that would specifically bind with an activating FcγR and not to an inhibitory receptor. In mice, IgG1 and IgG3 isotypes both bind with activating FcγRI. However, IgG1 also binds with inhibitory FcγRIIb, whereas IgG3 binds only with activating FcγRI.

We had previously found that anti-Rha antibodies generated in mice with Rha-Ficoll were more efficient at enhancing an immune response than those generated with Rha-Ovalbumin. Earlier studies revealed that knocking out the FcγRIIB receptor from mice or blocking of FcγRIIB through mAb resulted in the up-regulation of MHC II molecules. These studies motivated us to use the Fc domain from an IgG isotype that would specifically bind with an activating FcγR and not to an inhibitory receptor. In mice, IgG1 and IgG3 isotypes both bind with activating FcγR. However, IgG1 also binds with inhibitory FcγRIIB, whereas IgG3 binds only with activating FcγRI.

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We had previously found that anti-Rha antibodies generated in mice with Rha-Ficoll were more efficient at enhancing an immune response than those generated with Rha-Ovalbumin. The anti-Rha antibody generated in mice was mostly IgG3 when the mice were immunized with Rha-Ficoll and IgG1 in response to Rha-Ova. Because mouse IgG3 is supposed to bind only activating FcγRI, we targeted the Fc portion of the murine IgG3 isotype (Sino-Biological Inc.) to conjugate with liposomal Pam3-Cys-SK4-MUC1-Tn cancer vaccine.

For conjugation purposes, thiol residues were introduced into Fc by modification with the heterobifunctional cross-linker N-succinimidyl 3-(2-pyridyldithio)propionate. Next, deprotection was carried out by using dithiothreitol (DTT) and later the protein was conjugated with liposome
The freshly reduced thiolated Fc solution was added to dipalmitidoylphosphatidylocholine (DPPC) liposomes containing 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethyleneglycol)] DSPE-PEG-MAL (1% by wt). The mixture was stirred overnight at RT. The liposomal pellet was centrifuged down, washed twice with phosphate-buffered saline (PBS) buffer, and resuspended in the desired volume of PBS buffer for injections. Assay of the supernatant showed that approx. 90% of the thiolated Fc was conjugated to the liposomes.

After the conjugation process, we sought to determine whether Fc conjugation enhanced the antigen uptake and presentation by DCs. We compared this conjugation approach with the human anti-Rha antibody-targeting approach. As a control group, we used cancer antigen (MUC1-Tn) liposomes only. Three groups of liposomes were prepared for three groups of C57BL/6 mice (five per group). Stock solutions were prepared of DPPC, Cholesterol, DSPE-PEG-MAL, and Rha-TEG-Cholesterol. Aliquots from stock solutions were mixed to obtain a lipid solution of 30 mM in a total volume of 1 mL.

Group A received mouse IgG3-Fc-conjugated cancer vaccine, whereas group B received human anti-Rha antibody 1 h before immunization with Rha-containing vaccine. The last group (group C) received cancer antigen liposomes only. All mice received one priming and two boosts of liposomal vaccines i.p. after 14 and 28 days. Liposomes were formulated by the extrusion method with 100 nm polycarbonate membranes as previously described.17,32

**Group A:** IgG3-Fc-DSPE-PEG-MAL (1% by wt), PamCysSK4-MUC-1-Tn conjugate (0.2 μmol), DPPC (79%), and cholesterol (20%) in 100 μL.

Group B: Anti-Rha antibody (10 μg/mouse, 1 h earlier) + PamCysSK4-MUC-1-Tn conjugate (0.2 μmol), cholesterol-TEG-Rha (10%), cholesterol (10%), and DPPC (80%) in 100 μL.

Group C: PamCysSK4-MUC-1-Tn conjugate (0.2 μmol), DPPC (79%), cholesterol (20%), and DSPE-PEG-MAL (1% by wt) in 100 μL.

Mice were bled 7 days after the second boost for measuring antibody production. An ELISA assay was performed to determine the antigen-specific antibody production by screening the mice sera against the antigen, MUC1-Tn. The ELISA was conducted as described.15 The Fc-conjugated vaccine (group A) was found to have the best antibody production in comparison with group B. As predicted, group C had the lowest antibody production (Figure 2A). Therefore, it clearly shows that both Fc-conjugated antibody and human anti-Rha antibody targeting were efficient approaches compared to the control group (group C).

