Original Article

Delivery of gefitinib with an immunostimulatory nanocarrier improves therapeutic efficacy in lung cancer

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Background: Combining different cancer treatments represents a promising strategy to improve the therapeutic outcome for lung cancer patients with or without druggable gene alterations.

Methods: We previously developed a polyethylene glycol-based (PEG-based) immunostimulatory nanocarrier (PEG_{2k}-Fmoc-NLG919) which can efficiently co-deliver an indoleamine 2,3-dioxygenase-1 (IDO1) inhibitor and the chemotherapeutic agent, paclitaxel. This method was found to improve cancer therapy by simultaneously performing immuno- and chemo-therapy. However, whether this nanocarrier could deliver targeted drugs to implement targeted therapy together with immunotherapy remains unclear.

Results: Here, we report that the delivery of the classical tyrosine kinase inhibitor (TKI), gefitinib, with the optimized PEG_{5k}-Fmoc-NLG919 nanocarrier, increased the sensitivity of lung cancer cells to gefitinib *in vitro*. Gefitinib was gradually but sufficiently released from the nanocarrier with comparable capacity to inhibit epidermal growth factor receptor (EGFR) activity as using free gefitinib directly. More importantly, treatment with gefitinib-loaded PEG_{5k}-Fmoc-NLG919 could suppress lung tumor development more efficiently than gefitinib alone *in vivo* by inducing an immune active microenvironment with more functional $CD8^+$ T cells and less regulatory T cell infiltration.

Conclusions: Our study therefore demonstrates that delivery of small molecular targeted drugs with the immunostimulatory nanocarrier is a straightforward strategy for improving antitumor response for lung cancer therapy.

Keywords: Gefitinib; nanocarrier; immunostimulatory; lung cancer

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Introduction

Although chemotherapy, targeted therapy, and immunotherapy have improved cancer patients' prognosis in last few decades, lung cancer remains the leading cause of cancer-related death with the highest incidence among all cancer subtypes worldwide, with estimated 2,093,000 new and 1,761,000 death cases in 2018 (1). The classical platinum-based chemotherapy has been used for treating lung cancer patients for over 40 years, but the high toxicity, drug resistance, and limited therapeutic effect involved have hampered this approach as the primary selection for clinicians (2,3). Targeted therapy predominantly using tyrosine kinase inhibitors (TKIs) has been proven to significantly improve lung cancer patients' prognosis, but is limited to only those patients who carry genetic mutations such as epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK). According to a research enrolled 1,770 patients with non-small cell lung cancer, there were 50.3% patients harbor EGFR mutation and only 4.3% patients carry ALK fusion (4). Even worse, drug resistance is usually quick to establish in these patients, and other treatments are inevitably required (5,6). The newly emergent immunotherapy with immune checkpoint inhibitors, such as monoclonal antibody of programmed cell death protein 1 (PD-1), programmed cell death protein ligand 1 (PD-L1), and cytotoxic T lymphocyte-associated protein 4 (CTLA-4), can efficiently stimulate antitumor efficacy and thereby provide dramatic survival advantages for lung cancer patients (7,8). The latest results from the randomized, phase III trials Checkmate 017 and 057 showed a 5-year overall survival rate of 13.4% versus 2.6% for advanced non-small cell lung cancer patients who treated with nivolumab or docetaxel, respectively (9). However, immunotherapy is only effective in a small subpopulation of patients, and even these responsive patients may suffer severe side effects and subsequent cancer progression (10,11). Therefore, the development of novel treatment methods and combination strategies for lung cancer patients is urgently needed.

Recently, accumulating evidence has suggested that combination of different therapies is a promising strategy for improving lung cancer patient prognosis. For instance, chemotherapy has been found to trigger antitumor immune responses (12) and enhance tumor cell susceptibility to cytotoxic T lymphocyte (CTL)-mediated killing during cancer immunotherapy, which then substantially increases the response to immunotherapy (13). Ipilimumab, one of the antibodies that specifically blocks binding between CTLA-4 and its ligands (CD80/CD86) and subsequently augments T-cell activation and proliferation, has been shown to be recruited in many clinical immunochemotherapy treatment studies (14-16). Furthermore, TKI combined with pemetrexed and carboplatin-based chemotherapy was demonstrated to significantly increase EGFR-mutated Japanese lung cancer patients' overall survival as compared with targeted therapy using TKIs alone (17). However, no clear and solid clinical evidence has shown that combined targeted therapy with immunotherapy can bring substantive benefits for lung cancer patients so far, although some notable clinical trials have been launched to investigate this issue, including LUX-Lung IO, CheckMate 012, CheckMate 370, KEYNOTE-021 (18,19).

