Construction of PEI-EGFR-PD-L1-siRNA dual functional nano-vaccine and therapeutic efficacy evaluation for lung cancer

Guixue Yang | Dong Zhou | Yin Dai | Yanqi Li | Jiang Wu | Quanxing Liu | Xufeng Deng

1Department of Thoracic Surgery, Xinqiao Hospital, Army Medical University (Third Military Medical University), Chongqing, China
2Department of Information, Xinqiao Hospital, Army Medical University (Third Military Medical University), Chongqing, China

Correspondence
Xufeng Deng, Department of Thoracic Surgery, Xinqiao Hospital, Army Medical University (Third Military Medical University), No. 83, Xinqiao Zheng Street, Chongqing 400037, China.
Email: 15120100521@126.com
Quanxing Liu, Department of Thoracic Surgery, Xinqiao Hospital, Army Medical University (Third Military Medical University), No. 83, Xinqiao Zheng Street, Chongqing 400037, China.
Email: quanxing9999@qq.com

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Abstract
Background: PD-1/PD-L1 tumor immunotherapy shows effective anticancer in treatment of solid tumors, so PEI lipid nanoparticles (PEI-LNP)/siRNA complex (EPV-PEI-LNP-SiRNA) with the therapeutic function of PD-L1-siRNA and EGFR short peptide/PD-L1 double immune-enhancing function were constructed for the prevention and treatment of EGFR-positive lung cancer in this study.

Method: In this study, PEI lipid nanoparticles (PEI-LNP)/siRNA complex (EPV-PEI-LNP-siRNA) with the therapeutic function of PD-L1-siRNA and EGFR short peptide/PD-L1 double immune-enhancing function were constructed for the prevention and treatment of EGFR-positive lung cancer and functional evaluation was conducted.

Results: On the basis of the construction of the composite nano-drug delivery system, the binding capacity, cytotoxicity, apoptosis and uptake capacity of siRNA and EPV-PEI-LNP were tested in vitro, and the downregulation effect of PD-L1 on A549 cancer cells and the cytokine levels of cocultured T cells were tested. Lipid nanoparticles delivered siRNA and EGFR short peptide vaccine to non-small cell lung cancer (NSCLC), increasing tumor invasion and activation of CD8+ T cells. Combination therapy is superior to single target therapy.

Conclusion: Our constructed lipid nanoparticles of tumor targeted therapy gene siRNA combination had the ability to target cells in vitro and downregulate the expression of PD-L1, realizing the tumor-specific expression of immune-stimulating cytokines, which is a highly efficient and safe targeted therapy nano-vaccine.

KEYWORDS
lung cancer, PEI-EGFR-PD-L1-siRNA, therapy, vaccine

INTRODUCTION

Tumor immunotherapy, which includes monoclonal antibody immune checkpoint inhibitors, therapeutic antibodies, cancer vaccines, cell therapy and small molecule inhibitors, is a kind of therapy which can restore the body’s normal antitumor immune response by restarting and maintaining the tumor-immune cycle, so as to control and eliminate tumors.1,2 In recent years, PD-1/PD-L1 tumor immunotherapy has shown strong antitumor activity in the treatment of a variety of solid tumors, such as non-small cell lung cancer (NSCLC), kidney and prostate cancers. Several tumor immunotherapeutics have been approved by the Food and Drug Administration (FDA) for clinical use. However, the immunosuppressive tumor microenvironment (TME) limits the effects of immune therapy, and abnormal structure and function of tumor vasculature leads to highly heterogeneous and lack of oxygen TME. Thereby, it can promotes tumor and stromal cells release immunosuppression factor and promotes immunosuppression source sex cells of bone marrow infiltration, which limits the tumor infiltration of T cells and leads to inhibition of antitumor immune response and produce immune tolerance.3,4 Therefore, it is imperative to develop a feasible strategy to counter this immune tolerance.
and the efficacy of amplified immune checkpoint blocking (ICB) therapy.

