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ORIGINAL ARTICLE

Optimized lipid nanoparticles enable effective CRISPR/Cas9-mediated gene editing in dendritic cells for enhanced immunotherapy



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Abstract Immunotherapy has emerged as a revolutionary approach to treat immune-related diseases. Dendritic cells (DCs) play a pivotal role in orchestrating immune responses, making them an attractive target for immunotherapeutic interventions. Modulation of gene expression in DCs using genome editing techniques, such as the CRISPR-Cas system, is important for regulating DC functions. However, the precise delivery of CRISPR-based therapies to DCs has posed a significant challenge. While lipid nanoparticles (LNPs) have been extensively studied for gene editing in tumor cells, their potential application in DCs has remained relatively unexplored. This study investigates the important role of cholesterol in regulating the efficiency of BAMEA-O16B lipid-assisted nanoparticles (BLANs) as carriers of CRISPR/Cas9 for gene editing in DCs. Remarkably, BLANs with low cholesterol density exhibit exceptional mRNA

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uptake, improved endosomal escape, and efficient single-guide RNA release capabilities. Administration of BLAN_{mCas9/gPD-L1} results in substantial PD-L1 gene knockout in conventional dendritic cells (cDCs), accompanied by heightened cDC1 activation, T cell stimulation, and significant suppression of tumor growth. The study underscores the pivotal role of cholesterol density within LNPs, revealing potent influence on gene editing efficacy within DCs. This strategy holds immense promise for the field of cancer immunotherapy, offering a novel avenue for treating immune-related diseases.

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1. Introduction

Immunotherapy has emerged as a transformative and promising approach for the treatment of a variety of immune-related diseases, spanning from cancer and autoimmune disorders to viral infections¹⁻⁴. Among the key orchestrators of immune responses, dendritic cells (DCs) stand out as professional antigen-presenting cells with a pivotal role in this landscape. Their remarkable ability to initiate and modulate immune responses makes them of central importance in the realm of immunotherapy⁵. DCs possess the unique capacity to capture, process, and present antigens to T cells, thereby shaping the nature and vigor of ensuing immune responses⁶. Consequently, the functional status of DCs profoundly influences the overall responsiveness of the immune system. Harnessing the potential of DCs to modulate immune responses opens a promising avenue for treating a diverse range of diseases, spanning from cancer to autoimmune diseases and viral infections. By fine-tuning DC functionality, it becomes possible to enhance the capabilities of the broader immune system, thus laying the foundation for novel therapies targeting immune-related conditions.

The advent of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9) technology has unlocked novel avenues for precise gene regulation within the context of tumor immunotherapy⁷⁻⁹. However, the efficient and specific delivery of CRISPR-based therapies remains a significant technical challenge, constraining the therapeutic potential of this groundbreaking technology. Researchers have explored the utilization of lipid nanoparticles (LNPs) as delivery vehicles for CRISPR-based therapies¹⁰⁻¹⁶. While many investigations have concentrated on employing nanoparticles for delivering the CRISPR/Cas9 system to edit genes in tumors^{13,17-20}, gene editing within DCs to modulate their functions has received relatively limited attention. DCs, serving as professional antigen-presenting cells, possess a heightened capability to phagocytose and degrade exogenous substances compared to other cell types^{21,22}. This heightened nuclease activity within DCs can result in the rapid degradation of mRNA²³, impeding the functionality of the CRISPR/Cas9 system within these cells. Therefore, the effective delivery of Cas9 mRNA into DCs and its subsequent expression as Cas9 protein represent pivotal steps in achieving successful gene editing within DCs. Addressing the challenges associated with efficient and specific CRISPR-based therapy delivery to DCs is critical, given the central role of these cells in immune responses and their immense potential for enhancing disease immunotherapy. Further research and optimization of LNPs designed for DCs hold the promise of pioneering advancements in gene editing, unlocking the therapeutic potential of CRISPR/Cas9 in the realm of disease immunotherapy.

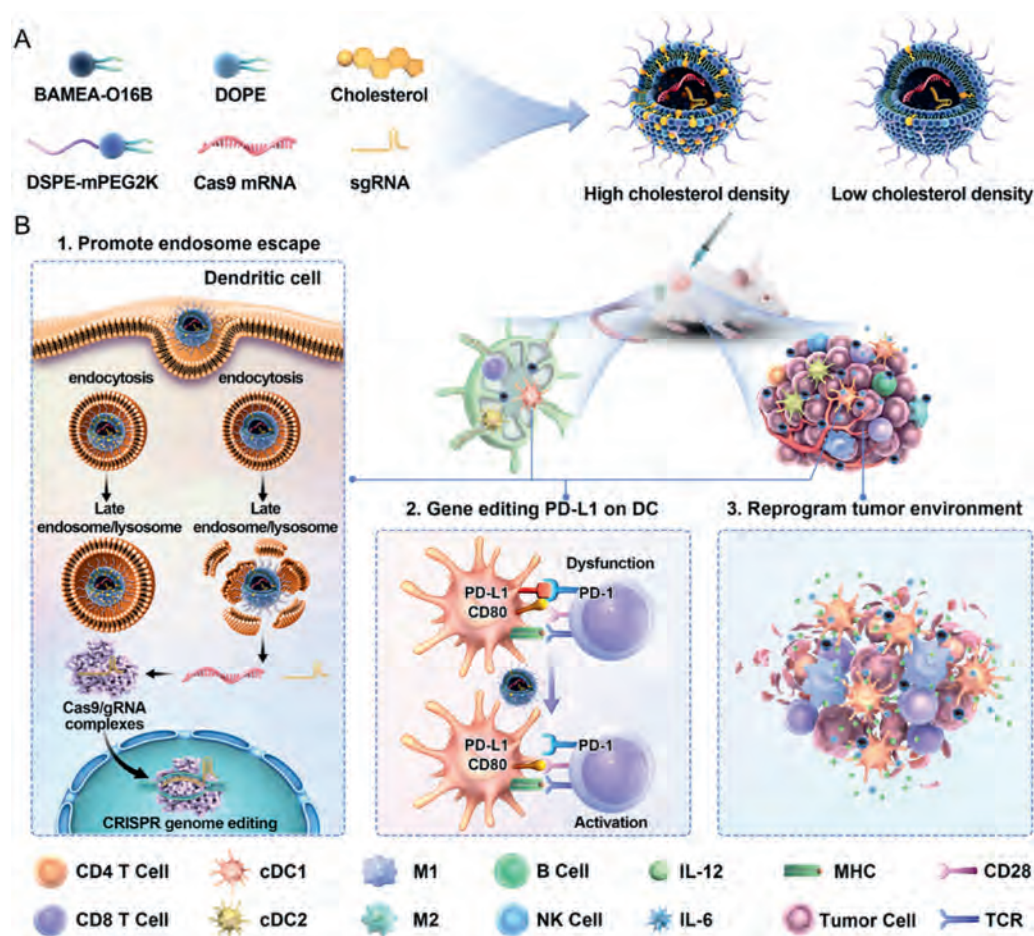
LNP formulations encompass a spectrum of lipid components, including ionizable cationic lipids, polyethylene glycol (PEG)-lipid

conjugates, phospholipids, and cholesterol²⁴. Cholesterol within LNPs plays a pivotal role in maintaining particle stability and preventing drug leakage, which are critical for efficient mRNA encapsulation and release, pivotal steps for successful gene editing^{25,26}. However, the precise impact of modulating cholesterol density within LNPs on mRNA release and CRISPR/Cas9 gene editing in DCs remains an enigma. In this study, we have devised optimized cationic lipid (BAMEA-O16B)-assisted nanoparticles (BLANs) with varying cholesterol densities to enhance mRNA delivery and gene editing specifically targeting DCs (Scheme 1). Our findings unveiled the pivotal role of cholesterol density within BLANs in governing mRNA expression within DCs. BLAN formulations with reduced cholesterol density exhibited superior cellular uptake, efficient release of single-guide RNA (sgRNA), and improved endosomal escape, thereby facilitating the effective *in vivo* delivery of the CRISPR/Cas9 system to conventional DCs (cDCs) in both tumors and draining lymph nodes (DLNs) (Scheme 1). Our study showcased how the utilization of BLANs amplifies immunotherapy by targeting PD-L1, a crucial surface molecule governing DC function. The administration of BLAN_{mCas9/gPD-L1} not only leads to the knockout of PD-L1 in cDCs but also enhances cDC1 enrichment and activation, subsequently promoting T cell activation and suppressing tumor growth. This study, which employs LNPs to deliver the CRISPR/Cas9 system for gene modulation within DCs, offers a compelling and effective strategy for elevating immunotherapy to new horizons.