Next, T-cell priming was tested among those groups to determine whether the Fc-conjugated approach helped in the production of MUC1-Tn-primed CD4+ T-cells. The proliferation was measured with varying concentrations (0–40 μg mL⁻¹) of the antigen. Three mice from each group were sacrificed 7 days after the second boost of vaccine. Spleen cell suspensions were prepared following the protocol described earlier.15 CD4+ T-cells were separated using Dynabeads FlowComp mouse CD4 positive isolation kit (Invitrogen). The rest of the cell suspension was kept for separating CD8+ T-cells. DCs prepared from murine bone marrow were first added to a 96 well cell culture plate (3 × 10⁵ cells in 90 μL) followed by the MUC1-Tn antigen at different concentrations (0, 10, 20, and 40 μg mL⁻¹) in 60 μL each.15 After 30 min, CD4+ T-cells were added to their individual wells (3 × 10⁵ cells in 50 μL) and incubated for 72 h at 37°C, 5% CO₂. Thus, the ratio of DCs and CD4 was kept at 1:10. [³H]-Thymidine was added (25 μL, 40 μCi mL⁻¹) and incubated for 24 h in the same condition. Cells were harvested, and filters dried overnight. Scintillation fluid (40 μL) was added to each well and thymidine incorporation was measured as the CPM value in a top count scintillation counter (Packard). The best proliferation was measured at 40 μg mL⁻¹. At this concentration, CD4+ T-cells from group A proliferated significantly more compared to the other two groups (B and C) (Figure 2C).

Because high levels of IFN-γ production are correlated with enhanced cytotoxicity, we measured the IFN-γ production among those three groups of mice. Purified CD8+ T-cells were obtained using the Dynabeads Flowcomp CD8+ isolation kit (Invitrogen) and distributed in a 12-well plate (1 × 10⁵ cells in 500 μL/well). Bone marrow-derived DCs were added (5 × 10⁴ cells in 500 μL) to make a ratio of 1:20. The combination was pulsed with the 40 μg mL⁻¹ concentration of 8 amino acid long CD8 peptide epitope previously reported.15 The plate was incubated for 24 h at 37°C and 5% CO₂. Next day, the plate was centrifuged, and supernatant was collected immediately for measuring IFN-γ. IFN-γ cytokine secretion was measured using a murine IFN-γ ELISA kit (Peprotech). Group A released more IFN-γ compared to group C, which proves that...
enhanced uptake and presentation leads to the secretion of more IFN-γ (Figure 3A).

To investigate the cytotoxic ability of CD8+ T-cells separated from different groups, we used a cytotoxicity assay called a JAM assay.33 A mouse lymphoma cell line (EL4) was used as a target cell line. One day before the experiment, EL4 cells were grown in a fresh T25 flask. [3H]-Thymidine was added (40 μCi/mL) and incubated for 4 h at 37 °C and 5% CO₂. Following two washings, cells were brought to 10⁵ cell/mL and divided into two equal halves. Only one-half was pulsed with the CD8 epitope. Both flasks were washed twice and 10⁵ EL-4 cells in 100 μL were added to each well of a U-bottom 96-well plate. The purified CD8+ T-cell concentration was brought to 10⁶ cells/mL and divided into two equal halves. Only one-half was pulsed with the CD8 epitope. Both flasks were washed twice and 10⁵ EL-4 cells in 100 μL were added to each well of a U-bottom 96-well plate. The purified CD8+ T-cell concentration was brought to 10⁶ cells/mL and 100 μL added to the plate at a ratio of E/T of 1:100. Stauroporine (2 μM) was added as a positive control instead of CD8+ T-cells in some wells. The plate was incubated for 6 h and harvested on a glass fiber filter plate using a cell harvester. The filter plate was then dried overnight and 40 μL of scintillation fluid was added to determine the CPM value using a Top Count scintillation counter. Cytotoxicity induced by primed CD8+ T-cells from group A was found to be higher compared to the control group (Figure 3B). Specific cytotoxicity was calculated according to the following formula

\[
\text{Specific cytotoxicity (\%)} = \frac{\text{cytotoxicity of peptide pulsed EL4} - \text{cytotoxicity of unpulsed EL4 (\%)}}{\text{spontaneous cpm} - \text{experimental cpm}}
\]

Here, spontaneous cpm denotes the cpm value of EL4 cells alone and experimental cpm denotes the cpm value of EL4 + CD8+ T-cells. Stauroporine cytotoxicity was considered as 100% and % specific cytotoxicity was calculated accordingly.

**STATISTICAL ANALYSIS**

Results were calculated as mean ± SD. Statistical analysis was performed using the GraphPad Prism 5 software. Tukey’s multiple comparison test was performed to determine the statistical significance among groups (both IFN-γ production and Jam assay) (*P < 0.1, **P < 0.01) (n = S, where, n = number of mice per group).

**CONCLUSIONS**

In summary, we have demonstrated that targeting a cancer vaccine with a specific Fc isotype domain might be a promising approach in terms of enhancing vaccine immunogenicity at both the humoral and cellular level. The liposomal Fc-conjugated vaccine was better for producing antigen-specific antibodies compared to the liposomal antigen only. The vaccine also primed CD4+ T-cells that help in producing both antibody and CD8+ effector T-cells responsible for killing tumor cells. Overall, this approach appears to be somewhat better or as good as targeting anti-rhamnose antibodies to DCs.
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