**[New Tumor Targeting Strategies in Cancer Immunotherapy Research]**

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**Immunotherapy is a method that uses immunotherapeutic drugs instead of antitumor drugs in the treatment of cancer because antitumor drugs are typically harmful towards both the cancer cells and normal cells within a cancer patient’s body. Immunotherapeutic drugs give the immune system a boost and enable it to be able to recognize tumor cells and kill them. Since this method is relatively new, researchers are studying potential strategies that would involve targeting the cancerous tumors to investigate which strategies exhibit high synergistic therapeutic efficacy and enhance antitumor immunity. It is important that these potential treatments are able to suppress tumor growth and promote the body’s natural immune responses. Recently, a team has developed a pH-responsive hybrid biomimetic membrane-camouflaged PLGA nanoparticle system that could prove to be a promising strategy when using combination therapy. Although there have been many studies investigating tumor targeting strategies for use in immunotherapy, there are still many mechanisms at play that are not fully understood. Until these mechanisms are studied more, it will remain difficult for researchers to develop successful strategies.**

**Introduction**

Immunotherapy is a relatively new approach to cancer treatment that aims to help the patient’s immune system to recognize tumor cells and kill them without harming the surrounding normal cells. Since the field of immunotherapy is so active, it is expected that immunotherapy is going to change the standard of care towards multiple different types of cancer (4). Within the last decade, there has been a lot of success surrounding immune checkpoint inhibitors (ICIs) and immune checkpoint (ICP)-targeting drugs. Although these drugs have displayed considerable success in some studies, there are still some major concerns surrounding these drugs (2). “Suboptimal efficacy is among the major concerns because previous trials suggest that the response rate to ICI monotherapy is limited, and the responses vary significantly across multiple tumors and among individuals” (5). Another article observed that the ICIs exhibited unsurpassed clinical efficacy, but that major challenges still remained regarding the “identification of predictive biomarkers and the development of resistance during or after immunotherapy” (4). Increasing numbers of studies looking at ICP-targeting drugs have found that the positive response rate among patients receiving these drugs remains quite low. Additionally, previous studies demonstrated that in most cases, the clinical benefit is commonly prevented by acquired resistance to the tumor (3). These challenges have encouraged researchers to begin studying other tumor targeting strategies, primarily focusing on targeting the tumor immune microenvironment (TME) and targeting the tumor-associated macrophages (TAMs) (3, 4, 5). Researchers have since found increasing evidence suggesting that the “tumor immune microenvironment plays a more significant role than ICPs in tumor immune surveillance and immunological evasion” (3). Recently, there has been an increase in using cell membrane-based biomimetic nanoparticles (NPs) for therapeutic and imaging applications. In today’s study, “a breast cancer model was used to evaluate the synergistic immunotherapy efficacy of short interfering RNA (siRNA) targeting FGL1 (siFGL1) and Metformin (Met).” Metformin is a known immune-metabolic modulator that reportedly is able to both directly and indirectly exert antitumor effects, while regulating the differentiation and activity of T cells. Met has an immune-mediated anticancer mechanism that gives it the potential to improve the therapeutic outcomes of immunotherapy. The goal of this study was to observe “the potential of hybrid biomimetic membrane camouflaged nanoparticles and combined Met-FGL1 blockade in breast cancer immunotherapy” (1).

