Targeting pan-tumor antigens to activating Fcγ receptors generates a novel dendritic cell tumor vaccine

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Abstract

Objective: Therapeutic tumor vaccines are eagerly awaited in clinic by patients with high expectations; however, very few clinically successful tumor vaccine has been developed thus far, and there remains no consensus on the generation of tumor vaccines. We hypothesized that targeted delivery of pan-tumor antigens instead of individual tumor-associated antigen (TAA) to dendritic cells via the activating receptor endocytic pathway (AREP) would provide an alternative avenue to develop potent personalized tumor vaccines. Methods: We first prepared biotin-tagged tumor antigens (B-TAgs) with mouse CT26. WT colorectal cancer cells by exploiting metabolic glycan labeling and bioorthogonal reaction methods; then, we prepared a bifunctional fusion protein containing streptavidin and a mouse IgG2a Fc fragment (SA-Fc), in which streptavidin was used for conjugation with B-TAgs, and Fc for mediating the interaction with the Fcγ receptor. Finally, conjugates (Fc-TAgs) of SA-Fc with B-TAgs were prepared based on affinity-guided noncovalent reaction. The phenotype of Fc-TAgs pulsed bone marrow-derived dendritic cells (BMDCs) was examined by flow cytometry. The therapeutic effects of Fc-TAgs pulsed BMDCs were observed in an established mouse CT26. WT colorectal cancer model. Results: The prepared B-TAgs covers almost all glycosylated tumor antigens. SA-Fc fusion protein exhibits biotin-binding activity as a homodimer. SA-Fc can effectively conjugate with B-TAg at a mixing ratio of 1:96 (w/w). Data of flow cytometry revealed that on Fc-TAgs pulsed BMDCs, the expression levels of surface molecules, such as CD80 and MHC II, were greatly increased. In the established murine colorectal cancer model, combination treatments with Fc-TAgs pulsed BMDCs and PD-1 blockade achieved significant therapeutic effects. Limitations: The strategy we proposed for the preparation of personalized tumor vaccine requires that the tumor be surgically removed from the patient. The rationality and validity of this strategy need to be proven by more preclinical

investigations. Conclusions: The novel strategy we proposed circumvents the necessities for neoantigen prediction and provides an alternative pathway to establish a flexible system for the preparation of personalized dendritic cell tumor vaccines. In the setting of checkpoint blockade-based immunotherapy, a novel DCV would improve antitumor immunity and benefit the eradication of tumor residues within the body of the cancer patients.

Full Text

Preamble

Targeting Pan-Tumor Antigens to Activating Fcγ Receptors Generates a Novel Dendritic Cell Tumor Vaccine

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Abstract

Objective: Therapeutic tumor vaccines are eagerly awaited in the clinic, yet very few have achieved clinical success, and no consensus exists on optimal vaccine generation. We hypothesized that targeted delivery of pan-tumor antigens—rather than individual tumor-associated antigens (TAAs)—to dendritic cells via the activating receptor endocytic pathway (AREP) could provide an alternative avenue for developing potent personalized tumor vaccines.

Methods: We first prepared biotin-tagged tumor antigens (B-TAgs) from mouse CT26.WT colorectal cancer cells using metabolic glycan labeling and bioorthogonal reaction methods. Next, we prepared a bifunctional fusion protein containing streptavidin and a mouse IgG2a Fc fragment (SA-Fc), where streptavidin served to conjugate with B-TAgs and the Fc domain mediated interaction with Fcγ receptors. Finally, conjugates (Fc-TAgs) of SA-Fc with B-TAgs were prepared through affinity-guided noncovalent binding. The phenotype of bone marrow-derived dendritic cells (BMDCs) pulsed with Fc-TAgs was examined by flow cytometry, and the therapeutic effects of these pulsed BMDCs were evaluated in an established mouse CT26.WT colorectal cancer model.

Results: The prepared B-TAgs covered nearly all glycosylated tumor antigens. The SA-Fc fusion protein exhibited biotin-binding activity as a homodimer and could effectively conjugate with B-TAg at a mixing ratio of 1:96 (w/w). Flow cytometry revealed that Fc-TAg-pulsed BMDCs showed greatly increased expression of surface molecules such as CD80 and MHC II. In the established murine colorectal cancer model, combination treatment with Fc-TAg-pulsed BMDCs and PD-1 blockade achieved significant therapeutic effects.