Previously, we developed a polyethylene glycol-based (PEG-based) immunostimulatory nanocarrier (PEG_{2k}-Fmoc-NLG919) for co-delivery of an indoleamine 2,3-dioxygenase-1 (IDO1) inhibitor (NLG919) and a chemotherapeutic agent, paclitaxel (PTX) (20). IDO1 also plays a vital role in establishing the immunosuppressive tumor microenvironment, however, the IDO1 inhibitor was poorly soluble under physiological conditions, while PEG_{2k} was used to dramatically increase the delivery efficiency. The Fmoc group was introduced to the carrier to increase the drug-loading capacity and formulation stability (21), and the nanocarrier PEG_{2k} was improved to PEG_{5k} for better delivery efficiency (22). The systemic delivery of PTX using this nanocarrier could induce significantly increased antitumor response in breast cancer and melanoma murine models (20); however, whether the delivery of TKIs using the PEG_{5k}-Fmoc-NLG919 can provoke synergistic antitumor effect remains unclear. In the present study, we aimed to explore whether the delivery of gefitinib using PEG_{5k}-Fmoc-NLG919 and gefitinib could provide improved prognosis for lung cancer with both in and ex vivo lung cancer models.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/tlcr-21-144).

Methods

Chemical materials

Gefitinib was purchased from LC labs (MA, USA), while RPMI 1640 medium, fetal bovine serum (FBS), and penicillin-streptomycin solution (100×) were all purchased

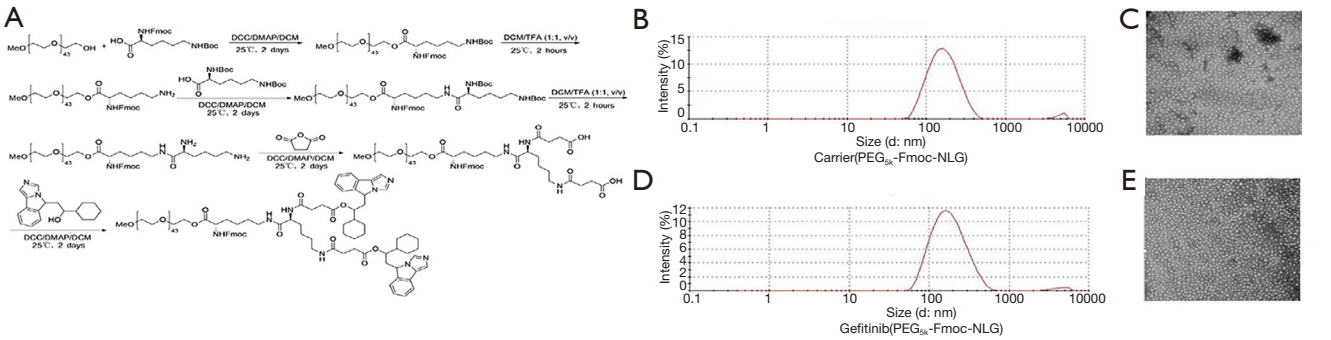

Figure 1 *In vitro* synthesis and characterizations of gefitinib/PEG_{5k}-Fmoc-NLG919 micelles. (A) Synthesis scheme of gefitinib/PEG_{5k}-Fmoc-NLG919. (B) Size distribution of drug-free PEG_{5k}-Fmoc-NLG919 micelles were examined by dynamic light. (C) Morphology of drug-free PEG_{5k}-Fmoc-NLG919 micelles were examined by TEM. (D) Size distributions of gefitinib-loaded PEG_{5k}-Fmoc-NLG919 micelles (carrier: drug, 2.5:1, m/m) were examined by dynamic light scattering. (E) Morphologies of gefitinib-loaded PEG_{5k}-Fmoc-NLG919 micelles (carrier:drug, 2.5:1, m/m) was examined by TEM. TEM, transmission electron microscope.

from Invitrogen (NY, USA). Monomethoxy PEG_{5K} and 4-dimethylaminopyridine (DMAP) were obtained from Sigma Aldrich (Germany).

Animals

C57BL/6 mice (6–8 weeks) were purchased from Charles River (CA). All animals were housed under pathogen-free conditions according to Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines. Experiments were performed under a project license (NO.: NFEC-2020-094) granted by institutional ethics board of Nanfang Hospital, Southern Medical University, in compliance with Chinese national or institutional guidelines for the care and use of animals.

Cell culture

Lewis lung carcinoma (3LL) murine lung cancer cells and A549 human lung cancer cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37 ℃ in a humidified environment with 5% CO_2. All cell lines used in this work were obtained from American Type Culture Collection (ATCC, VA, USA).

Synthesis of PEG_{5k}-Fmoc-NLG919 conjugate

PEG_{5k}-Fmoc-NLG919 conjugate was synthesized and purified following a published protocol and is outlined in *Figure 1* (22).