The aim of this study was to use PD-L1 combined with tumor neoantigen vaccine to enhance the strategy of lung cancer immunotherapy, and to verify the efficacy function at the cellular level. Of lung cancer patients, 85% are NSCLC, with the main subtype being adenocarcinoma of the lung. The EGFR gene mutation rate in NSCLC has been reported to reach over 60%, and mutation sites are mostly concentrated in the 18–21 exon region.\(^5\) After taking tyrosine kinase inhibitors (TKIs), a mutation of T790M in exon 19 has been found to be present in more than 90% of patients who are resistant to treatment. Therefore, novel antigen screening for the mutant peptide produced by the EGFR mutant gene can break down individual differences, thus combating the immune tolerance induced by ICB therapy, resulting in a better therapeutic effect and making the immunotherapy of lung cancer universal.

Cationic liposome nanocarriers are an effective gene delivery system,\(^6\)–\(^8\) which can load exogenous genes\(^9\)–\(^10\) through encapsulation, electrostatic adsorption and chemical bonding, and transfer them into cells through cytophagocytosis, resulting in successful gene expression.\(^11\) Liposome complex and polyethylenimine (PEI) with modified by other active materials such as anti PD-L1 antibody, have the advantages of diverse functions, good biocompatibility and easy surface modification.\(^12\)–\(^14\) Herein, we designed the encapsulation of therapeutic PD-L1-siRNA and EGFR short peptide combination into positive PEI lipid nanoparticles, which have the function of enhancing stability, controlled release characteristics and increasing transfection activity.\(^15\)\(^,\)\(^16\) The siRNA-loaded lipid nanoparticles have immune-checkpoint PD-L1 targeting siRNA and EGFR short peptide vaccine activating T cell vaccine function, thereby jointly enhancing antitumor ability against EGFR-mutated NSCLC.

**METHODS**

**Reagents and instruments**

All the cell-culture medium, FBS, and phosphate-buffered saline (PBS) were purchased from Gibco Life Technologies. All the culture consumables needed for cell culture were supplied by Corning. DAPI, dioleoylphosphatidylcholine (DOPC), polyethyleneimine (PEI, 25 kDa) and cholesterol (Chol) were purchased from Sigma-Aldrich. Biotin functionalized 1,2-dioleoyl-m-glycerol-3-phosphoethanolamine-poly (ethylene glycol)-2000 (DSPE-PEG-biotin) was purchased from Ponsure Biotech. DNA and RNA sequences were synthesized and purified by the Sangon Biotechnology Company. All the chemicals purchased for the present research met the reagent grade standards. A Millipore Milli-Q system was used for the creation of ultrapure water (18 MΩ.cm). Testing the hydrodynamic diameters and concentrations of suspended purified exosomes, Fe₃O₄@PDA, and Exo-Fe₃O₄@PDA was evaluated using a transmission electron microscope (8H-7000FA; Hitachi) and Nanosight (Malvern Instruments, UK). Lastly, the evaluation of the UV–Vis absorption spectra was made with a spectrophotometer (UV2550; Shimadzu). Fourier-transform infrared (FTIR) analyses were conducted in terms of pure exosomes at the speed of 2 mm/s by and in the range of 400–4000/cm (VERTEX 70; Bruker). qRT-PCR was conducted with an ABI 7500 quantitative PCR System (Thermo Fisher Scientific). The sequence of siRNA was (Sense) 5'–CCAGCACACUGAGAUA-CAATT–3' and (anti-Sense) 5'–UUGAUUCUCAGUG UGTT–3'. PD-L1 and control siRNA ( scramblesiRNA, siSCR) were purchased from Shanghai GenePharma Co., Ltd.