2. Materials and methods

2.1. Materials

DSPE-mPEG₂₀₀₀ was purchased from Seebio (Shanghai, China). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was purchased from Avanti Lipids (Alabaster, AL, USA). Cholesterol, sodium acetate, and L-glutamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute 1640 (RPMI 1640), collagenase IV, Lipofectamine™ 2000, and trypan blue were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Gemini (Woodland, CA, USA). Penicillin–streptomycin, Transl-T1 phage chemically competent cell, and FastPfu PCR supermix were purchased from Transgen (Beijing, China). T7 Endonuclease I was purchased from Vazyme (Nanjing, China). GXL DNA polymerase was purchased from TaKaRa (Kusatsu, Shiga, Japan). Cas9 mRNA was prepared using mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. PD-L1 sgRNA and NC sgRNA were designed and cloned into pX458, then prepared by T7 RiboMAX™ Express Large



Scheme 1 Schematic illustration of cholesterol in BLAN_{mCas9/gRNA} regulating mRNA delivery and gene editing within dendritic cells. (A) Conceptual depiction of the formulation process for BLAN_{mCas9/gRNA} with different cholesterol densities. (B) Mechanistic overview demonstrating the effects of BLAN_{mCas9/gPD-L1} with reduced cholesterol density. This configuration enhances the internalization and endosomal escape of dendritic cells, leading to efficient PD-L1 gene knockout. Subsequently, this process triggers T cell activation and culminates in the inhibition of tumor growth.

Scale RNA Production System Kit (Promega, Madison, WI, USA). The Cy5-Cas9 mRNA was purchased from APEX BIO (Houston, TX, USA). It had a Cap 1 structure and was modified with 5-moUTP and Cy5-UTP.

2.2. Cell culture

The murine colon cancer line CT26, mouse dendritic cell line DC1.2, and mouse macrophage cell line RAW264.7 were purchased from the American Type Culture Collection (Rockville, MD, USA). These cells were maintained in DMEM or RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin–streptomycin, and incubated at 37 °C incubator with 5% CO₂.

2.3. Generation of PD-L1 deficiency CT26 cell line

Wild-type CT26 cells were transfected with PD-L1 sgRNA and Cas9 mRNA by Lipofectamine™ 2000. Three days later, cells were harvested and PD-L1-negative single-cell lines were sorted by BioProtect IV with Influx Flow Cytometer (Baker, BP-504-5, Germany). Then these monoclonal cells were treated with 20 ng/mL

IFN- γ for 32 h for further validation. PD-L1 expression levels were determined by flow cytometry. Cell clones without PD-L1 expression were used for the following experiments.

2.4. Preparation and characterization of mRNA encapsulated BLAN

The synthesis of BAMEA-O16B lipid was performed and characterized as our previously described¹¹. Briefly, head amine and acrylate in a molar ratio of 1:3.3 were added to a 5 mL Teflon-lined glass screw-top vial followed by a further 3 days of stirring at 75 °C. After cooling, the crude product was purified using flash chromatography on silica gel. The lipid structure was confirmed by ¹H NMR (Bruker Fourier 300, Fällanden, Switzerland). The integral ratio of the methyl group in hydrophilic amine (~2.22 ppm) to hydrophobic tail methyl groups (~0.88 ppm) is 3:9, indicating that the lipid has three hydrophobic tails and also verifying the correctness of its structure. (¹H NMR (400 MHz, CDCl₃) δ 4.34 (q, J = 6.8 Hz, 6H, –COOCH₂), 2.94–2.86 (m, 8H), 2.80 (t, J = 7.2 Hz, 4H), 2.69 (q, J = 6.7, 5.9 Hz, 8H), 2.55 (dt, J = 6.8, 4.6 Hz, 4H), 2.46 (h, J = 6.2 Hz, 7H), 2.22 (s, 3H, –CH₂CH₂CH₂–), 1.72–1.52 (m, 15H), 1.27 (d, J = 4.5 Hz,

53H), 0.88 (t, $J = 6.8$ Hz, 9H, $-\text{CH}_2\text{CH}_3$). BAMEA-O16B lipid and DOPE ratios are kept constant (BAMEA-O16B/DOPE = 4/1, w/w), regulating cholesterol density from 0% to 40%. The BAMEA-O16B and DOPE were mixed with cholesterol in chloroform in a glass bottle; the mixture was dried overnight in a fume hood. Then lipid film was hydrated using a mixed solution of ethanol/sodium acetate buffer and added dropwise to another glass bottle which contained DSPE-mPEG₂₀₀₀ with 26.6 μg Cas9 mRNA and 26.6 μg sgRNA or 26.6 μg RFP mRNA. The solution was dialyzed in a dialysis bag (MWCO 10,000) with phosphate buffered saline (PBS) to remove excessive ethanol and sodium acetate. The diameter and zeta potential of lipid nanoparticles were determined using a Zeta-sizer Nano-ZS90 (Malvern, Worcs, UK).

2.5. *In vitro* mRNA expression and gene editing with BLAN

DC1.2 cells were seeded into 24-well plates with suitable cell density and cultured overnight. For RFP mRNA expression, the cells were transfected with PBS, Lipo_{mRFP}, unoptimized BLAN_{mRFP} or BLAN_{mRFP} with different cholesterol densities for 6 h. The RFP mRNA concentration was 5 $\mu\text{g}/\text{mL}$. Twenty-four hours after transfection, cells were harvested to detect RFP fluorescence by flow cytometry. For gene editing, cells were transfected with BLAN_{mCas9/gPD-L1} with 40%, 5%, and 0% cholesterol densities at the final concentration of 5 or 10 $\mu\text{g}/\text{mL}$ Cas9 mRNA for 6 h. Three or six days after incubation, cells were collected and the expression of PD-L1 was determined by flow cytometry.

2.6. Cellular uptake study

DC1.2 cells were seeded in 48-well plates at a density of 5.5×10^4 cells/well with 400 μL complete 1640 medium and incubated overnight at 37 °C incubator with 5% CO₂. BLAN_{Cy5-mCas9} suspended in Opti-mem medium was added into each well. The final concentrations of Cy5-Cas9 mRNA were 1.25, 2.5, 5, and 10 $\mu\text{g}/\text{mL}$. The cells were incubated at 37 °C for 2 h and washed twice with PBS. The Cy5-positive cells were analyzed by flow cytometry.

For fluorescence microscopy observation, DC1.2 cells were seeded into 24-well plates containing a coverslip overnight. DC1.2 cells were cultured with BLAN_{Cy5-mCas9} at a concentration of 10 $\mu\text{g}/\text{mL}$ mRNA for 2 h and incubated for an additional 6 h in complete 1640 medium at 37 °C. The cells were washed twice with PBS, followed by fixation with 4% paraformaldehyde for 20 min and perforation with 0.1% Triton X-100 for 30 min at room temperature. After washed with PBS, the cells were incubated with Cy3-conjugated anti-LAMP1 antibody (Abcam, Cambridge, UK) overnight at 4 °C. The cells were further stained with Alexa FluorTM 488 Phalloidin (Molecular Probes, St. Louis, MO, USA) for cytoskeleton. Finally, coverslips were mounted with DAPI-Fluoromount-G clear mounting media (SouthernBiotech, Birmingham, AL, USA). The cellular uptake and intracellular localization of nanoparticles were visualized by a laser scanning confocal microscope (Zeiss, LSM 880, Jena, Germany).

2.7. Encapsulation efficiency and mRNA release kinetics

The encapsulation efficiency of mRNA was determined by a Quant-iTTM RiboGreen[®] RNA reagent kit (Thermo Fisher Scientific, Waltham, MA, USA). BLAN or dilutions of free mRNA at known concentrations were diluted in a final volume of 100 μL in

RNase-free TE buffer (10 mmol/L Tris-HCl, 20 mmol/L EDTA) with or without 1% Triton X-100. The particles in Triton X-100 buffer were incubated for 15 min at 40 °C to allow particles to become permeabilized. Then the nanoparticle sample was mixed with an equal volume of the Ribogreen solution in a 96-well black plate. The plates were incubated for 4 min after shaking. The fluorescence (Ex: 480 nm, Em: 530 nm) was measured using a plate reader (Tecan, Spark, Männedorf, Zürich, Switzerland), and the concentration of each sample was calculated using a standard curve. Encapsulation efficiency was calculated from the content of free mRNA (without Triton X-100) and total mRNA (with Triton X-100), by the following Eq. (1):

$$\text{EE} (\%) = (\text{Total mRNA} - \text{Free mRNA}) / (\text{Total mRNA}) \times 100 \quad (1)$$

The release of mRNA from BLAN_{mRNA} was measured in TE buffer. Briefly, 0.4 mL BLAN_{mRFP} was dispersed in 0.6 mL PBS. The mixture was divided into 6 parts into 1.5 mL tubes, then the tubes were placed into a 37 °C water bath under shaking at 80 rpm (Jinghong, Shanghai, China). Samples were collected for measurement at 0, 12, 24, 36, and 48 h. Free mRNA and total mRNA were measured with a Quant-iTTM RiboGreen[®] RNA reagent kit and the release rate of RNA was calculated.