Preparation of gefitinib/PEG_{5k}-Fmoc-NLG919 micelles

Gefitinib-loaded PEG_{5k}-Fmoc-NLG919 micelles were prepared via a film-hydration method. Gefitinib [2.5 mmol in dehydrated culture media (DCM)] and PEG_{5k}-Fmoc-NLG919 (10 mmol) were mixed at different ratios in glass tubes. Saline was added after removing the organic solvent. Other micelles were similarly prepared. The particle size of these micelles was measured by dynamic light scattering (DLS), and the morphologies were measured by transmission electron microscope (TEM).

Gefitinib release assay

The kinetics of gefitinib *in vitro* release from gefitinib/PEG_{5k}-Fmoc-NLG2 was studied using a dialysis method. Briefly, 1 mL of gefitinib/PEG_{5k}-Fmoc-NLG2 micelles containing 1 mg of gefitinib were placed in a clamped dialysis bag (MWCO 3.5 kDa) and immersed in 25 mL of phosphate-buffered saline (PBS) solution containing 0.5% (w/v) Tween. The experiment was performed in an incubation shaker at 37 ℃ at 100 rpm. At selected time intervals, both 10 μL of gefitinib/PEG_{5k}-Fmoc-NLG2 micelle solution in the dialysis bag and 1 mL of medium outside the dialysis bag were withdrawn while the same amount of fresh medium was added for replenishment. For comparison, free gefitinib dissolved in 2% dimethyl sulfoxide (DMSO) was included as free diffusion control. The gefitinib released from micelles was also measured by Waters e2695 HPLC system equipped with a Waters 2489 UV detector (MA, USA). Then, 100 mM of ammonium

acetate (pH =5) and acetonitrile (60:40, v/v) was used for the mobile phase. Gefitinib was detected at a 248 nm wavelength.

In vitro MTT assay

A549 and 3LL lung cancer cell lines (5×10^3 cells/well) were seeded in 96-well plates and incubated for 24 hours. Cells were then treated with carrier alone, gefitinib, or gefitinib/PEG_{5k}-Fmoc-NLG919 in different concentrations. The cell viabilities were determined by MTT assay.

Western blotting

A549 or 3LL lung cancer cell lines (7×10^3 cells/well) were seeded in 6-well plates and incubated for 24 hours. Cells were then treated with blank medium, carrier alone, gefitinib, or gefitinib/PEG_{5k}-Fmoc-NLG919 for 48 hours. Cells and the tumors after homogenization were incubated with radioimmunoprecipitation assay buffer (RIPA) in a 4 ℃ room for 1 hour. These samples were centrifuged at 12,500 rpm for 10 minutes, and the precipitation was discarded. Loading buffer with one-fourth of the supernatant volume was added, and the samples were heated at 95 ℃ for 5 minutes. The expression of EGFR and p-EGFR in these total protein samples were evaluated by western blotting. The EGFR [4267] and p-EGFR antibody [2235] used were acquired from Cell Signal Technology, Inc.

In vivo antitumor activity

To investigate antitumor activity of gefitinib-loaded PEG_{5k}-Fmoc-NLG919 micelles, C57BL/6 mice were inoculated with 3LL tumor cells on the right flank area, and randomly divided into four groups (n=4): control, blank carrier, free gefitinib, and gefitinib/PEG_{5k}-Fmoc-NLG919 (with a molar ratio of 1:10). Mice were treated with various drugs after the tumor volume reached about 50 mm^3, once every 3 days 5 times through tail vein injection (dosage of gefitinib: 10 mg/kg). The tumor volume and body weighs of mice were monitored. Tumor sizes were measured with the digital caliper every 3 days following the initiation of the treatment and calculated by the following formula: $(L \times W^2)/2$, where L is the longest diameter and W is the shortest diameter of tumor (mm). At the end of the experiment, tumors were collected for hematoxylin and eosin (HE) and immunohistochemical (anti-Ki67) staining analysis.

Quantification of tumor-infiltrating lymphocytes

Tumor-bearing mice were intravenously administered with various agents once every 3 days 3 times. Tumor tissues were harvested at 24 hours after the final treatment, and then the single cell suspension was collected and stained with various antibodies (CD8, CD4, granzyme B, IFN-γ, and PD-1) for fluorescence-activated cell sorting (FACS) evaluation.

The immune cell populations in the tumor tissues were analyzed by flow cytometry. Cell suspensions from tumor tissues were filtered, and red blood cells were lysed. For extracellular staining, cells were incubated with the indicated combinations of antibodies (CD8, CD4, and CD45). For intracellular staining, cells were fixed and permeabilized immediately after cell surface staining according to the manufacturer's instructions. Briefly, fixation buffer was diluted 4 times, and incubated with cell for 20mins. Then, combinations of antibodies (FoxP3, IFN-γ, and granzyme B) were added to cells in permeabilization buffer. For IFN-γ and granzyme B staining, cells were stimulated with phosphomolybdic acid (PMA, 5 ng/mL) and ionomycin (500 ng/mL) in the presence of 10 μg/mL brefeldin A (BFA) for 4 hours followed by extracellular and intracellular staining. All antibodies were purchased from BD Biosciences, and data were collected on an LSRFortessa (BD Biosciences). The data were analyzed by FlowJo software.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Differences between groups were assessed using ANOVA, and a P value <0.05 was considered statistically significant.