**Synthesis of PD-L1-siRNA loaded EPV-PEI-LNP (EPV-PEI-LNP-siRNA)**

PEI (Mw = 2000) and stearic acid were mixed into a solution of water and ethanol with a molar ratio of 1: 3. EDC was added into PEI (Mw = 2000) and stearic acid. The mixture was heated and stirred at 50°C for 24 h. Freeze-dried PEI-stearic acid (PEI-SA) was prepared. Liposomes were prepared by reverse emulsification. The matrix materials PEI-SA, DOPC, cholesterol (Chol) (mass ratio 3: 2: 4) were dissolved in dichloromethane as oil phase, and PBS (0.1 m) with twice the volume of water phase was added after fully dissolved. After mixing well, the solution was fully emulsified using a probe ultrasonic instrument with a power of 100 W, ultrasonic 2 s, interval of 1 s, total time of 3 min, and temperature of 25°C. At the end of ultrasound, uniform emulsion was formed, and the organic solvent of dichloromethylethylene was removed by rotary evaporator. PEI-SA/ DOPC/cholesterol nanoparticles (PEI-LNP) were obtained after 3 h dialysis with PBS solution.

In order to prepare the EGFR short peptide vaccine adjuvant (EPV), the PEI-LNP prepared above was dispersed in PBS solution (0.1 m) to form a suspension (mass concentration of PEI-LNP 2 mg/ml). It was then blended with EDC and NHS in equal molar ratio to PEI, and fully dissolved. Then EGFR short peptide (CLTSTVQLET, Mw = 1112.3) with molar ratio of 2 times was added and stirred for 8 h. After the reaction, dialysis (molecular weight 1000) the unreacted EDC and NHS was removed, and the remaining solution was centrifuged and concentrated with an ultrafiltration tube and stored for later use.

Then, 3 mg/ml EPV-PEI-LNP was placed in a centrifuge tube with 1 ml PBS solution, and the volume kept constant to 1.5 ml. siRNA 20, 40, 60, 80, and 100 μg, respectively was then added. After fully dispersing, the mixture was placed into a thermostatic oscillator and vibrated for 3 h. Drug loaded lipid nanoparticles of EPV-PEI-LNP-siRNA were obtained. Figure 1 shows the liposome preparation and cell uptake process.

**Characterization of EPV-PEI-LNP**

The PEI-LNP and EPV-PEI-LNP were formulated as described above and resuspended in deionized water. The
morphology of the NPs was characterized by TEM (H-7500; Hitachi High-Tech). The NPs were dropped on dried formvar coated 100-mesh copper grids at room temperature. All grids were further dried for 4 h in the oven at 37°C before imaging. The particle size and surface charge were examined using a Zetasizer system (Zetasizer NanoZS; Malvern Instruments Ltd) at room temperature.

**Drug release experiment of PEI-LNP/siRNA**

Standard solutions of siRNA, PEI-LNP-siRNA and EPV-PEI-LNP-siRNA in a series of concentration gradients were prepared. The maximum absorption wavelength of siRNA was determined by scanning the full spectrum with an ultraviolet spectrophotometer, and the regression equation was obtained by using the standard curves of concentration (C) and absorbance (ABS). Then, 1 mg PEI-LNP and EPV-PEI-LNP were dispersed into 10 centrifuges containing 1 ml PBS solution, and siRNA of 10, 20, 40, 60, 80 and 100 μg were added, respectively. After incubation at room temperature for 30 min, siRNA release test was performed in a thermostatic oscillator (50 RPM, 6 h). The supernatant was centrifuged to test its absorbance, and the amount of siRNA loaded by PEI-LNP was calculated according to the regression equation.

The material with the best experimental effect was selected for the release experiment. Then, 0.5 ml siRNA, PEI-LP-siRNA and EPV-PEI-LP-siRNA (siRNA concentration 50 μg/ml) were absorbed and placed into dialysis bags, respectively, and then placed into EP tubes containing 5 ml PBS buffer with pH values of 5, 7 and 9. The sample was placed in a thermostatic oscillator with the temperature set at 37°C and the speed set at 50 rpm. Three parallel samples were set for each group, and the time points were set as 0, 2, 4, 6, 8, 12, 18, and 24 h, 2, 3, 4, 5, 6, and 7 days. Then, 1 ml PBS solution was taken from the EP tube at each time point to measure the absorbance, and then poured again into an EP tube after the measurement. The release rate of siRNA was calculated.