2.8. T7E1 assay and sequencing

DC1.2 cells were seeded into 6-well plates and cultured overnight. Then cells were transfected with BLAN_{mCas9/gPD-L1} at the final concentration of 5 or 10 $\mu\text{g}/\text{mL}$ Cas9 mRNA. After transfection, the genomic DNA of DC1.2 was extracted using the AxyPrepTM multisource genomic DNA miniprep kit (Axygen, Silicon Valley, CA, USA) according to the manufacturer's instructions. The flanking region of the gPD-L1 targeting sequence was amplified by PCR. Purified PCR products were diluted in T7 endonuclease I reaction buffer and denatured at 95 °C for 5 min. Then denatured PCR product were reannealed at 95–85 °C (ramp rate: -2 °C/s) and 85–25 °C (ramp rate: -0.1 °C/s). After cooling down, T7 endonuclease I was added and incubated at 37 °C for 1 h. The fragmented PCR products were analyzed with 2% agarose gel electrophoresis. The genomic region-flanking PCR products of gPD-L1 were ligated to clone vector using pEASY[®]-Blunt Zero Cloning Kit (Transgen, Beijing, China) according to the manufacturer's instructions. The ligated products were transformed into Trans-T1 competent cells, and colonies were plated overnight. Single colony solutions were used for genome sequencing.

2.9. Animals and tumor model

Female BALB/c mice (8 weeks of age) were purchased from Charles River (Beijing, China). All mice were raised in a specific pathogen-free environment with free access to food and water. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the First Hospital of Jilin University, and all experiments were performed in accordance with the protocols (approval number: 2021-273). To set up the tumor-bearing mouse model, a total of 1×10^6 PD-L1 KO CT26 cells in 100 μL PBS were inoculated subcutaneously into the right dorsal flanks of mice. Tumor volumes (V) were determined using length (a) and width (b), and calculated as Eq. (2):

$$V = ab^2/2 \quad (2)$$

2.10. *In vivo biodistribution of BLAN_{mCas9/gRNA}*

When the tumor volumes were about 500 mm³, the tumor-bearing mice were administrated with DiD-labeled BLAN_{mCas9/gNC} or PBS by intratumoral injection. The dose of Cas9 mRNA was 363 µg/kg. Twenty-four hours after injection, mice were euthanized and the main organs were harvested. The fluorescent signal was imaged on the IVIS Spectrum *In Vivo* Imaging System (PerkinElmer, Waltham, MA, USA). The tumor and draining lymph node tissues were isolated into single cell suspensions. These cells were stained with antibodies, and cellular uptake of BLAN_{mCas9/gRNA} was determined by flow cytometry.

To confirm the presence of BLAN_{mCas9/gRNA} in organs, tissue sections were prepared and imaged using a laser scanning confocal microscope. Briefly, tumors and draining lymph nodes were fixed in 4% paraformaldehyde and immersed using 30% sucrose solution. Tissue blocks were embedded into O.C.T. compound and co-sectioned (4 µm) on a Cryostat instrument HM550 (Thermo Fisher Scientific, Waltham, MA, USA). The tissue slices were stained with Alexa Fluor[®] 594-anti-mouse CD11c (Biolegend, San Diego, CA, USA) and DAPI before confocal microscopy imaging.

2.11. *Tumor growth and treatment*

PD-L1 KO CT26 tumor-bearing mice were randomly grouped into treatment groups when tumor volumes were less than 50 mm³. These mice received intratumoral injections of PBS, BLAN_{mCas9/gNC}, or BLAN_{mCas9/gPD-L1} every other day for 10 times. The dose of Cas9 mRNA administered was dependent on the tumor volume: 1) The tumor volume was less than 50 mm³, Cas9 mRNA was 72.5 µg/kg. 2) The tumor was between 50 and 150 mm³, Cas9 mRNA was 145.5 µg/kg. 3) The tumor volume was 150–300 mm³, Cas9 mRNA was 218 µg/kg. 4) The tumor volume was 300–500 mm³, Cas9 mRNA was 290.5 µg/kg. 5) The tumor volume was 500–800 mm³, Cas9 mRNA was 363 µg/kg. 6) The tumor volume was more than 800 mm³, Cas9 mRNA was 435.5 µg/kg. Tumor volumes and the mouse weights were measured every other day.

2.12. *In vivo immune response analysis*

PD-L1 KO CT26 tumor-bearing mice were administered PBS, BLAN_{mCas9/gNC}, or BLAN_{mCas9/gPD-L1} treatment for 6 times by intratumoral injection. Twenty-four hours after the last administration, tumors, draining lymph nodes, and spleens were harvested to analyze the phenotypes of immune cells by flow cytometry.

2.13. *Cell isolation from tissues*

Tumor tissues were clipped and digested with 1 mg/mL collagenase IV at 37 °C for 40 min, then passed through a 70 µm filter to remove large pieces of undigested tumor. Tumor-infiltrating cells were washed twice with PBS. Spleen tissues were triturated and passed through a 70 µm filter. Then cells were added with 1 mL RBC lysing buffer (Solarbio, Beijing, China) to remove erythrocytes. Draining lymph node tissues were triturated and passed through a 70 µm filter.

2.14. *Flow cytometric analysis*

For flow cytometric analysis, cells were first incubated with anti-CD16/32 (Biolegend) for 10 min in FACS buffer, followed by staining

with fluorochrome-conjugated antibody panels for 30 min. CD206 were stained intracellularly by using Foxp3/transcription factor staining buffer set kit (eBioscience, San Diego, CA, USA) following the manufacturer's instructions. Propidium iodide (PI) and LIVE/DEAD fixable aqua dead cell stain kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to exclude dead cells. The following mouse antibodies used in our study were purchased from Biolegend and eBioscience: CD45 (30-F11), CD11b (M1/70), CD11c (N418), I-A/I-E (M5/114.15.2), XCR1 (ZET), CD103 (2E7), CD80 (16-10A1), CD86 (GL-1), CD40 (3/23), PD-L1 (10F.9G2), CD8 (53-6.7), CD4 (GK1.5), CD3 (17A2), CD44 (IM7), CD62L (MEL-14), CD206 (C068C2), F4/80 (BM8). Data were collected on Aurora (Cytek Biosciences, Fremont, CA, USA) and FACSCelesta (BD Biosciences, Franklin Lakes, NJ, USA), and analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

2.15. *Histopathology, immunohistochemistry and immunofluorescence*

The main organs hearts, lungs, livers, spleens, and kidneys were harvested from tumor-bearing mice after 10 times administration and fixed in 4% paraformaldehyde. Paraffin sections were prepared and stained with hematoxylin and eosin (H&E). Immunohistochemistry (IHC) was performed on paraffin-embedded sections of mouse tumor tissues to stain for IL-6 and IL-12. Briefly, tissue sections were stained with anti-mouse IL-6 and IL-12 antibodies from Abcam (Cambridge, UK). All the stained slides were visualized and photographed with optical microscope (Olympus, Tokyo, Japan). For analysis of apoptosis, tumor paraffin sections were deparaffinized, and apoptotic cell death was detected using Click-iT Plus terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The tumor tissues were incubated with Ki-67 and PD-L1 antibodies (Abcam), and then Alexa Fluor 594 goat anti-rabbit was used as the secondary antibody. The tumor tissue and draining lymph node were stained with CD8, CD103, CD68, and DAPI using Opal 7-Color Manual IHC kits following the manufacturer's instructions. Fluorescent images were visualized by a Zeiss LSM 880 confocal laser scanning microscope.

2.16. *Statistical analyses*

Data are shown as mean ± standard deviation (SD). Statistical analysis was performed by unpaired Student's two-tailed *t* test for two groups and one-way analysis of variance (ANOVA) for multiple groups to compare the unpaired analyses. Statistical analysis was performed on the GraphPad Prism 8 software (GraphPad, San Diego, CA, USA). DNA gel images were acquired by ChemiDoc XRS + Image Lab (Bio-Rad, Hercules, CA, USA). A value of *P* < 0.05 was considered statistically significant.