Results

Characterization of blank and gefitinib/PEG_{5k}-Fmoc-NLG919 micelles

The blank and gefitinib-loaded PEG_{5k}-Fmoc-NLG919 micelles were prepared using a film hydration method (*Figure 1A*). The critical micelle concentration (CMC), particle size, and morphologies of PEG_{5k}-Fmoc-NLG919 micelles were measured according to a previously described method (20). The average size of PEG_{5k}-Fmoc-NLG919 and gefitinib/PEG_{5k}-Fmoc-NLG919 as revealed by the DLS had a major peak of 175.5 and 191.8 nm, respectively (*Figure 1B,C*). The morphology of micelles with and

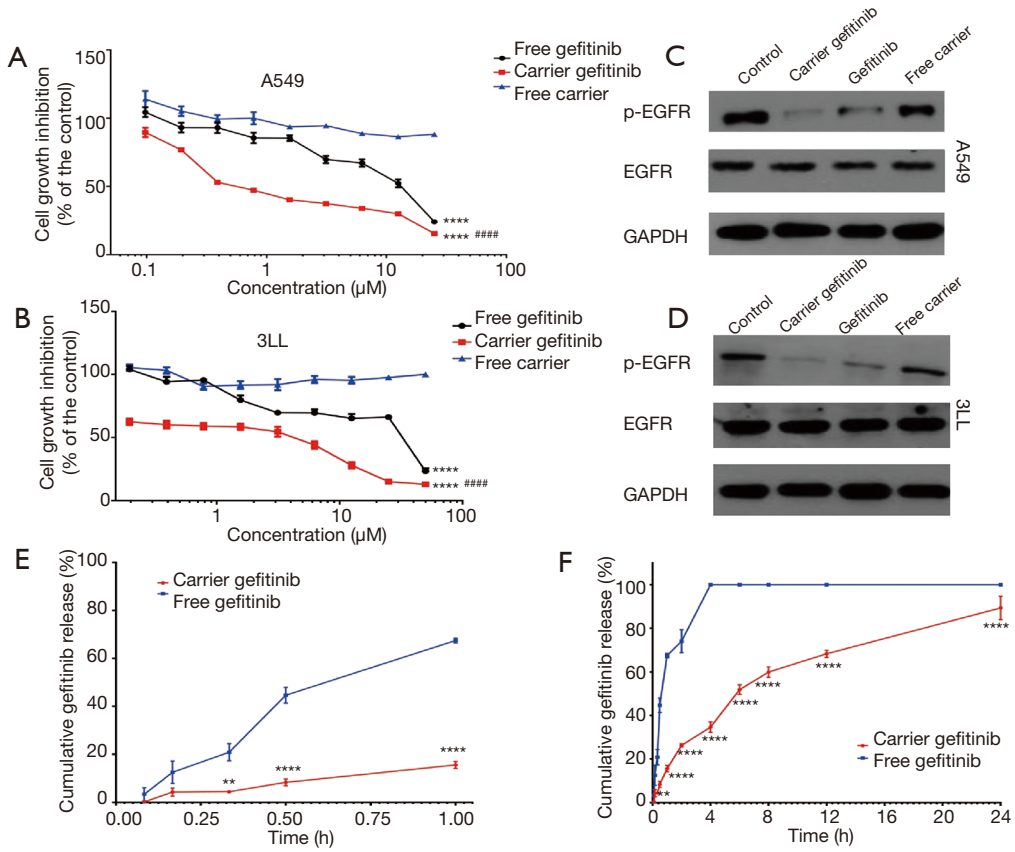

Figure 2 *In vitro* evaluation of anti-lung cancer efficacy of gefitinib-loaded PEG_{5k}-Fmoc-NLG919. (A) Cytotoxicity analysis PEG_{5k}-Fmoc-NLG919 (carrier) alone, free gefitinib, and gefitinib/PEG_{5k}-Fmoc-NLG919 (carrier gefitinib) against A549 human lung cancer cell line. ****, P<0.0001 (free gefitinib, carrier gefitinib *vs.* free carrier group); ####, P<0.0001 (carrier gefitinib *vs.* gefitinib group). (B) Cytotoxicity evaluation of PEG_{5k}-Fmoc-NLG919 alone, free gefitinib, and gefitinib/PEG_{5k}-Fmoc-NLG919 in 3LL mouse lung cancer cell line (3LL). ****, P<0.0001 (free gefitinib *vs.* carrier gefitinib *vs.* free carrier); ####, P<0.0001 (carrier gefitinib *vs.* free gefitinib). (C) Immunoblot assay of EGFR activity in A549 cells treated with PEG_{5k}-Fmoc-NLG919 alone, free gefitinib, and gefitinib/PEG_{5k}-Fmoc-NLG919, with GAPDH used as the loading control. (D) Western blot analysis of EGFR activity in 3LL cells treated with PEG_{5k}-Fmoc-NLG919 alone, free gefitinib, and gefitinib/PEG_{5k}-Fmoc-NLG919. (E) The 1-hour drug releasing assay of gefitinib/PEG_{5k}-Fmoc-NLG919, with free gefitinib used as the control. **, P<0.01; ****, P<0.0001. (F) The 24-hour drug releasing assay of gefitinib/PEG_{5k}-Fmoc-NLG919, with free gefitinib used as the control. **, P<0.01; ****, P<0.0001.

without gefitinib-loaded PEG_{5k}-Fmoc-NLG919 analyzed by TEM showed that both the carrier and gefitinib/PEG_{5k}-Fmoc-NLG919 produced an even-size spherical morphology (*Figure 1D,E*).

In vitro cytotoxicity evaluation of gefitinib/PEG_{5k}-Fmoc-NLG919 micelles

The cytotoxicity of blank carrier and gefitinib-loaded micelles was examined by the MTT assay. The carrier alone showed very little cytotoxicity in both A549 and 3LL cell lines (*Figure 2A,B*). The IC_{50} of gefitinib/PEG_{5k}-Fmoc-NLG919 and free gefitinib were 1.107 and 10.53 μM in the A549 cell line, respectively (*Figure 2A*); similarly, the IC_{50} value of gefitinib/PEG_{5k}-Fmoc-NLG919 was 1.826 μM, as compared to that of 23.67 μM of gefitinib alone in murine lung cancer 3LL cells (*Figure 2B*); this suggested a significant increase of the killing effect in lung cancer cells for gefitinib when delivered with PEG_{5k}-Fmoc-NLG919. Furthermore, we compared the EGFR activity in both A549 and 3LL cells which were treated with carrier, gefitinib, and gefitinib/PEG_{5k}-Fmoc-NLG919. In line with