**Cytocompatibility analysis of EPV-PEI-LNP**

The frozen human umbilical vein endothelial cell (HUVEC) cell lines were resuscitated and added into the cell culture flask with 90 ml 1640 medium and 10 ml fetal bovine serum. The cells were incubated at 37°C and contained 5% CO2 for subculture. HUVEC cells in good cell state were digested with trypsin and counted. Then, 1 × 10⁴ HUVEC cells were added to each well of 96-well plates, and 200 μl of 1640 medium was added to culture for 24 h. After cell adherence, the medium was replaced. PBS was added and siRNA, PEI-LP-siRNA and EPV-PEI-LP-siRNA at concentrations of 10, 30, 50 and 80 μg/ml were cocultured for 24 h, 2, 4 and 7 days, respectively. When the time was up, 20 μl MTT (5 μg/ml) was added to each well, they were incubated for 4 h, and the MTT was discarded. Then, 200 μl DMSO was added to each well to fully dissolve the formazan before testing. The absorbance value was measured by ELISA and cell survival rate was calculated. At the same time, AO/PI fluorescence staining was used to qualitatively determine the biocompatibility of PEI-LNP-siRNA and EPV-PEI-LNP-siRNA (80 μg/ml) cocultured with HUVEC for 2, 4 and 6 days.

**Lung cancer cell inhibition experiment**

A549 cells in good cell state were digested with trypsin and counted, then 5 × 10⁴ A549 cells/well were added to 96-well plates, and 200 μl DEME with high sugar medium was added for 24 h until the cells adhered to the bottom of the culture plate. Then, siRNA, PEI-LP-siRNA and EPV-PEI-LP-siRNA with a concentration of 5–15 μg/ml prepared by the medium were added, and incubated in the cell incubator.
for 48 h. Then, 20 μl MTT (5 μg/ml) was added to each well, and MTT was discarded after incubation for 4 h, and 200 μl DMSO was added to each well to dissolve formazan. The absorbance value was measured by ELISA and the cell survival rate was calculated.

Cell apoptosis

After the A549 cells in good cell state were digested with trypsin and counted, 5 × 10⁵ A549 cells/well were added to the 6-well plate, and 1 ml DEME with high-sugar medium was added to culture for 24 h until the cells adhered to the wall. siRNA, PEI-LNP-siRNA and EPV-PEI-LNP-siRNA were added to the medium at a dose of 20 μg per well. After coculture for 48 h, the medium was discarded, and the cells were digested with trypsin without EDTA and PBS cleaning. After centrifugation at 1200 RPM (rotate speed per minute) for 4 min, the supernatant was discarded, and the cells were resuspended with precooled PBS. They were then centrifuged at 2000 RPM for 5 min, the supernatant discarded, and 300 μl 1 × binding buffer was added for cell suspension. Annexin-FITC 5 μl was added and mixed, and the cells were incubated at room temperature without light for 15 min. Before feeding, 5 μl PI was added and stained. Flow cytometry was performed after 5 min of incubation. At the same time, the qualitative analysis of apoptosis was carried out using AO/PI fluorescence staining. In addition, protein was extracted from each group of cells cultured for 0, 12, 24 and 48 h, and BCA was quantified. After quantification, 12% SDS-PAGE separation gel was used for protein electrophoresis. After antibody incubation, an infrared imaging film scanning system was used for imaging film scanning, and the relative expression of related proteins was analyzed by gray value.

Qualitative analysis of endocytosis

After the A549 cells in good cell state were digested with trypsin and counted, 1 × 10⁵/well A549 cells were added to the 24-well plate, and 1 ml DEME with high-sugar medium was added to culture for 24 h until the cells adhered to the wall. siRNA, PEI-LNP-siRNA and EPV-PEI-LNP-siRNA were added after the medium was aspirated. The siRNA was labeled with carboxyl fluorescein reagent. After culture for 12 h, 20 μl DAPI (0.5 μg/ml) was added. After incubation for 15 min, DAPI was discarded, they were cleaned with PBS three times, and observed under a fluorescence microscope.