Limitations: Our proposed strategy for preparing personalized tumor vaccines requires surgical tumor removal from patients, and its rationality and validity require further preclinical investigation.

Conclusions: Our novel strategy circumvents the need for neoantigen prediction and provides an alternative pathway for establishing a flexible system to prepare personalized dendritic cell tumor vaccines. In the context of checkpoint blockade-based immunotherapy, this novel DCV improves antitumor immunity and facilitates eradication of tumor residues in cancer patients.

Keywords: [Dendritic cell tumor vaccine]; [Fcγ receptor]; [Metabolic labeling]; [Bioorthogonal reaction]; [Colorectal cancer]

1. Introduction

For decades, dendritic cell tumor vaccines (DCVs) have represented a promising strategy for eradicating malignancies. Numerous clinical trials have demonstrated their safety, immunogenicity, and relevance to clinical outcomes, yet few clinically successful DCVs have been developed, highlighting the need to refine vaccine generation strategies and reconsider immunotherapy regimens.

It is now widely accepted that cancer is a highly heterogeneous, personalized disease, necessitating personalized DCVs. However, no consensus exists on DCV generation. Previous studies have shown encouraging results by targeting antigens to DC surface receptors. Gil M. et al. generated a recombinant protein containing an IgG2a Fc fragment and a GD2 ganglioside mimotope, delivering the antigenic cassette to activating Fcγ receptors on DCs and eliciting antitumor immune responses. Kato Y. et al. and Li J. et al. demonstrated that targeting antigen to Clec9a induced robust cellular and humoral immunity in animal models. Additionally, DEC205-targeting antibodies have successfully induced immune responses against both cancer and pathogen antigens. Although focused on individual TAAs, these findings strongly suggest that antigen targeting to DCs represents a viable vaccination strategy.

Conversely, Wendy W. J. Unger et al. showed that sialic acid-modified tumor antigens (Sia-TAgs) induce tolerance by inhibiting T-cell proliferation and promoting regulatory T cell (Treg) induction. Sialic acid on glycosylated TAgs interacts with Siglecs (sialic acid-binding Ig-type lectins) on DCs, mediating TAg uptake and conferring regulatory properties. Preventing TAg entry via the Siglec pathway would presumably enhance antitumor immunity.

Here, we aimed to deliver TAgs to DCs via the activating receptor-mediated endocytic pathway (AREP) while avoiding Siglec-mediated entry. To our knowledge, no such attempt has been made previously. Our approach leverages three key technologies: affinity-guided noncovalent streptavidin-biotin interaction, metabolic glycan labeling, and bioorthogonal click chemistry. We first prepared a recombinant SA-Fc fusion protein containing streptavidin and mouse IgG2a Fc; next, we prepared biotin-tagged TAgs (B-TAgs) with biotin at glycan termini; finally, we used SA-Fc/B-TAg conjugates (Fc-TAgs) as loading antigens for DCV preparation. In combination with PD-1 blockade, our DCV conferred significant therapeutic efficacy against murine colorectal cancer, suggesting that this strategy may be applicable for personalized DCVs, particularly for patients with surgically resectable tumors.

2. Methodology (Design/Approach)

Our objectives were to prepare a novel dendritic cell tumor vaccine by delivering tumor antigens to DCs via the activating Fc receptor endocytic pathway and to evaluate its therapeutic potency in an implanted animal tumor model.

First, we conducted key preparatory steps: (1) Using metabolic glycan labeling and click chemistry, we prepared biotin-tagged tumor antigens (B-TAgs) by tagging biotin onto sialic acid residues at the nonreducing termini of cell-surface glycans on mouse CT26.WT cells; (2) We prepared a bifunctional linker-bridged fusion protein containing streptavidin (SA) and mouse IgG2a Fc segments; (3) Since these conjugates would serve as antigen sources for DC loading, we analyzed the optimal conjugation ratio of these reagents; and (4) We examined the phenotype of bone marrow-derived dendritic cells pulsed with the conjugates, SA-Fc, and B-TAg.