the cytotoxicity assay, when delivered with PEG_{5k}-Fmoc-NLG919, gefitinib showed better efficiency in suppressing EGFR activity (*Figure 2C,D*). These data collectively indicated that the use of PEG_{5k}-Fmoc-NLG919 micelles was a powerful system to wrap and deliver small molecular drugs, such as gefitinib.

Gefitinib was gradually but sufficiently released from the gefitinib/PEG_{5k}-Fmoc-NLG919 micelles

To evaluate how gefitinib is released from gefitinib/PEG_{5k}-Fmoc-NLG919, we performed a dialysis assay to determine the kinetics of gefitinib's release in a time-dependent fashion. A 1-hour short-period experiment indicated that roughly 20% of the gefitinib could be gradually released from the gefitinib/PEG_{5k}-Fmoc-NLG919, which was dramatically slower than the free-gefitinib release (*Figure 2E*). Similarly, in a 24-hour period assay, we also observed that gefitinib could be slowly unloaded from the micelles as compared to free gefitinib, but about 90% of the gefitinib was released from the micelles after 24 hours, suggesting that PEG_{5k}-Fmoc-NLG919 was a robust vector to satisfactorily release the small molecular drugs (*Figure 2F*).

Gefitinib/PEG_{5k}-Fmoc-NLG919 micelles show improved antitumor effect over gefitinib alone

In order to evaluate the *in vivo* antitumor effect of carrier loaded gefitinib, we established a mouse lung cancer xenograft model using 3LL cells. Mice bearing 3LL tumors received different treatments via tail vein injection once every 3 days for a total of 3 times. Compared to the control group mice which received saline injection, mice treated with PEG_{5k}-Fmoc-NLG919 did not show any antitumor effects while the mice treated with gefitinib alone only showed mild effects in delaying tumor progression; however, when mice were treated with the gefitinib/PEG_{5k}-Fmoc-NLG919 micelles, the tumor development was significantly inhibited as revealed by the tumor growth curve (*Figure 3A*). In line with this, tumor size evaluation suggested that only gefitinib/PEG_{5k}-Fmoc-NLG919 treatment could dramatically suppress the tumor formation as compared to all other treatments (*Figure 3B*). We then analyzed the EGFR activity in the tumors isolated from each group, and, not surprisingly, gefitinib treatment could efficiently block EGFR activity, while a comparable inhibition effect could be achieved by gefitinib/PEG_{5k}-Fmoc-NLG919 treatment (*Figure 3C*), further supporting PEG_{5k}-Fmoc-NLG919 micelles as an efficient system to deliver gefitinib both *ex vivo* and *in vivo*. Moreover, histology examination indicated that tumors treated with gefitinib/PEG_{5k}-Fmoc-NLG919 consistently exhibited markedly increased apoptotic figures or necrotic areas (*Figure 3D*), while proliferation of lung tumors as detected by Ki67 staining also suggested that gefitinib/PEG_{5k}-Fmoc-NLG919 could substantially suppress lung tumor cell proliferation (*Figure 3E*).