3. Results

3.1. *Cholesterol density affects the expression efficiency of mRNA delivered by BLAN within DCs*

The biocompatible cationic lipid BAMEA-O16B, comprising hydrophobic tails containing disulfide bonds, was prepared following a previously reported method¹¹. The representative ¹H-NMR (proton nuclear magnetic resonance) spectra of

BAMEA-O16B lipid were shown in Supporting Information Fig. S1. The BAMEA-O16B lipid-assisted nanoparticles were formulated by combining BAMEA-O16B, cholesterol, DOPE, and DSPE-mPEG_{2k}, and had been previously used for mRNA delivery to the liver¹¹. However, its efficiency in delivering mRNA to DCs remains unknown. Here, we employed the BLAN system to simultaneously deliver Cas9 mRNA and PD-L1 sgRNA into DCs. Initially, we evaluated the mRNA delivery efficiency of BLAN using red fluorescent protein (RFP) mRNA (BLAN_{mRFP}) within DCs *in vitro*. Notably, compared to the commercial transfection reagent Lipofectamine™2000, BLAN_{mRFP}-treated cells exhibited lower RFP expression (Fig. 1A and Supporting Information Fig. S2), indicating the suboptimal mRNA delivery efficiency of BLAN into DCs. To enhance mRNA expression efficiency in DCs, we optimized the BLAN formulation by adjusting the cholesterol content. BLAN with different cholesterol densities (ranging from 40% to 0%, w/w) were prepared, while keeping the proportions of the other three components constant (BAMEA-O16B/DOPE/DSPE-mPEG_{2k} = 4/1/2, w/w/w). BLAN_{mRFP} with different cholesterol densities exhibited a gradual increase in size and zeta potential as the cholesterol density decreased (Supporting Information Fig. S3A and S3B). Subsequently, we evaluated mRNA expression in DCs after incubating with BLAN_{mRFP} containing different cholesterol densities. The percentage of RFP-positive cells in BLAN_{mRFP} containing 40% cholesterol-treated cells was only $28.0 \pm 1.6\%$, whereas the frequencies of RFP⁺ cells in cells treated with BLAN_{mRFP} containing 0.62% and 0% cholesterol were $58.1 \pm 1.5\%$ and $63.4 \pm 1.7\%$, respectively. The proportion of RFP⁺ cells, as well as the mean fluorescence intensity (MFI), significantly increased with decreasing cholesterol density in BLAN_{mRFP} (Fig. 1B and C). Additionally, we investigated whether this phenomenon is consistent across different cell types by transfecting BLAN_{mRFP} with different cholesterol densities into macrophages and tumor cells, respectively. For macrophages, RFP expression levels declined as the cholesterol density decreased within the range of tested cholesterol densities (40%–1.25%, Supporting Information Fig. S4A–S4C). In contrast, the percentage of RFP⁺ cells was approximately 76% when cholesterol density was reduced to 20%, and subsequent changes in cholesterol density did not affect RFP expression in tumor cells (Fig. S4D–S4F). These results collectively suggest that cholesterol density in BLAN influences mRNA expression in dendritic cells, demonstrating a negative correlation with cholesterol density. This observation highlights the importance of cholesterol optimization for enhancing mRNA delivery and expression in DCs, a critical step in improving the efficacy of gene-editing strategies for immunotherapy.

We further investigated the impact of BLAN with different cholesterol densities on the encapsulation efficiency and release profile of mRNA, considering the substantial molecular weight difference between Cas9 mRNA (~4500 nt) and sgRNA (~100 nt). For the low molecular weight mRNA (RFP mRNA), the encapsulation efficiencies of BLAN_{mRFP} with different cholesterol densities were consistently above 95% (Fig. 1D). In the release assay, 0% cholesterol BLAN_{mRFP} exhibited the highest release rate, 5% cholesterol BLAN_{mRFP} demonstrated an intermediate release rate, and 40% cholesterol BLAN_{mRFP} showed the lowest release (Fig. 1E). Regarding the encapsulation efficiency of large molecular weight mRNA (Cas9 mRNA), BLAN effectively maintained high encapsulation efficiency, all above 94% (Fig. 1F). Interestingly, we observed no difference in the release rate of mRNA among BLAN with 40%, 5% and 0% cholesterol density

(Fig. 1G). These results demonstrate that neither the mRNA weight nor the cholesterol density significantly affects the RNA loading capacity of BLAN. However, cholesterol content plays a crucial role in governing the release of low molecular weight mRNA, where the higher cholesterol density leads to a slower release rate of RNA.

Next, we investigated the function of BLAN with different cholesterol densities in DCs. BLAN with 40%, 5%, and 0% cholesterol density were successfully utilized to simultaneously load Cas9 mRNA and sgRNA (BLAN_{mCas9/gRNA}). Notably, the BLAN_{mCas9/gRNA} variants with different cholesterol densities displayed sizes ranging from 116 to 135 nm and zeta potential ranging from 5.4 to 6.4 mV (Fig. 1H and I). The polydispersity of different cholesterol content BLAN_{mCas9/gRNA} ranged from 0.2 to 0.3 (Fig. 1J). Interestingly, BLAN_{mCas9/gRNA} with 0% cholesterol density exhibited a slightly larger size. Importantly, no discernible disparity was observed in the ability of the BLAN with different cholesterol densities to deliver both Cas9 mRNA and sgRNA, with all encapsulation efficiencies exceeding 94% (Fig. 1K). To assess the efficacy of BLAN in entering DCs, we employed fluorescent dye-labeled mRNA (Cy5-Cas9 mRNA)-encapsulated BLAN *in vitro*. Following co-incubation with DC1.2 for 2 h, internalization was examined by flow cytometry. At low concentrations, the proportion of Cy5-positive cells gradually increased with decreasing cholesterol density (Supporting Information Fig. S5). Moreover, at higher concentrations, the MFI of Cy5 significantly increased in the 0% cholesterol BLAN_{Cy5-mCas9} group, even though the proportion of Cy5⁺ cells remained constant across BLAN with different cholesterol densities (Fig. 1L). Notably, as the cholesterol density decreased, the MFI of Cy5 steadily increased. These results suggest that DCs exhibit higher efficiency in internalizing BLAN with low cholesterol density.

We conducted further experiments to confirm the endosomal escape capability of BLAN with different cholesterol densities following internalization by DCs. BLAN_{Cy5-mCas9} with varying cholesterol densities was incubated with DC1.2 for 2 h, followed by an additional 6-h culture period. The co-localization of Cy5-mRNA with the late lysosomal marker, lysosomal-associated membrane protein 1 (LAMP1), was examined using confocal microscopy. We observed that Cy5-mRNA mainly co-localized with LAMP1 in DCs treated with 40% and 5% cholesterol BLAN_{Cy5-mCas9}. However, this co-localization was significantly reduced in DCs treated with 0% cholesterol BLAN_{Cy5-mCas9} (Fig. 1M). These results indicate that cholesterol in BLAN inhibited mRNA escape from the endosomes. Taken together, our findings demonstrate that BLAN with low cholesterol density exhibits higher mRNA expression ability due to enhanced uptake and endosomal escape capabilities.

The gene editing capability of BLAN_{mCas9/gRNA} in DCs was thoroughly investigated. The proportion of PD-L1^{hi} cells was markedly reduced in DC1.2 cells treated with 0% cholesterol BLAN_{mCas9/gPD-L1}, while there was no significant effect observed in cells treated with 40% or 5% cholesterol BLAN_{mCas9/gPD-L1} compared to PBS control (Fig. 1N). As a result, we selected 0% cholesterol BLAN_{mCas9/gPD-L1} for further investigation in subsequent experiments.