Expression of PD-L1 and IL-2

A549 cells were inoculated into 6-well plates with a density of 2 × 10⁵ cells per well, and siRNA, PEI-LNP-siRNA and EPV-PEI-LNP-siRNA were added at the same amount of 100 nmol siRNA, respectively. After two days, the cells in each group were collected and lysed with RIPA to extract proteins. Protein samples were concentrated and separated by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a PVDF membrane. After the antibody was incubated, western blot imaging was performed on PVDF membrane by chemiluminescence method. The coculture supernatant of PEI-LP-siRNA and EPV-PEI-LP-siRNA groups was collected and the expression of IL-2 was detected by ELISA.

Establishment of A549/activated T cell coculture model

Peripheral blood mononuclear cells (PBMCs) of healthy individuals were separated by gradient density centrifugation (Sigma-Aldrich reagent). PBMCs were inoculated with 2 × 10⁵ cell lines per well on 96-well plates and stimulated with anti-CD3 (10 g/l) and anti-CD28 (2 g/l) for 48 h. A549 cells were pretreated with PEI-LNP-siRNA and EPV-PEI-LNP-siRNA cultures for 12 h, and the purified activated T cells were cocultured with A549 cells in a ratio of 10:1 for 48 h. The expression levels of TNF-α, IFN-γ and IL-10 in coculture supernatant were detected by ELISA.

RESULTS

Material characterization analysis

The Zeta potential and particle size results of PEI-LNP and EPV-PEI-LNP prepared in the experiment are shown in Figure 2a,b. PEI-LNP is positively charged with an average particle size of 118.3 nm. After binding with negatively charged PD-L1-siRNA, the particle size of the complex gradually increases and zeta potential decreased (Figure 2c,d). The results indicated that PEI-LNP could load siRNA to form a nanocarrier siRNA complex through electrostatic action. As shown in Figure 2e, PEI-LNP is regular spherical under transmission electron microscopy and is coated with the amphiphilic cation PEI-SA. After PEI-LNP is combined with siRNA, the siRNA is mainly adsorbed and aggregated on the surface of nanoparticles. With the carrier particle as the center, the siRNA concentration became higher and higher, and closer to the center, which indicated that PEI-LNP can carry a large amount of siRNA and be used as the transfer vector of foreign RNA.

Analysis of loading and release test results

Figure 3a shows the standard curve of PD-L1-siRNA. The excitation wavelength of siRNA was 260 nm, and the correlation coefficient R² reached more than 99.9%, indicating a high fitting degree of the straight line. Therefore, the regression equation was used to calculate the subsequent
experiments, and the results obtained were highly reliable. When loading siRNA, the unit load increased gradually with the increase of the ratio of drug to carrier. The maximum unit load of PEI-LNP for siRNA was 89 μg/mg, and that of EPV-PEI-LNP for siRNA was 80 μg/mg (Figure 3b). The release rate of PD-L1-siRNA is shown in Figure 3c. Within
the first 24 h, the release curve is steep, but gradually flattens in the following time. The release rate of the loaded system under acidic conditions is faster than that under pH = 7 and 9, and the release rate of siRNA can reach 92.5% under acidic conditions. This may be because the protonation reaction of tertiary amine group on PEI occurred in an acidic environment, resulting in weakening of the positive electricity of polymer nanospheres and the weakening of the electrostatic interaction between PEI-LNP nanocarriers and siRNA, resulting in more drug release.

Results of cytocompatibility experiments

Figures 4a–c show the cell growth and proliferation of the loading system after 24 h, 2, 4 and 7 days coculture with HUVEC by the MTT method. As can be seen from the figure, the survival rate of PEI-LNP-siRNA and EPV-PEI-LNP-siRNA after treatment was above 90%, indicating that the loading system had no effect on normal cells and had good biocompatibility. Figure 4c shows the growth and proliferation of HUVEC cells after 24 h, 2, 4 and 7 days coculture with the loading system. It can be seen from AO/PI fluorescence staining that the cells after coculture all had good growth and complete cell morphology, with no difference from the control group. Also, with the increase of concentration and the passage of time, the number of cells increased, with good cell proliferation. This indicates that the loading system had no effect on the growth and proliferation of normal cells, and that biological toxicity is low. This is consistent with the experimental results of MTT.