Gefitinib/PEG_{5k}-Fmoc-NLG919 micelles reshaped immune cell compositions in the tumor microenvironment

Since the PEG_{5k}-Fmoc-NLG919 nanocarrier harbors an IDO1 inhibitor (NLG919), we then examined whether gefitinib/PEG_{5k}-Fmoc-NLG919 micelles could affect the immune cell infiltration into the tumors. Tumor tissues from mice with different treatments were collected and digested for immune cell population assay using flow cytometry. Intriguingly, treatment with gefitinib/PEG_{5k}-Fmoc-NLG919 resulted in the highest infiltration of functional granzyme B-positive $CD8^+$ T cells in the tumor microenvironment (*Figure 4A,B*); in contrast, the infiltration of regulatory T cells (Treg) which were identified as $CD4^+$ and $FoxP3^+$ were significantly decreased in the gefitinib/PEG_{5k}-Fmoc-NLG919-treated tumors (*Figure 4C,D*), suggesting that gefitinib/PEG_{5k}-Fmoc-NLG919 could not only inhibit EGFR activity as free gefitinib *in vivo*, but could also synergistically stimulate the local antitumor immune response via inhibiting IDO activity.

Discussion

Increasing evidence has shown that combining different cancer therapies is a promising strategy to improve lung cancer patients' prognosis (23-26). For instance, it has been previously reported that TKI could augment the expression of MHC classes I and II genes and thus promote the antigen presentation (27); besides, TKI could enhance the production of interferon-γ and inhibit the apoptosis of T cells in the tumor microenvironment (12), indicating combination of targeted and immunotherapy might improve therapeutic efficacy for lung cancer patients, however, it is still unclear whether such combination strategy could induce severe adverse effect. Our previously developed PEG-NLG-based immunostimulatory nanocarrier (PEG_{2k}-Fmoc-NLG919) for simultaneously delivering IDO inhibitor