To study the properties of Cas9 mRNA and sgRNA co-loaded into BLANs with 0% cholesterol, we assessed the sustained gene editing efficiency at the genomic level using the CRISPR/Cas9 system. We specifically targeted PD-L1, as it plays a crucial role in regulating T cell activation when expressed on DCs, and blocking PD-L1 on DCs offers an enticing avenue for potentiated

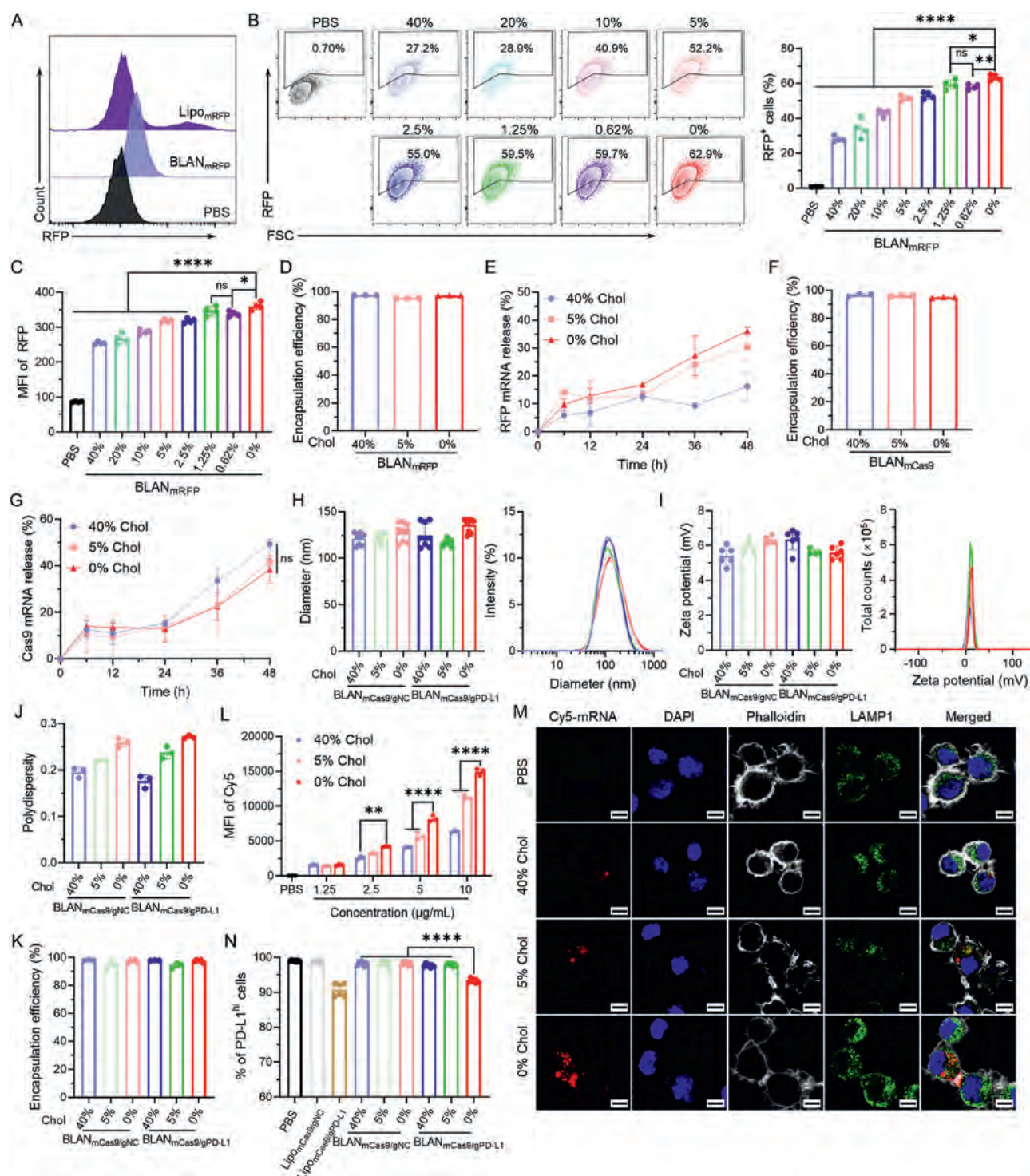


Figure 1 Influence of cholesterol in BLAN on mRNA expression in DCs. (A) Representative flow cytometry analysis of the RFP expression in DC1.2 cells treated with PBS, Lipo_{mRFP} or BLAN_{mRFP} (B, C) The percentage of RFP⁺ cells (B) and MFI of RFP (C) in DC1.2 cells treated with BLAN_{mRFP} containing different cholesterol densities for 24 h assayed by flow cytometry ($n = 4$ per group). (D, E) The encapsulation efficiency (D) and release profile (E) of RFP mRNA of BLAN_{mRFP} with 40%, 5%, and 0% cholesterol. (F, G) The encapsulation efficiency (F) and release profile (G) of Cas9 mRNA of BLAN_{mCas9} with 40%, 5%, and 0% cholesterol. (H–J) Particle size (H), zeta potential (I), and polydispersity (J) distributions for BLAN_{mCas9/gNC} and BLAN_{mCas9/gPD-L1} with different cholesterol densities measured by dynamic light scattering. (K) Comprehensive mRNA encapsulation efficiency (Cas9 mRNA + sgRNA) for 40%, 5%, and 0% cholesterol BLAN_{mCas9/gRNA}. (L) Quantitative evaluation of cellular uptake of Cas9 mRNA by DC1.2 cells following a 2-h incubation with BLAN_{Cy5-mCas9} containing different cholesterol densities using flow cytometry. The Cy5-Cas9 mRNA concentration was 1.25, 2.5, 5, or 10 $\mu\text{g/mL}$ ($n = 3$ per group). (M) Confocal laser scanning microscopy (CLSM) images of DC1.2 cells treated with PBS, 40% cholesterol BLAN_{Cy5-mCas9}, 5% cholesterol BLAN_{Cy5-mCas9}, or 0% cholesterol BLAN_{Cy5-mCas9}.

tumor immunotherapy²⁷⁻³¹. After transfecting DC1.2 cells with BLAN_{mCas9/gPD-L1} for 6 days, the frequency of PD-L1^{hi} cells and MFI of PD-L1^{low} cells were substantially reduced when cells were treated with BLAN_{mCas9/gPD-L1}, in contrast to PBS or BLAN_{mCas9/gNC} treatments (Fig. 2A–C). This indicates that BLAN delivering Cas9 mRNA and PD-L1 sgRNA achieves prolonged and efficient gene editing. To validate the targeted disruption efficiency of the endogenous PD-L1 gene, we performed the T7 endonuclease I (T7E1) cleavage assay and next-generation sequencing (Fig. 2D). The results showed that gene mutation occurred in the target PD-L1 gene, with a mutation frequency of 16.6% for BLAN_{mCas9/gPD-L1} (Fig. 2E). Notably, the degree of gene editing was higher when using BLAN_{mCas9/gPD-L1} compared to Lipo_{mCas9/gPD-L1} treatment, which resulted in a 7.9% mutation frequency. The PD-L1 gene underwent efficient editing at specific loci in response to BLAN_{mCas9/gPD-L1} treatment (Fig. 2F). These results demonstrate that BLAN_{mCas9/gPD-L1} enables effective and precise gene editing of PD-L1 in DCs *in vitro*, highlighting its potential as a robust tool for targeted gene modulation in immunotherapeutic strategies.

3.2. Targeted delivery of BLAN_{mCas9/gRNA} to dendritic cells *in vivo*

In our *in vitro* experiments, BLAN_{mCas9/gRNA} showed promising uptake by DCs and successful gene editing. To further assess the *in vivo* biodistribution and targeting capabilities of BLAN_{mCas9/gRNA}, we employed a mouse CT26 colon cancer model. We labeled BLAN_{mCas9/gNC} with DiD (BLAN_{mCas9/gNC}@DiD) and administered it *via* intratumoral injection in CT26 tumor-bearing mice. After 24 h, strong fluorescent signals were detected in the tumor and DLN. The fluorescence intensity was significantly higher in mice injected with BLAN_{mCas9/gNC}@DiD in both the tumor (Fig. 3A) and DLN (Fig. 3G). However, little or no fluorescent signal was observed in other organs, including the brain, heart, liver, spleen, lung, and kidney (Supporting Information Fig. S6A). These results indicate the efficient accumulation of BLAN_{mCas9/gRNA} within tumor and DLN, while minimizing distribution in other organs, thus enhancing the safety of this nanomedicine.