**Recent Progress**

In a recent study, the synergistic immunotherapy effects between Met and siFGL1 were tested in a breast cancer model by preparing Met and siFGL1-loaded hybrid biomimetic membrane and camouflaged nanoparticles. The researchers hypothesized that “Met and siFGL1 would exert a synergistic immunotherapy effect.” The “synergistic immunotherapy effects were further investigated using in vitro and in vivo” (1). There were so many techniques used and tests performed in this experiment that ensure that the results are accurate in every step. The experiment began by preparing and characterizing the MC-PLGA@Met-CO2/siFGL1 NPs then viewing them using transmission electron microscopy and immunogold staining to be able to confirm that the hybrid membrane had formed correctly. Agarose gel electrophoresis was performed next to assess the siRNA condensation ability of Met-CO2. In a cellular uptake assay, A flow cytometer was used to measure the cellular uptake of FAM-siFGL1 by PLGA NPs with and without the hybrid membrane coating. To ensure that the results from this test were accurate, this test was performed three times. Confocal laser scanning microscopy (CLSM) was then used to observe the distribution of FAM-siFGL1 mediated by PLGA NPs with and without the hybrid membrane coating in the cells. In an intracellular lysosome escape assay, the cells were successfully stained but the two-dimensional CLSM images were obtained and then used to produce three-dimensional CLSM images. For the in vitro release of Met, they investigated the Met release from MC-PLGA@Met-CO2/siFGL1 NPs at different pH values (pH = 7.4, 6.0, and 5.0) by using dialysis against PBS at 37°C. After dialysis, the concentration of released free Met was determined using a UV spectrophotometer at 233 nm. Cell cycle analysis was performed using a cell cycle staining kit and after the cells were stained according to the kit, flow cytometry was used to analyze the cell-cycle distribution. A cytocompatibility assessment was performed by using a cell viability assay to determine the toxicity of the blank nanomaterials without Met and SiFGL1 against 4T1 cells. These results were calculated as the means±standard deviation of at least 5 independent experiments. A tumor cell killing assay mediated by co-culture was used to determine the rate of tumor cell apoptosis. After PBS was used to remove the lymphocytes and cell debris, the cancer cells were collected and stained with an Annexin V-PI apoptosis kit and then detected with flow cytometry. “Western blotting was then used to evaluate protein expression of the AMPK signaling pathway in tumor cells influence by Met” (1). After the membranes were washed and visualized with enhanced chemiluminescence, a GE Image Quant Las 4000 mini was used to obtain images of the membranes. When it was time to investigate the in vivo antitumor effect of MC-PLGA@Met-Co2/siFGL1 NPs, cancer cells were injected subcutaneously into the right breast of mice. The mice were then randomly divided into six groups, based on what drugs they would receive. The drugs were injected into the tail vein every four days, four consecutive times. The team also regularly monitored tumor volumes and animal body weights. After tumor specimens were cut into small pieces and red blood cell lysis had occurred, the resulting cell suspension was stained with three different antibodies and flow cytometry was used to analyze the T cells. An enzyme-linked immunosorbent assay (ELISA) was used to analyze the levels of tumor-associated IFN- γ and interleukin-2 (IL-2) in tumor samples obtained from the mice after treatment. Immunofluorescence was used to assess the hypoxic area of the tumor tissues from each of the 6 groups of mice. After staining, the sections were observed under CLSM and all images were acquired using LSM soiware. At the end of treatment, the major organs and tumor tissues were collected from the mice and a histological examination was performed using H&E staining. “Statistical analysis tells us the data is expressed as the mean±standard deviation (SD). Analysis of variance (ANOVA) was used to evaluate the differences among the six groups, and a p-value of <0.05 was considered to be statistically significant” (1). Overall, this experiment technically consisted of about 13 smaller experiments that were performed in order to ensure that each step taken was performed correctly. By breaking this experiment down into 13 sections, it is easier to make sure that the experiment is carried out correctly and if someone were to copy their methods, they could use the results from each section to ensure they are performing each step correctly. The results of the synthesis and characterization of MC-PLGA@Met-CO2/siFGL1 NPs found that the hybrid membrane-coated NPs were stable at a pH of 7.4. They also found that the size of hybrid membrane-coated NPs synthesized using Met without CO2 was not significantly affected at a pH of 6.0 or 5.0, but when Met-CO2 was used their size increased to 225.7±10.2 nm and deformation was observed on some of the hybrid membrane-coated NPs at pH 6.0. When the pH was lowered to 5.0, the size of the NPs increased to 340.9±19.5 and defects were observed on all of the hybrid membrane-coated NPs. The results from the agarose gel electrophoresis showed that “the migration of siFGL1 into the gel was almost completely inhibited by nanoparticle encapsulation with negligible release at pH 7.4” (1). They also observed that at pH 6.0 there was a smaller electrophoresis band, indicating a slower release of the siRNA, and at pH 5.0 a strong electrophoresis band was observed for MC-PLGA@Met-CO2/siFGL1 NPs. These findings show that the low-pH-triggered nano-bomb effect of the MC-PLGA@Met-CO2/siFGL1 NPs could result in a pH-sensitive release of the encapsulated siFGL1. This test “concluded that the MC membranes protect the siRNA from RNase A degradation in the negatively charged PLGA NPs and the guanidine group of Met could condense and protect siRNA” (1). The cellular uptake assay results showed that in free FAM-siFGL1 there was hardly any cell uptake which “indicates that FAM-siFGL1 cannot be taken up by cells unless it is loaded using the hybrid membrane-coated NPs or PLGA NPs (1).” There was barely any green fluorescence observed for free FAM-siFGL1, but MC-PLGA@Met-CO2/FAM-siFGL1 NPs showed stronger green fluorescence than PLGA@Met-CO2/FAM-siFGL1. This finding reflected the results from flow cytometry. The results from the intracellular lysosome escape assay found that CO2 facilitates the escape of FAM-siFGL1 from the lysosomes. The results of the in vitro release profiles of Met from the hybrid membrane-coated NPs found that “in the first 6 hours, nearly 37% and 48% of Met was released at pH 5.0 and 6.0 and MC-PLGA@Met-CO2/siFGL1 NPs exhibited a slow release of 3% within 6 hours at pH 7.4. These results indicate that the hybrid membrane-coated NPs are more stable under physiological conditions” (1). Flow cytometry found that “Met formulations on cell cycle arrest in 4T1 cells cause a non-obvious upsurge in the accumulation of G1 phase cells among 4T1 cells with MC-PLGA@Met-CO2/siFGL1 NPs which means that siFGL1 had no obvious cell cycle arresting effect on 4T1 cells” (1). The results from the cytocompatibility assessment showed that there was a good biocompatibility of the NPs because the cells still had a viability above 85% after a 24-hour and 48-hour incubation. The tumor cell killing assay mediated by co-culture found that “the delivery of Met-CO2 NPs and siFGL1 mediated by hybrid membrane-coated NPs had a synergistic antitumor immune killing effect, causing a higher level of apoptosis” (1). The western blot analysis “demonstrated that MC-PLGA@Met-CO2/siFGL1 NPs could suppress the expression of FGL1 in 4T1 cells as the concentration of siFGL1 continued to rise to 100 nM” (1). The results of the antitumor effect in vivo showed that “the overall inhibitory rate by tumor volume was 2.66%, 56.1%, and 97.3% in the siFGL1-treated, MC-PLGA@siFGL1 NP-treated, and MC-PLGA@Met-CO2/siFGL1 NP-treated groups. This illustrates the strong synergism due to the MC hybrid biomimetic membrane-camouflaged PLGA NP-mediated delivery. These in vivo results were consistent with the in vitro results” (1). It was also found that neither group experienced a significant loss of body weight which indicates that the systemic toxicity of this therapeutic regimen is low. Most importantly, this study found that “treatment with MC-PLGA@Met-CO2 NPs and MC-PLGA@siFGL1 NPs showed slightly improved survival rates, whereas co-loaded MC-PLGA@Met-CO2/siFGL1 NPs enhanced the survival period by nearly 40 days” (1). The results from flow cytometry analysis and ELISA “collectively indicated the synergistic immunotherapy efficacy of siFGL1 and Met.” This is because they found that “MC-PLGA@Met-CO2/si FGL1 NPs increased the ratio of CD8+ T cells to CD4+ T cells compared to MC-PLGA@Met-CO2 NPs and MC-PLGA@siFGL1 NPs.” It was also observed that “the cytokine levels were significantly higher in MC-PLGA@Met-CO2/siFGL1 NPs groups than in MC-PLGA@Met-CO2 NPs and MC-PLGA@siFGL1 NPs groups.” Their final finding from this test was that “the MC-PLGA@Met-CO2/siFGL1 NP-treated groups showed decreased expression of Ki-67 in tumor tissues, indicating a remarkable decrease in tumor cell proliferation” (1). The results from the immunofluorescence evaluation found that “compared to that in the MC-PLGA@Met-CO2 NP-treated and MC-PLGA@siFGL1 NP-treated groups, the percentage of intratumoral CD8+ T cells was significantly upregulated relative to the total number of cells in tumors in the MC-PLGA@Met-CO2/siFGL1 NP-treated group. These results are consistent with the results from the flow cytometry analysis” (1). Finally, the results from the histologic analysis of the main organs showed that no groups experienced any obvious abnormalities, “indicating that the systemic toxicity of this therapeutic regimen is low” (1).