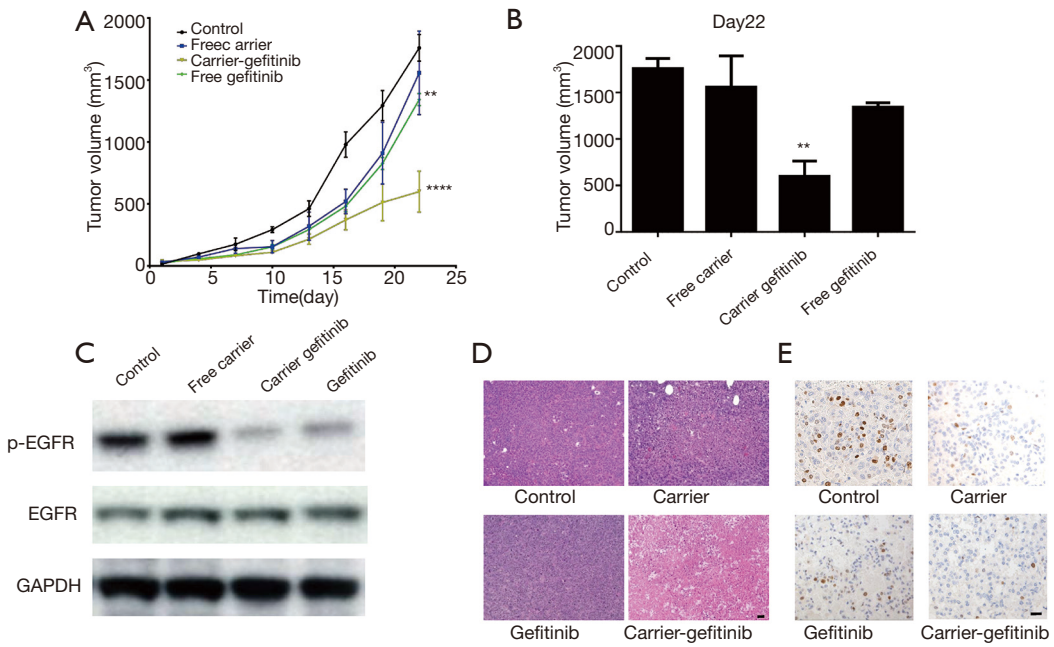

Figure 3 *In vivo* anti-lung cancer assay of gefitinib/PEG_{5k}-Fmoc-NLG919. (A) Tumor growth curve of tumor-bearing mice treated with PBS, PEG_{5k}-Fmoc-NLG919 alone, free gefitinib, and gefitinib/PEG_{5k}-Fmoc-NLG919. **, P<0.01 (free gefitinib vs, control); ****, P<0.0001 (carrier-gefitinib *vs.* control). (B) Tumor volume evaluation of tumor-bearing mice on the day 22 after treating with PBS, PEG_{5k}-Fmoc-NLG919 alone, free gefitinib, and gefitinib/PEG_{5k}-Fmoc-NLG919. **, P<0.01 (carrier-gefitinib *vs.* free gefitinib). (C) Immunoblot assay of EGFR activity in the tumor tissues isolated from the mice treated with PEG_{5k}-Fmoc-NLG919 alone, free gefitinib, and gefitinib/PEG_{5k}-Fmoc-NLG919. (D) Histological analysis (hematoxylin and eosin staining) of lung tumors collected from mice treated with PBS, PEG_{5k}-Fmoc-NLG919 alone, free gefitinib, and gefitinib/PEG_{5k}-Fmoc-NLG919; scale bars: 100 μm. (E) Proliferation assay (Ki67 staining) of lung tumors isolated from mice treated with PBS, PEG_{5k}-Fmoc-NLG919 alone, free gefitinib, and gefitinib/PEG_{5k}-Fmoc-NLG919; scale bars: 50 μm.

(NLG919) and a chemotherapeutic agent, PTX, has shown great advantage in stimulating antitumor response in breast cancer and melanoma murine models (20). Unlike many other drug carriers which are "inert", the PEG-Fmoc-NLG is a pro-drug that exhibits immunostimulatory activity by suppressing IDO activity. This property can induce additional tumor-suppressing effects by not only targeting the tumor cells directly, but also by modulating local immune response in the tumor microenvironment. Indeed, as indicated by the immunological analysis, there were increased functional $CD4^+$ and $CD8^+$ T cells, but decreased Treg and myeloid-derived suppressor cells infiltrated in the tumor tissues of mice treated with PEG_{2k}-Fmoc-NLG919, compared with vehicle treated mice (20,22).

Some studies have investigated the efficacy of combination of anti PD-1/PD-L1-based immunotherapy and targeted therapy in clinic; unfortunately, according to recent reports, the efficacy of such strategies is limited by severe adverse effects (19,28,29). Thus, whether combining targeted and immune therapy is still a feasible method to achieve better antitumor performance needs to be urgently resolved. Importantly, it should be noted that IDO, in addition to PD-1/PD-L1/CTLA-4, plays a vital role in establishing the immunosuppressive microenvironment (30-32). To our knowledge, there are no ongoing clinical studies that are aimed at exploring the potential therapeutic effect of using IDO inhibitor in addition to small molecular targeted drugs.

In our study, we used the optimized PEG_{5k}-Fmoc-NLG919 micelles as the carrier for loading the classical EGFR inhibitor, gefitinib to treat lung cancer. Surprisingly, the IC_{50} evaluation showed that gefitinib/PEG_{5k}-Fmoc-NLG919 could kill lung tumor cells more efficiently than free gefitinib; furthermore, it was observed that the EGFR activity in gefitinib/PEG_{5k}-Fmoc-NLG919-treated cells was lower than that of free gefitinib–treated cells. This could be