To achieve effective CRISPR/Cas9-based gene editing in DCs, it is crucial to deliver the CRISPR/Cas9 system to DCs within

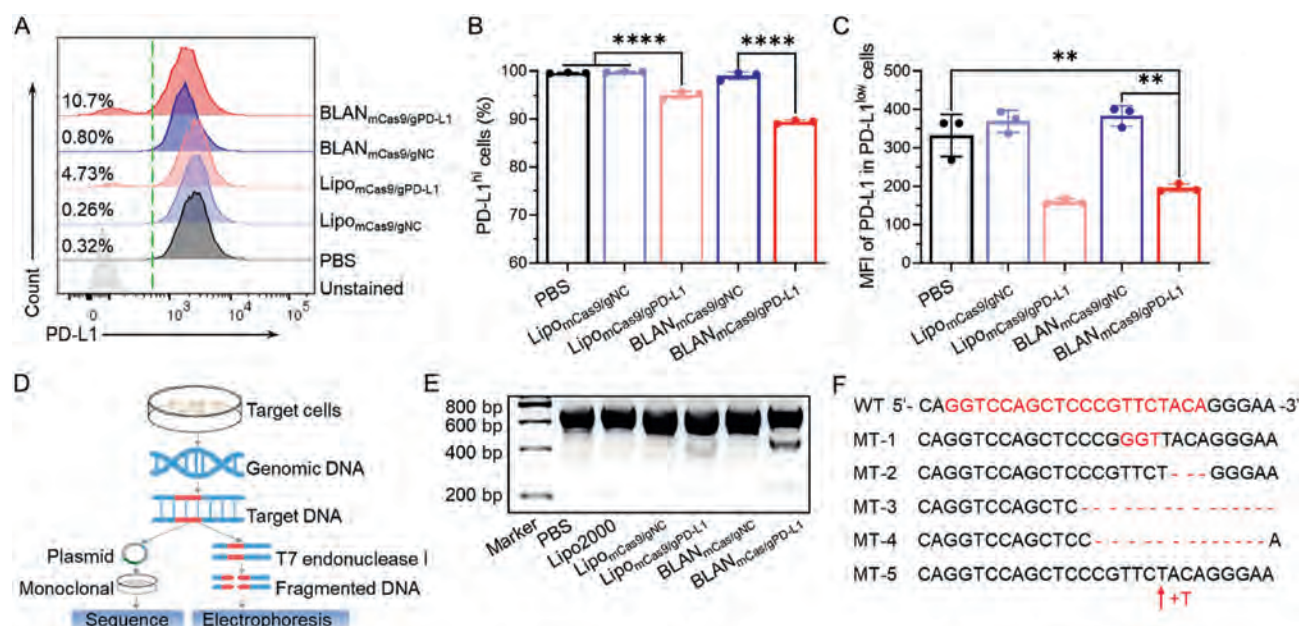


Figure 2 BLAN_{mCas9/gPD-L1} effectively knockdowns PD-L1 expression in DCs. (A) Representative flow cytometry analysis demonstrating PD-L1 expression in DC1.2 cells following a 6-day incubation with 0% cholesterol BLAN_{mCas9/gPD-L1} or other controls. (B, C) Quantification of PD-L1^{hi} cell frequency (B) and PD-L1 MFI (C) on DC1.2 cells treated with 0% cholesterol BLAN_{mCas9/gPD-L1} or other controls for 6 days. The Cas9 mRNA concentration was 10 μ g/mL ($n = 3$ per group). (D) Schematic illustration of the T7E1 assay and DNA sequencing procedure for evaluating PD-L1 gene editing in DC1.2 cells under BLAN_{mCas9/gPD-L1} treatment. (E) T7E1 cleavage assay was performed on DNA isolated from DC1.2 cells upon treatment with BLAN_{mCas9/gPD-L1} or other controls for 72 h. (F) Sequencing results of PD-L1 gene editing in DC1.2 cells treated with BLAN_{mCas9/gPD-L1} for 72 h. Data are presented as the means $\pm$ SD. ** $P < 0.01$; **** $P < 0.0001$.

BLAN_{Cy5-mCas9} at 37 $^{\circ}$ C for 2 h, followed by removal of nanoparticles and additional 6-h culture in complete DMEM. The Cas9 mRNA was labeled with Cy5 (red), endosome was stained with LAMP1 (green), cell nuclei were stained with DAPI (blue), and the cytoskeleton was stained with AF488 phalloidin (white). The dose of Cy5-Cas9 mRNA was 5 μ g/mL. Scale bar = 5 μ m. (N) The expression of PD-L1 in DC1.2 cells upon treatment with BLAN_{mCas9/gPD-L1} or other controls for 72 h was assayed by flow cytometry. The Cas9 mRNA concentration was 10 μ g/mL ($n = 6$ for Lipo_{mCas9/gNC} or Lipo_{mCas9/gPD-L1} group, and $n = 7$ for other groups). Data are presented as the means $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$; ns, not significant.

tumor and lymph node tissue after administration. We analyzed the distribution of BLAN_{mCas9/gRNA} in DCs using CLSM. The DCs exhibited high fluorescence in both the tumor (Fig. 3B) and DLN (Fig. 3H) in the BLAN_{mCas9/gNC}@DiD group. We further measured the ability of cDCs to uptake BLAN_{mCas9/gNC}@DiD using flow cytometry. There was no significant change in the proportion of cDC in the tumor (Fig. 3C). However, the percentage of DiD-positive cDC significantly increased in BLAN_{mCas9/gNC}@DiD-treated tumor (Fig. 3D). Conventional DCs can be further divided into two distinct subtypes: cDC1 and cDC2³². The percentages of DiD-positive cDC1 and cDC2 in the tumor were 49.00% and 68.16%, respectively (Fig. 3E and F). We also measured the distribution of BLAN_{mCas9/gNC}@DiD in tumor cells, and the results showed in Fig. S6B and C revealed that more than 20% of tumor cells exhibited DiD fluorescence signals. In comparison with the PBS-treated group, the number of cDC in the BLAN_{mCas9/gNC} groups was increased, with a 2.53-fold higher proportion in the DLN compared to the PBS group (Fig. 3I). Furthermore, cDC in the DLN also exhibited uptake of BLAN_{mCas9/gRNA} (Fig. 3J). The frequencies of DiD-positive cDC1 and cDC2 in the DLN were approximately 1.74% and 12.72%, respectively (Fig. 3K and L). These data demonstrated that BLAN carrying Cas9 mRNA and gRNA can be effectively taken up by cDC1 and cDC2 in both tumor and DLN tissues. In addition to cDCs, we also analyzed the distribution of BLAN_{mCas9/gNC}@DiD in other immune cells in tumor and DLN. As shown in Supporting Information Fig. S7A, S7C, and S7D, macrophages in both tumor and DLN were able to uptake BLAN_{mCas9/gNC}@DiD. While T cells, B cells, and NK cells in tumors were capable of uptaking BLAN_{mCas9/gNC}@DiD, their uptake efficiency is significantly lower than that of cDCs (Fig. S7B). Furthermore, there was little enrichment of BLAN_{mCas9/gNC}@DiD in T cells, B cells, and NK cells in DLNs (Fig. S7E). These findings highlight the potential of BLAN_{mCas9/gRNA} as a targeted nanomedicine for gene editing in DCs in the context of tumor immunotherapy.

3.3. BLAN_{mCas9/gPD-L1} significantly enhances antitumor immunity via DC modulation

To assess the impact of BLAN_{mCas9/gRNA} on tumor immunotherapy via gene editing in DCs, we employed a PD-L1 knockout (KO) CT26 colon cancer model. Stimulation of PD-L1 KO CT26 tumor cell lines with IFN- γ confirmed the complete knockout of PD-L1 expression in tumor cells (Supporting Information Fig. S8). The PD-L1 KO CT26 tumor-bearing mice received intratumoral injections of PBS, BLAN_{mCas9/gNC}, or BLAN_{mCas9/gPD-L1} every other day for a total of 10 injections. PD-L1 expression on DCs was evaluated post-treatment. Mice treated with BLAN_{mCas9/gPD-L1} showed a significant decrease in the proportion of PD-L1⁺ cells and MFI of PD-L1 on cDC1 in the DLN compared to the PBS- or BLAN_{mCas9/gNC}-injected controls (Supporting Information Fig. S9A and B). There was a significant decrease in the proportion of PD-L1⁺ cDC in tumor microenvironment of mice treated with BLAN_{mCas9/gPD-L1}. This finding was notably absent in mice treated with PBS or BLAN_{mCas9/gNC} (Fig. S9C). Immunohistochemistry analyses further confirmed a marked reduction in PD-L1 expression within tumors from mice treated with BLAN_{mCas9/gPD-L1} compared to counterparts receiving BLAN_{mCas9/gNC} or PBS injections (Fig. S9D). However, there was no difference in the proportion of PD-L1⁺ macrophages in tumors and DLNs after

BLAN_{mCas9/gPD-L1} treatment (Fig. S9E and S9F). These results collectively confirm that BLAN_{mCas9/gPD-L1} primarily targets and gene-edits DCs to reduce PD-L1 expression *in vivo*.