**Discussion**

These researchers were able to successfully create a hybrid biomimetic system that exhibited a synergistic immunotherapeutic effect both in vitro and in vivo. This system was able to improve the tumor immunosuppressive microenvironment in vivo “by effectively alleviating tumor hypoxia and inducing M1-type differentiation of tumor-related macrophages” (1). The experiments mentioned in this paper were performed very carefully so nothing could interfere with the results. The results obtained by this group for these experiments were typically consistent with the findings of similar studies and they turned out to be almost exactly like the team had hypothesized. For these reasons, we know that the results obtained in this experiment are valid and that we can trust that this new hybrid membrane could work for treating cancer. As a whole, their results indicated that “MC-PLGA@Met-CO2/siFGL1 NPs have the potential to become a promising strategy to optimize cancer immunotherapy using combination therapy with multiple ICIs” (1). This finding is significant because this new method of combination therapy has shown a lot of potential as a new immunotherapeutic treatment. Since there are so many different kinds of cancer and different stages of severity of the disease, it is important that we find new medicines that can be used for treatment. As we continue to develop more drugs, it is becoming easier for us to treat certain types of cancer so our survival rates are increasing. For instance, if a patient had been diagnosed with breast cancer in 2010 their prognosis may have looked grim, but if a patient was diagnosed with breast cancer today there are so many more treatments we have that have proven to be effective in treating breast cancer. Most of the treatments used for breast cancer are immunotherapy based that came from research like this. Unfortunately, advancements like this have not been made for all kinds of cancer and that is why we have to continue researching these new tumor targeting strategies. In the future, further research experiments surrounding targeting the tumor microenvironment need to be conducted so that we can understand the mechanisms behind it better and use that knowledge to create more treatments to be used in cancer immunotherapy.

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