Next, we established a murine CT26.WT colorectal cancer model by subcutaneously injecting tumor cells into the flank of each mouse. One week after inoculation, when tumors became measurable, tumor-bearing mice were randomized into groups (n=5 per group).

Our immunotherapy plan comprised two components: monotherapy with the DCV and combination therapy with DCV plus anti-PD-1 antibody. Control DCVs were prepared using unaltered tumor antigens. The DCV was administered subcutaneously via peritumoral injection, while anti-PD-1 antibody was delivered intraperitoneally. All groups received treatments on the same schedule, and tumor growth was monitored individually. To evaluate immunotherapy impact on antitumor immunity, we examined lymphocyte subsets in peripheral blood and spleens.

Animal numbers, statistical tests, and experimental replicates are described in figure legends. All data including outliers are presented. Researchers were not blinded during data collection or analysis.

3. Results

SA-Fc Fusion Protein Exhibited Biotin-Binding Activity as a Homodimer

To deliver TAgs to DCs via AREP, we required a complex guide that could both interact with a specific activating receptor on DCs and conjugate with pretagged tumor antigens. We selected the mouse FcγIIa receptor, a confirmed activating endocytic receptor, as the "gateway" for tumor antigen entry into DCs, and chose streptavidin for its potent biotin-binding capacity. We constructed a recombinant pFUSE plasmid encoding mouse IgG2a Fc, streptavidin, and a flexible linker between them (Fig. 1 [Figure 1: see original paper] A, upper and left, and Fig. S1), then expressed the fusion protein in a eukaryotic system. Coomassie blue staining showed the nonreduced protein' s molecular weight was nearly twice that of the reduced form, indicating homodimeric structure in its native state (Fig. 1 A, middle). Western blotting confirmed the Fc component (Fig. 1 A, right), and fluorescence microscopy demonstrated effective biotin binding (Fig. 1 B). These results confirmed the protein met our experimental requirements.

SA-Fc Effectively Conjugated with B-TAg

We next added biotin labels to the terminal ends of glycans on glycosylated TAgs using a two-step strategy (Fig. 2 [Figure 2: see original paper] A). TAgs were first labeled with azido-sugar via unnatural sugar metabolic incorporation, then azido-TAgs were further labeled with biotin via click reaction. Using Ac4ManNAz as precursor, we replaced sialic acid residues at glycan termini with azido-sialic acid. To optimize metabolic incorporation, we dynamically investigated azido-Sia expression on CT26.WT cells with DIBO-FITC under various conditions. FITC signal increased with Ac4ManNAz concentration, but cell numbers decreased at 3 mM (Fig. 2 B), suggesting high concentrations affected proliferation. Flow cytometry revealed optimal incorporation occurred at approximately 16 h (Fig. 2 C), and fluorescence microscopy confirmed azido-sugar expression (Fig. 2 D). Based on these observations, we adopted the following protocol: tumor cells were preconditioned for 24 h with 0.5 mM Ac4ManNAz, followed by 24 h culture with 2.0 mM Ac4ManNAz.

To obtain B-TAgs, we reacted thermally denatured CT26.WT cell lysates labeled with azido-sugars with DIBO-biotin. B-TAg formation was confirmed by Western blot (Fig. 2 E). We also examined representative cell surface glycoproteins in B-TAg precipitates pooled by streptavidin resins, including CCR6, E-cadherin, and IGF-1R. Detected glycoprotein amounts were nearly equal between B-TAgs and control TAgs (Fig. 2 F), indicating our methodology enabled selective biotin labeling of most glycosylated TAgs.

For Fc-TAg conjugate preparation, we investigated the optimal SA-Fc to B-TAg mixing ratio using immunoprecipitation and Western blot. A 1:96 ratio (SA-Fc:B-TAgs, w/w) proved optimal, as this maximally conjugated input B-TAgs

while leaving less than 1/8 unconjugated (Fig. 2 G). These results addressed key preparation steps for further investigations.