DCs, as professional antigen presenting cells, play a key role in the initiation and maintenance of anti-tumor T cell immunity^{33,34}. To elucidate the changes in DCs following BLAN_{mCas9/gPD-L1} treatment, experiments were conducted in PD-L1 KO CT26 tumor-bearing mice. These mice were treated with PBS, BLAN_{mCas9/gNC}, or BLAN_{mCas9/gPD-L1} through intratumoral injection for a total of 6 injections. Twenty-four hours after the final treatment, we harvested the tumor, DLN, and spleen to analyze the population, maturation, and antigen presentation ability of DCs using flow cytometry (Fig. 4A and Supporting Information Fig. S10). In tumors, the proportion of cDC significantly increased in mice treated with BLAN_{mCas9/gPD-L1}, but not in those receiving PBS or BLAN_{mCas9/gNC} (Fig. 4B). Specifically, cDC2, which plays a crucial role in the antitumor immune response, exhibited a significant increase in mice treated with BLAN_{mCas9/gPD-L1} ($31.10 \pm 2.60\%$) compared to those injected with PBS ($19.33 \pm 1.72\%$) or BLAN_{mCas9/gNC} ($24.24 \pm 2.90\%$) (Supporting Information Fig. S11A). Additionally, BLAN_{mCas9/gPD-L1} treatment promoted the maturation of DCs in tumors, as evidenced by the increased frequency of CD40⁺ cDC and CD86⁺ cDC compared to PBS or BLAN_{mCas9/gNC} (Fig. 4C). CD80⁺ cDC also showed a tendency toward an increase in tumor after BLAN_{mCas9/gPD-L1} treatment (Fig. S11B). Similarly, the frequencies of CD40⁺ cDC2, CD80⁺ cDC2, and CD86⁺ cDC2 in tumors significantly increased in BLAN_{mCas9/gPD-L1} group (Fig. S11C). Furthermore, BLAN_{mCas9/gPD-L1} administration modulated the immunosuppressive tumor microenvironment, as evidenced by a significant decrease (2.13-fold) in M2 macrophages and a concurrent increase (1.91-fold) in M1 macrophages (Fig. 4D and E). This shift from M2 to M1 macrophages can inhibit tumor growth and support antitumor immunity. We also analyzed the effect of BLAN_{mCas9/gPD-L1} on tumor-infiltrating T cells. The frequency of CD8⁺ T cells was increased to $4.29 \pm 0.96\%$, which was 2.09-fold and 1.98-fold higher than those in the PBS ($2.05 \pm 0.60\%$) or BLAN_{mCas9/gNC} ($2.17 \pm 0.51\%$) groups, respectively (Fig. 4F). Moreover, significant increases in activated CD8⁺ T cells and activated CD8⁺/CD4⁺ T cell ratios were detected in tumors treated with BLAN_{mCas9/gPD-L1}, compared to PBS- and BLAN_{mCas9/gNC}-injected groups (Fig. 4G and H). In addition, the expression of cytokines in the tumors was analyzed, revealing a notable increase in IL-12 but a significant decrease in IL-6 in mice treated with BLAN_{mCas9/gPD-L1} compared to those treated with PBS or BLAN_{mCas9/gNC} (Fig. S11D). These findings collectively indicate that co-delivery of Cas9 mRNA and PD-L1 sgRNA by BLAN_{mCas9/gPD-L1} results in promoting DC maturation, modulating the tumor microenvironment, and enhancing antitumor immune responses.

To further investigate the influence of BLAN_{mCas9/gPD-L1} treatment on leukocytes in the DLN and spleen, we analyzed their composition, activation status, and function. In the DLN, we observed a significant increase in cDC (Fig. 4I and J), cDC1 (Fig. 4K), and cDC2 (Supporting Information Fig. S12A) after BLAN_{mCas9/gPD-L1} treatment. Compared to the PBS and BLAN_{mCas9/gNC}, BLAN_{mCas9/gPD-L1} treatment resulted in a higher number of CD40⁺ cDC, CD80⁺ cDC (Fig. 4L), CD86⁺ cDC (Fig. S12B), CD3⁺ T cells (Fig. 4M), and CD8⁺ T cells (Fig. 4N) in the DLN. Furthermore, we detected an amplified proportion and numerical increase of CD40⁺ cDC1,

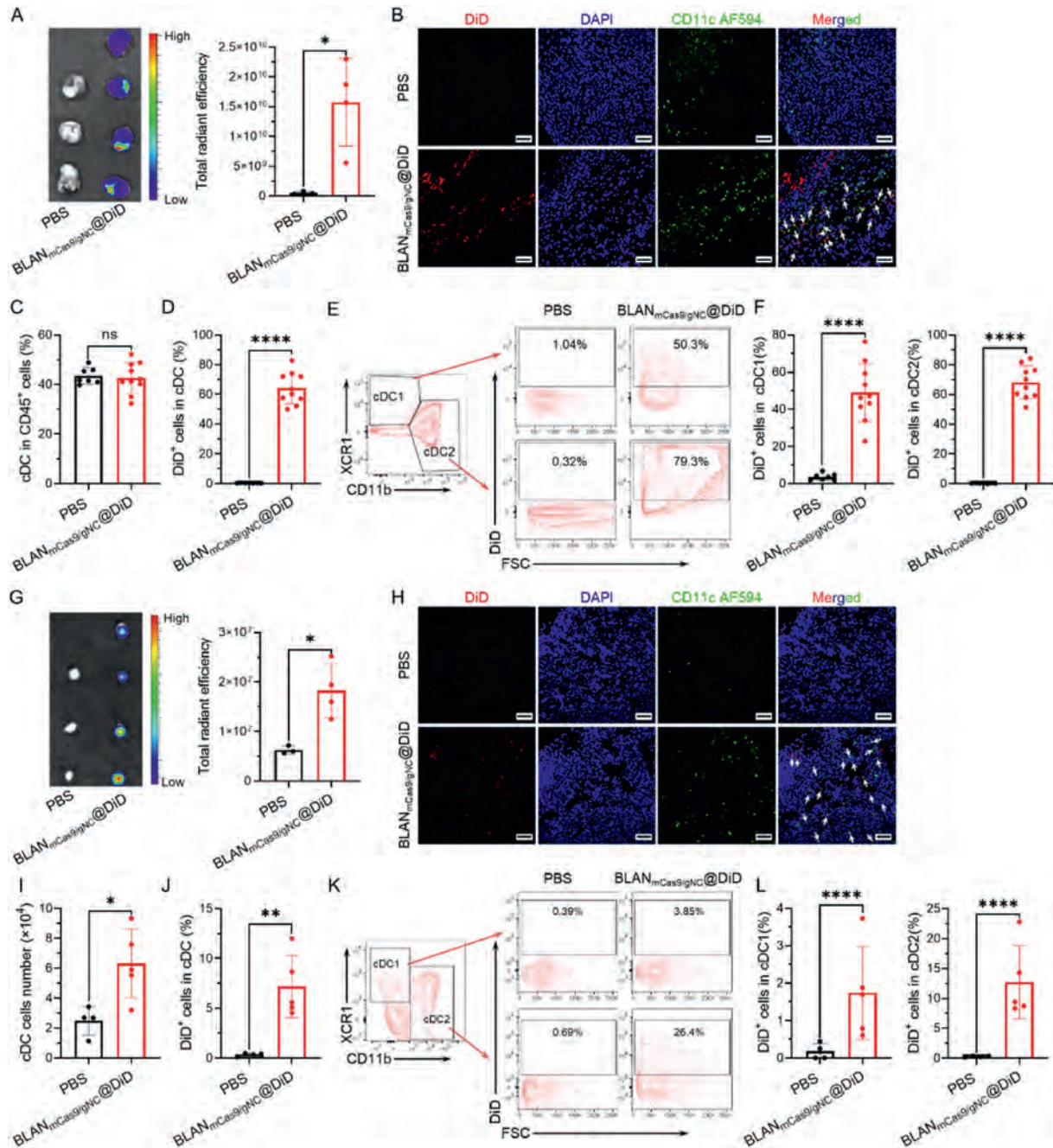
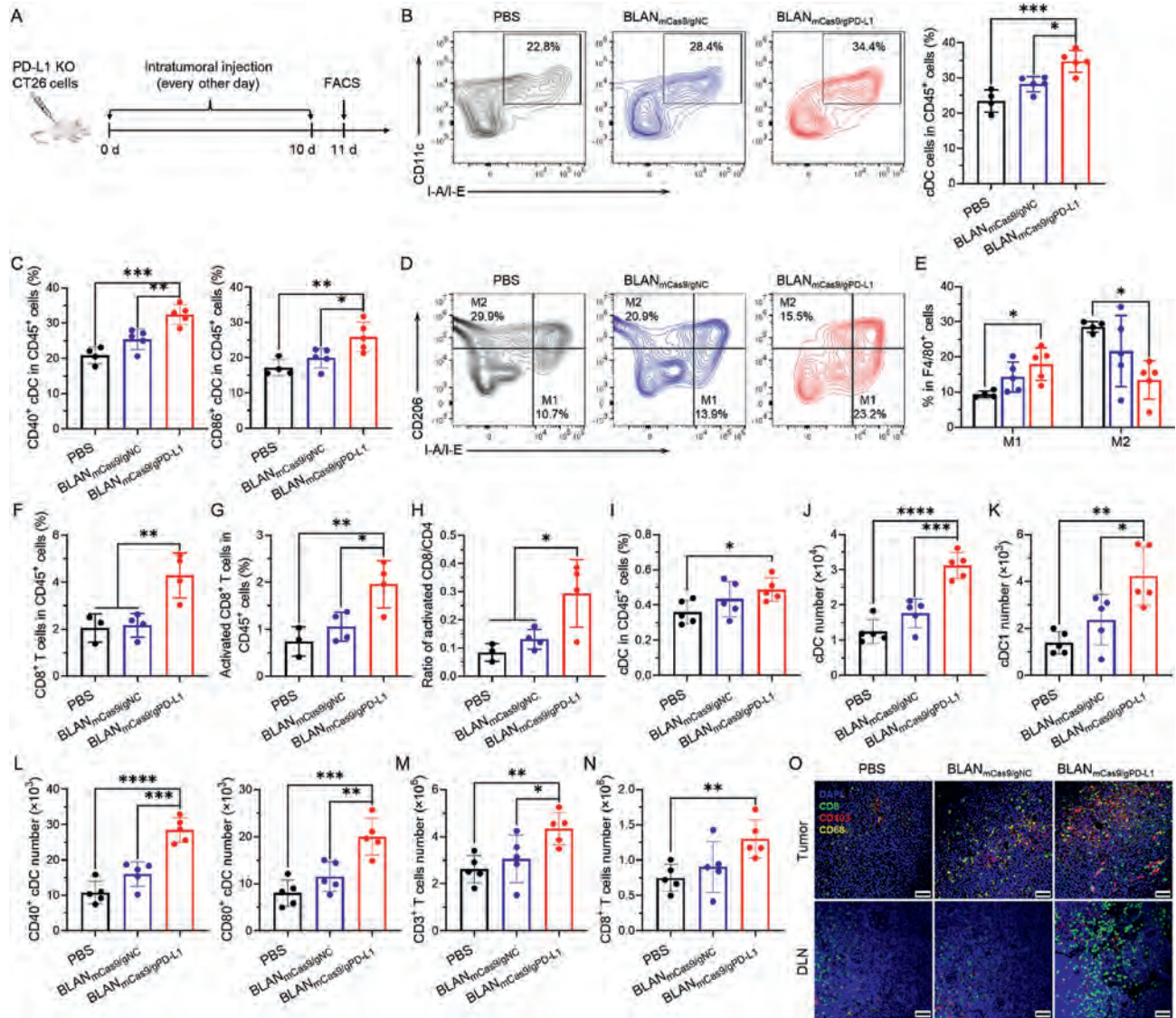