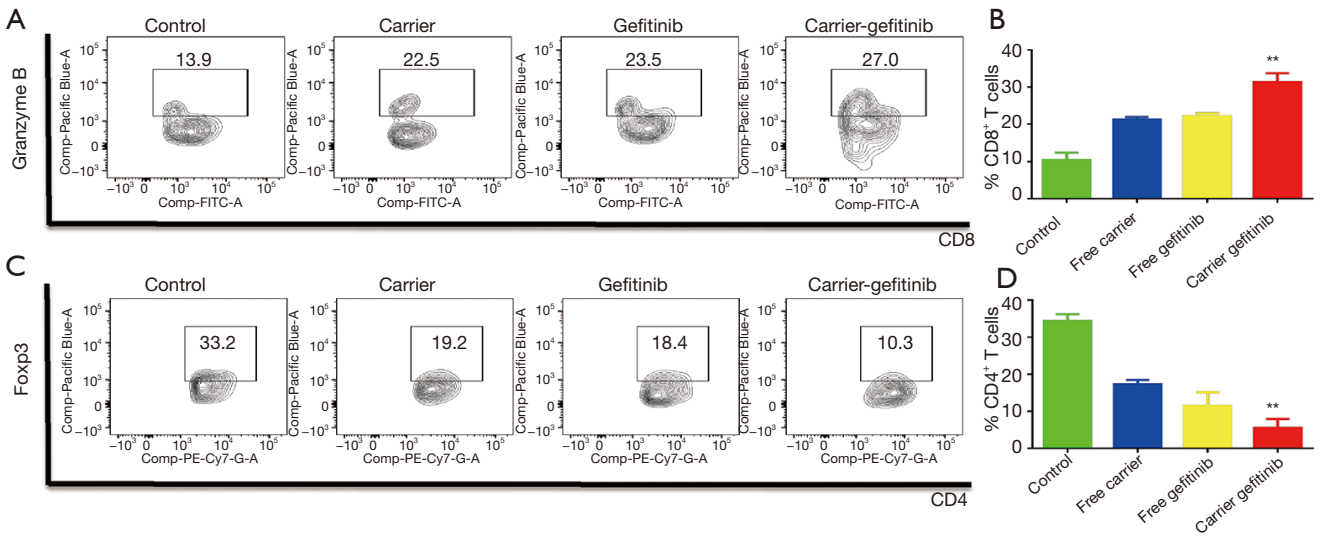

Figure 4 The tumor microenvironment evaluation after gefitinib/PEG$_{5k}$-Fmoc-NLG919 treatment. (A,B) The FACs analysis of functional CD8$^+$ cytotoxic T cell infiltration in the lung tumors treated with PBS, PEG$_{5k}$-Fmoc-NLG919 alone, free gefitinib, and gefitinib/PEG$_{5k}$-Fmoc-NLG919 (A) and the quantification (B). **, P<0.01 (carrier-gefitinib *vs.* control). (C,D) The FACs analysis of regulatory T cell infiltration in the lung tumors treated with PBS, PEG$_{5k}$-Fmoc-NLG919 alone, free gefitinib, and gefitinib/PEG$_{5k}$-Fmoc-NLG919 (C) and the quantification (D). **, P<0.01 (carrier-gefitinib *vs.* control).

partially explained through use of the cumulative gefitinib releasing assay, which indicated that gefitinib was gradually and slowly released from the micelles, providing a constant proliferation stress for lung tumor cells. Moreover, the long-term cumulative releasing assay revealed that roughly 90% of the gefitinib could be released from the PEG$_{5k}$-Fmoc-NLG919 after 24 hours, suggesting that delivery of gefitinib with our nanocarrier did not impair the final dose compared to administering gefitinib directly.

Another major concern is whether the transport of gefitinib using PEG$_{5k}$-Fmoc-NLG919 could facilitate a synergistical anti–lung cancer effect *in vivo*. For this purpose, we optimized the nanocarrier PEG$_{2k}$ to PEG$_{5k}$ for better delivery efficiency (22); the Fmoc group was introduced to increase the drug-loading capacity and formulation stability (21). Additionally, the IDO inhibitor, NLG919, was also included, which endowed the carrier with immune-stimulating capacity. Using the classical lung cancer xenograft mouse model, we repeatedly confirmed that gefitinib/PEG$_{5k}$-Fmoc-NLG919 exhibited better antitumor efficacy than gefitinib or carrier alone. By evaluating the immune cell subpopulations in the tumor microenvironment after different treatments, we demonstrated that gefitinib/PEG$_{5k}$-Fmoc-NLG919 could induce an immune-active microenvironment with more functional CD8$^+$ T cells and less Treg infiltration. Thus, we concluded that delivery of gefitinib using our optimized PEG$_{5k}$-Fmoc-NLG919 could kill tumor cells by not only inhibiting EGFR activity directly, but also by rewiring the tumor microenvironment to form a more hostile surrounding that indirectly suppresses tumor cell proliferation. Of note, the slow-releasing capacity of gefitinib/PEG$_{5k}$-Fmoc-NLG919 might also contribute to its better antitumor performance by sustaining a continuous drug concentration *in vivo*. Nevertheless, the possible advantages of deliver gefitinib using PEG$_{5k}$-Fmoc-NLG919 micelles includes several aspects, first of all, the PEG-Fmoc-NLG is a pro-drug that exhibits immunostimulatory activity by suppressing IDO activity given that IDO plays a vital role in establishing the immunosuppressive microenvironment in tumor. Secondly, gefitinib could be gradually released from the micelles, providing a constant proliferation stress for lung tumor cells and reduce the IC$_{50}$, finally, it could induce an immune-active microenvironment with more functional CD8$^+$ T cells and less Treg infiltration.

Taken together, our study has demonstrated that delivering small molecular targeted drugs with our modified immunostimulatory micelles can achieve a synergistic and improved antitumor effect. Since gefitinib is a first-generation TKI, determining whether delivery of new

generation TKIs with PEG_{5k}-Fmoc-NLG919 could also provide prognostic advantage and whether this method could result in severe side effects, requires further investigation. Finally, we have proven that the concept of combining targeted and immune therapy is a promising strategy to improve the prognosis for cancer patients, and that using immunostimulatory nanocarriers to distribute targeted drugs provides an ideal approach for ultimately achieving simultaneous targeted and immune therapy.

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Footnote

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