DCs Pulsed by Fc-TAgs Displayed a Phenotype Favoring Immune Response

Dendritic cells are the most potent antigen-presenting cells responsible for priming immune responses. Adaptive immunity requires three signals: (1) antigen peptide-MHC interaction with T cell receptors, (2) costimulatory molecule interactions, and (3) cytokine participation. By flow cytometry, we found Fc-TAg-pulsed DCs showed markedly different immune molecule expression profiles compared to unaltered TAg-pulsed DCs. Relative to unstimulated DCs, Fc-TAg-pulsed DCs showed significantly increased CD80 and MHC II expression, while CD86, CD64, CCR7, and MHC I remained unchanged. In contrast, TAg-pulsed DCs showed increased CD64 and CCR7, decreased CD86, and unchanged MHC I, MHC II, and CD80. These results indicated Fc-TAg-pulsed DCs might evoke effective antitumor responses as a vaccine.

Combination Therapy with Fc-TAg DCs and PD-1 Blockade Achieved Potent Therapeutic Effects in a Murine Colorectal Cancer Model

Given the phenotype of Fc-TAg-pulsed DCs (Fc-TAg DCs), we hypothesized these mature cells could serve as a novel vaccine to induce antitumor immunity. We inoculated CT26.WT cells into BALB/c mice to establish a tumor model. When tumors became measurable, mice were randomized into six groups: three receiving monotherapy (PBS, TAg-pulsed DCs, or Fc-TAg-pulsed DCs) and three receiving combination therapy (PBS + anti-PD-1 antibody, TAg DCs + anti-PD-1 antibody, or Fc-TAg DCs + anti-PD-1 antibody) (treatment schedule shown in Fig. 4 [Figure 4: see original paper] A). Tumor growth was monitored every three days.

Tumors in all monotherapy groups grew progressively (Fig. 4 B, upper three rows), with no significant differences in tumor volume at day 29 (Fig. 4 C, left). In the PBS + anti-PD-1 group, only one mouse showed complete regression, while four exhibited growth similar to monotherapy groups. TAg DCs + anti-PD-1 treatment also yielded complete regression in one mouse and slightly delayed growth in four others, but the expansion trend remained unchanged. Notably, Fc-TAg DCs + anti-PD-1 treatment caused complete regression in three of five mice and remarkably delayed growth in the remaining two (Fig. 4 B, lower three rows). Statistical analysis of day 29 tumor volumes showed significant differences between combination therapy groups (Fig. 4 C, right).

Combination Therapy Restored Circulating Treg Balance in Tumor-Bearing Mice

Effective antitumor immunity underlies immunotherapy success. We therefore analyzed lymphocyte subsets in peripheral blood mononuclear cells (PBMCs) and spleens. PBMC analysis showed CT26.WT colorectal cancer had no significant effect on overall CD3+CD4+ or CD3+CD8+ T cell proportions (Fig. S4 C

and D). However, CD4+CD25+ and CD4+Foxp3+ subpopulations were significantly reduced (Fig. 4 D), manifesting circulating regulatory T cell (Treg) imbalance. Fc-TAg DCs + anti-PD-1 treatment substantially prevented this Treg decline, likely correlating with tumor regression. Other treatments, whether monotherapy or combined, failed to do so. No significant lymphocyte population differences were detected in spleens between groups (Fig. S4).

4. Discussion

We have developed a novel strategy for preparing personalized DCVs for cancer immunotherapy. Compared to conventionally prepared DCVs, this approach shows greater potential for inducing antitumor immunity, particularly in combination with immune checkpoint inhibitors such as anti-PD-1 antibodies. The vaccine is straightforward to manufacture and eliminates the need for expensive, time-consuming, laborious neoantigen prediction. However, this approach requires surgical tumor removal from patients.

We selected whole tumor cells as our antigen source to encompass a broad antigen spectrum, including unknown neoantigens and damage-associated molecular patterns (DAMPs). Using metabolic incorporation and click chemistry, we prepared B-TAgs. Sialic acid capping on TAgs normally mediates DC internalization via the Siglec pathway, leading to inhibitory immune responses. Our modifications likely redirect internalization through our designed pathway, largely abolishing these inhibitory effects.

Candidate AREPs for tumor antigen delivery include Fc receptors (FcγRI/CD64 and FcγRII/CD32), integrins (αv$ $3 or αv$ 5), $Clec9A, C-typelectinreceptors(CLRssuchasmannoserecepto$ $FcthatservesbothasFc$ $ receptor ligand and B-TAg conjugate. More direct conjugation options exist, such as covalent linking of azido-TAgs to alkyne-modified Fc or other ligands, and such investigations are ongoing in our laboratory.