Inhibition and apoptosis of lung cancer cells

The cytotoxicity test results are shown in Figure 5a. The results showed that neither PEI-LNP nor EPV-PEI-LNP had significant cytotoxicity to A549 cells in the experimental concentration range. The inhibition rate of PEI-LP-siRNA on lung cancer cells was significantly lower than that of EPV-PEI-LNP-siRNA (p ≤ 0.05), and the inhibition rate of PEI-LNP-siRNA on lung cancer cells was significantly higher than that of pure siRNA (p ≤ 0.05). This indicates that PEI modified positive vector can effectively deliver more siRNA into cells and take effect. The modification of EGFR short peptide not only enables PEI-LNP to function as an immune adjuvant, but also plays a role of targeted mediating in lung cancer cells, thus enabling more PEI-LNP-siRNA to enter cells and release siRNA drugs. Figure 5b shows the fluorescence microscopy results after 48 h coculture of the nanodrug-carrying complex with A549 cells. According to the figure, A549 cells in the PBS control group grew well, but A549 cells in the four experimental groups showed different degrees of apoptosis. Among them, apoptosis of A549 cells in the EPV-PEI-LP-siRNA group was the largest. According to the results of flow cytometry apoptosis (Figure 5c,d), the apoptosis rate of siRNA and PEI-LNP-siRNA groups reached 23.3 and 39.6%, respectively, while the apoptosis rate of EPV-PEI-LNP-siRNA groups reached 76.2%.
As can be seen from Figure 5e, the expression of antiapoptotic protein Bcl-2 (26 kDa) in EPV-PEI-LNP-siRNA cocultured cells gradually decreased with the extension of treatment time of drug-loaded nanocomplex. The expression of proapoptotic protein BAD (25 kDa) increased gradually. The expression of pro-caspase-3 (32 kDa) decreased gradually. Cleaved caspase-3 (17 kDa) expression increased gradually. This indicates that the EGFR short peptide-targeted vaccine adjuvant system that we constructed has a superior therapeutic effect after loading siRNA.

Analysis of endocytosis

Figure 6 shows fluorescence microscope effect of endocytosis of A549 cells 12 h after PEI-LNP-siRNA and EPV-PEI-LNP-siRNA were treated with A549 cells (siRNA modified by FITC showed green fluorescence). As can be seen from the figure, the fluorescence intensity of the EPV-PEI-LNP-siRNA group was significantly higher than that of the PEI-LNP-siRNA group. This indicated that the PEI lipid nanoparticles prepared in this study were modified with EGFR short peptide, and the targeted recognition ability of the nanoparticles to EGFR-highly expressed cell A549 was enhanced. After 12 h of endocytosis, more siRNA was delivered into the cell interior.

Expression of PD-L1 and IL-2

Western blot results are shown in Figure 7a. PD-L1 protein was highly expressed in A549 cells in the blank
group, and the expression of PD-L1 was downregulated in the siRNA treated cells. PD-L1 expression in the PEI lipid microspheres with siRNA treated cells was significantly lower than that in the blank group and the siRNA alone treated group. These results indicate that PD-L1-siRNA can inhibit the expression of PD-L1, and
siRNA loaded by PEI lipid microspheres can enhance the downregulation of PD-L1, while the addition of EGFR short peptide can further enhance the drug therapy function of drug-loaded microspheres. The expression of IL-2 in A549 was detected by ELISA and it was found that IL-2 expression was significantly upregulated after siRNA loading by EPV-PEI-LNP.