Our DCV' s efficacy was demonstrated in a murine colorectal cancer model. Fc-TAg DCs plus PD-1 blockade caused complete tumor regression in three of five mice and significantly delayed growth in the other two. Notably, tumor surfaces remained intact without ulceration. In contrast, monotherapy groups and most other combination therapy groups showed expansive tumor growth with central crater-like ulceration. These results suggest the novel DCV offers advantages specifically in combination with checkpoint inhibitors, but not as monotherapy.

In this model, established tumors markedly altered lymphocyte population distribution. Peripheral blood CD4+CD25+ and CD4+Foxp3+ subpopulations (generally considered Tregs) were significantly reduced compared to normal, while CD3+CD4+ and CD3+CD8+ proportions remained largely undisturbed. These data highlight Treg imbalance importance in colorectal cancer pathology. Although Treg roles in cancer remain controversial, our context clearly shows Treg loss leads to immune anergy in tumor surveillance. Understanding the

mechanism underlying this Treg decline would be valuable, though beyond our current scope.

Under PD-1 blockade combination therapy, Fc-TAg DCs successfully prevented Treg loss, while TAg DCs only partially restored Tregs. Neither TAg DCs nor PD-1 blockade alone rebalanced Tregs in tumor-bearing mice. Notably, Fc-TAg DCs alone also failed to prevent Treg decline. Mechanistically, PD-1 blockade abrogates PD-1/PD-L1-mediated immune inhibition, allowing uninhibited cells to function. PD-1 blockade alone likely failed due to lack of effector immune cells in tumor-bearing mice, possibly resulting from DC inability to prime protective antitumor responses. Monotherapy failure with TAg DCs or Fc-TAg DCs may partly reflect PD-1/PD-L1 involvement, as both DCVs showed therapeutic effects when combined with anti-PD-1, particularly Fc-TAg DCs. We therefore propose that Treg restoration (or prevention of decline) may contribute to combination immunotherapy efficacy against colorectal cancer, but only achievable in combination settings. Further mechanistic investigations are warranted.

Although preliminarily verified in a murine colorectal cancer model, our strategy' s rationality and validity require additional preclinical validation. Nevertheless, our roadmap for DC-based tumor vaccine preparation may prove useful for personalized DCV generation. Given tumor heterogeneity, personalized DCVs produced by our method may yield different immune cell molecular patterns and cytokine profiles in body fluids or the tumor microenvironment. However, in checkpoint blockade-based immunotherapy settings, this novel DCV should improve antitumor immunity and facilitate eradication of tumor residues in cancer patients.

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Figure Legends

Fig. 1. Expression and characterization of the bifunctional protein of interest. (A) The protein contains two functional domains, streptavidin and mouse IgG2a Fc fragment, bridged by a linker (top). The expression plasmid harboring the coding gene was constructed using pFUSE (left). Protein expressed in Expi293 cells was purified with Protein A/G resins. Reduced and nonreduced samples were resolved by SDS-PAGE followed by Coomassie blue staining (middle). The Fc domain was characterized by Western blot (right). (B) FITC-biotin binding to the protein of interest on Protein A/G resins was observed by fluorescence microscopy (magnification, $×$200).

Fig. 2. Preparation and characterization of Fc-TAg conjugates. (A) Schematic of the Fc-TAg conjugate preparation strategy. (B) Dynamic observation of metabolic glycan labeling on CT26.WT. Cells were cultured with Ac4ManNAz at indicated concentrations for 24 h (left) or with 3 mM Ac4ManNAz for indicated times (right). After DIBO-FITC labeling, positive cell percentages and mean fluorescence intensity (MFI) were quantified by flow cytometry. (C) CT26.WT cells on coverslips were cultured with Ac4ManNAz or sialic acid (Sia) for 24 h, then DIBO-FITC labeled and observed by fluorescence microscopy (magnification, $×400). $(D) Characterization of biotin - labeled TAg (B - TAg). Thermal - denatured lysates from azido - sugar - labeled CT26.WT were reacted with DIBO−biotin, ethanol−precipitated, and subjected to Western blotting with a pooled precipitates or untreated CT26.WT lysates were examined with respective antibodies. Inputs were assessed$ actin antibody. (F) Analysis of optimal SA-Fc to B-TAg mixing ratio. SA-Fc and B-TAgs were mixed at 1:96, 1:48, 1:24, or 1:12 ratios (w/w), and conjugates were pooled with protein A/G resins. B-TAgs in precipitates and supernatants were examined by Western blotting. SA-Fc inputs were assessed by anti-streptavidin antibody.

Fig. 3 [Figure 3: see original paper]. DCs pulsed with Fc-TAgs displayed a phenotype favoring immune response. (A) Schematic of DC isolation, activation, antigen loading, and maturation. (B) Detection and analysis of DC surface markers by flow cytometry. Aliquots from TAg-pulsed DCs, Fc-TAg-pulsed DCs, or nonpulsed DCs (normal control) were stained with respective antibodies. Flow cytometry histograms are shown in left four columns, and statistical analysis of mean fluorescence intensity (MFI) for positive cells is displayed as bar graphs in right column. Data represent three independent experiments. *, $p < 0.05$; ns, not significant.

Fig. 4. Combination immunotherapy with Fc-TAg-pulsed DCs and

PD-1 blockade achieved significant therapeutic efficacy in a murine colorectal cancer model. (A) Schematic of animal experiment timing and treatment schedules. (B) Morphology and dynamic tumor growth. Photos were taken on day 29 and arranged by tumor size. Numbers represent mouse random IDs (left columns). Tumor volumes were measured and calculated every three days. Growth curves for mice in each group are shown in right column. (C) Tumor volume comparison between monotherapy (left) and combination therapy (right) groups. Data were collected from five mice per group on day 29. Statistical analyses used one-way ANOVA with Newman-Keuls multiple comparison test. *$p < 0.05$ vs. TAg DC+anti-PD-1 group. (D) Flow cytometry analysis of peripheral blood lymphocyte subsets. Lymphocytes from mouse peripheral blood (day 50, n=4 per group) were isolated using Mouse Lymphocyte Separation Solution, stained with corresponding antibodies, and analyzed by flow cytometry. Statistical analyses used one-way ANOVA with Newman-Keuls multiple comparison test.* $p < 0.05$ vs. PBS group (CD4+CD25+ subset) or vs. TAg DC+anti-PD-1 group (CD4+Foxp3+ subset).

Supplementary Figure Legends

Fig. S1. The sequence of the gene encoding streptavidin-mIgG2aFc (SA-Fc) fusion protein. The sequence contains four domains: interleukin-2 (IL-2) signal peptide, streptavidin, linker, and mouse IgG2a Fc fragment, marked by different colors.

Fig. S2. Structures of synthetic reagents. (A) Ac4ManNAz (tetraacetylated N-Azidoacetyl-D-Mannosamine). (B) DIBO-FITC (Dibenzocyclooctynol Fluorescein Isothiocyanate). (C) DIBO-biotin (Dibenzocyclooctynol-biotin).

Fig. S3. Flow cytometry analysis of peripheral blood lymphocyte subsets. Lymphocytes from mouse peripheral blood (day 50) were isolated with Mouse Lymphocyte Separation Solution, stained with corresponding antibodies, and analyzed by flow cytometry. Left panels in A, B, C, and D show percentages of double-stained cells (n=4 per group). Right tables show one-way ANOVA with Newman-Keuls multiple comparison test results. *$p < 0.05$,* ***$p < 0.01$,*** $p < 0.001$.

Fig. S4. Flow cytometry analysis of spleen lymphocyte subsets. Spleens from mice (day 50) were processed into single-cell suspensions, and lymphocytes were isolated by Mouse Lymphocyte Separation Solution. Cells were stained with corresponding antibodies and analyzed by flow cytometry. Left panels in A, B, C, and D show percentages of double-stained cells (n=4 per group). Right tables show one-way ANOVA with Newman-Keuls multiple comparison test results. *$p < 0.05$,* ***$p < 0.01$,*** $p < 0.001$.

Figure 1. Figure 2. Figure 3. Figure 4. Supplementary Figure S1. Supplementary Figure S2. Supplementary Figure S3. Supplementary Figure S4.

Note: Figure translations are in progress. See original paper for figures.