**Effect of EPV-PEI-LNP-siRNA on T cell function**

To determine whether siRNA loaded with PEI lipid microspheres affects T cell function by inhibiting PD-L1 expression, we established an A549/activated T cell coculture model. ELISA showed that IFN-γ and TNF-α levels were significantly increased after T cells were cocultured with EPV-PEI-LNP-siRNA pretreated A549 cells, while IL-10 levels were significantly decreased (Figure 8).

**DISCUSSION**

PD-L1-siRNA and EGFR short peptide combined lipid nanoparticles were prepared by PEI-LNP, and the binding ability of siRNA and EGFR short peptide with nanoparticles was tested by in vitro experiments. The release rate of the loading system was faster under acidic conditions than that under pH = 7. The release rate of PD-L1-siRNA can reach 80%. AO/PI fluorescence staining showed that the loading system had no effect on the growth and proliferation of normal cells and had no biological toxicity. The loaded combination of EPV-PEI-LNP-siRNA can effectively deliver siRNA and EGFR short peptide into cells. IFN-γ and TNF-α levels were significantly increased after T cells were cocultured with EPV-PEI-LNP-siRNA pretreated A549 cells, while IL-10 levels were significantly decreased. EPV-PEI-LNP-siRNA is a tumor-selective therapeutic gene vector that blocks immune checkpoints and provides costimulatory molecules, while also reducing the side effects of immunotherapy. Cancer cells or stromal cells also play an important role in regulating immune cell phenotypes in TME, inhibiting T cell activation and enhancing immune tolerance, thereby inhibiting anticancer immunity.1,17

Several previous clinical trials have demonstrated the safety and efficacy of polyclonal antibodies. However, PD-L1 is not only expressed in tumor cells, but is widely expressed in hematopoietic and nonhematopoietic cells, and PD-1/PD-L1 and B7-1/PD-L1 play a key role in regulating the function of effector T cells and limiting the autoimmune response. Nontargeted blocking of these interactions may lead to related adverse reactions in addition to therapeutic effects, and the mechanisms are not fully revealed.18 In order to explore a more efficient and specific blocking method for PD-1/PD-L1, this study used chemically modified cationic polymer nanocertifers to realize interference on PD-L1.

RNA interference is a specific mechanism of post-transcriptional gene silencing, which can cause the corresponding mRNA to be degraded by enzymes. Through this mechanism, siRNA, consisting of 21 to 25 base pairs, can specifically silence gene expression. PD-L1-siRNA therapy blocking the PD-1/PD-L1 interaction is safe and readily available. At present, the barrier of using siRNA gene therapy lies in that naked siRNA is easily degraded by nuclease in the environment and lysosome in the cell. The negative charge carried by siRNA and the rejection of cell membrane make it difficult for siRNA to penetrate cell membrane and enter cells. At this point, the nanocarriers prepared in this study are positively charged and can load the negatively charged nucleic acid through electrostatic action. Moreover, the liposome nanocarriers prepared in this study have good compatibility with the cell membrane, which is conducive to the uptake of the therapeutic system by cells.

In this study, EPV-PEI-LNP lipid particles showed excellent biocompatibility and showed effective tumor immunotherapy. The expression of PD-L1 in cells treated with EPV-PEI-LNP-siRNA was significantly lower than that in the blank group and the PD-L1-siRNA group, suggesting that PD-L1-siRNA can inhibit the expression of PD-L1, and the downregulation of PD-L1 can be enhanced by loading siRNA through liposomes. We used EPV-PEI-LNP-siRNA to deliver EGFR short peptides to immune cells rather than cancer cells, which can enable stromal cells to continuously produce immune-stimulating cytokines, regulate immunosuppressive TME, and achieve long-term antitumor immunity.18 PD-L1-siRNA nanotechnology delivery targeting the combination of immunosuppressive factor and EGFR short peptide is safe, efficient, and selectively targeted to TME, and is suitable as a next-generation immunotherapy strategy for cancer. Further in vitro and in vivo functional experiments will be carried out in this project to clarify the application prospect of this polymer.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**ORCID**

Xufeng Deng https://orcid.org/0000-0002-8321-8535

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