Figure 3 BLAN_{mCas9/gRNA} simultaneously delivers CRISPR/Cas9 into DCs *in vivo*. (A) IVIS fluorescent images and quantification of DiD signals in CT26 tumors harvested at 24 h after intratumoral injection of BLAN_{mCas9/gNC}@DiD or PBS control ($n = 4$ for BLAN_{mCas9/gNC}@DiD group, and $n = 3$ for PBS group). (B) CLSM images showed the distribution and cellular uptake of BLAN_{mCas9/gNC}@DiD (red) by DCs in tumor tissues after administration. DCs were stained with anti-CD11c antibodies (green), and cell nuclei were stained with DAPI (blue). White arrows indicate colocalization of BLAN_{mCas9/gNC}@DiD with DCs. Scale bar = 50 μ m. (C, D) Frequencies of cDC (CD11c⁺ I-A/I-E⁺) (C) and DiD⁺ cDC (D) in tumors after intratumoral injection of BLAN_{mCas9/gNC}@DiD or PBS control ($n = 10$ for BLAN_{mCas9/gNC}@DiD group, and $n = 7$ for PBS group). (E, F) Representative flow cytometry plots of DiD⁺ cDC1 (XCR1⁺ CD11b⁻) and DiD⁺ cDC2 (XCR1⁻ CD11b⁺) (E), and frequencies of DiD⁺ cells in cDC1 and cDC2 (F) in the tumor tissues detected by flow cytometry ($n = 10$ for BLAN_{mCas9/gNC}@DiD group, and $n = 7$ for PBS group). (G) IVIS fluorescent images and quantification of DiD signals in DLN after intratumoral injection of BLAN_{mCas9/gNC}@DiD or PBS control ($n = 4$ for BLAN_{mCas9/gNC}@DiD group, and $n = 3$ for PBS group). (H) CLSM images showed the distribution and cellular uptake of BLAN_{mCas9/gNC}@DiD (red) by DCs in DLN. DCs were stained with anti-CD11c antibodies (green), and cell nuclei were stained with DAPI (blue). White arrows indicate colocalization of BLAN_{mCas9/gNC}@DiD with DCs. Scale bar = 50 μ m. (I, J) The cell number of cDC (I) and frequency of DiD⁺ cells in cDC (J) in DLN after intratumoral injection of BLAN_{mCas9/gNC}@DiD or PBS control ($n = 5$ for BLAN_{mCas9/gNC}@DiD group, and $n = 4$ for PBS group). (K, L) Representative flow cytometry plots of DiD⁺ cDC1 and cDC2 (K) and frequencies of DiD⁺ cells in cDC1 and cDC2 (L) in DLN ($n = 5$ for BLAN_{mCas9/gNC}@DiD group, and $n = 4$ for PBS group). Data are shown as the means $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$; ns, not significant.



CD80⁺ cDC1, and CD86⁺ cDC1 in mice subjected to BLAN_{mCas9/gPD-L1} treatment (Fig. S12C). Immunofluorescence microscopy corroborated these findings, revealing a substantial increase of CD8⁺ T cells and cDC1 in both tumor tissues and

DLN in mice received BLAN_{mCas9/gPD-L1} treatment (Fig. 4O). These results demonstrate that enhanced activation of cDCs likely contributes to the promotion of T cells anti-tumor immune responses.

Similarly, in the spleen, we found that cDC1 were accumulated after BLAN_{mCas9/gPD-L1} treatment (Supporting Information Fig. S13A and S13B). Although there was no difference in cDC2 among the groups, the ratio of cDC1/cDC2 was significantly increased in BLAN_{mCas9/gPD-L1}-treated mice (Fig. S13C and D). Moreover, the proportion of CD80⁺ cDC (Fig. S13E) and MFI of CD80 (Fig. S13F) were significantly increased in mice treated with BLAN_{mCas9/gPD-L1} compared to PBS-injected control. The frequencies of CD80⁺ cDC1 and CD80⁺ cDC2 were also markedly increased by BLAN_{mCas9/gPD-L1} treatment (Fig. S13G). Analyzing the changes in T cells, we found that the percentage of CD8⁺ T cells (Fig. S13H) and the ratio of CD8/CD4 (Fig. S13I) in the spleen were significantly increased in mice treated with BLAN_{mCas9/gPD-L1}, but not with BLAN_{mCas9/gNC}, compared with the PBS. These results collectively illustrate that BLAN_{mCas9/gPD-L1} treatment promotes DC enrichment and maturation in both the DLN and spleen.

3.4. BLAN_{mCas9/gPD-L1} suppresses tumor growth by targeting PD-L1 on cDCs

We investigated the antitumor efficacy of BLAN_{mCas9/gPD-L1} *in vivo* using the PD-L1 KO CT26 tumor model. Tumor-bearing mice received intratumoral injection of PBS, BLAN_{mCas9/gNC}, or BLAN_{mCas9/gPD-L1} every other day for a total of 10 injections. The impact on tumor growth was assessed. As shown in Fig. 5A and B, treatment with BLAN_{mCas9/gNC} had minimal effect on tumor growth compared to the PBS group. In contrast, mice treated with BLAN_{mCas9/gPD-L1} exhibited significant tumor growth inhibition. At the end of the therapeutic experiment, the tumor weight was the smallest in the BLAN_{mCas9/gPD-L1} group compared to the control groups (Fig. 5C and Supporting Information Fig. S14). TUNEL staining of tumor sections showed increased apoptosis of tumor cells after BLAN_{mCas9/gPD-L1} therapy (Fig. 5D). Immunofluorescence analysis further demonstrated that the decelerated tumor

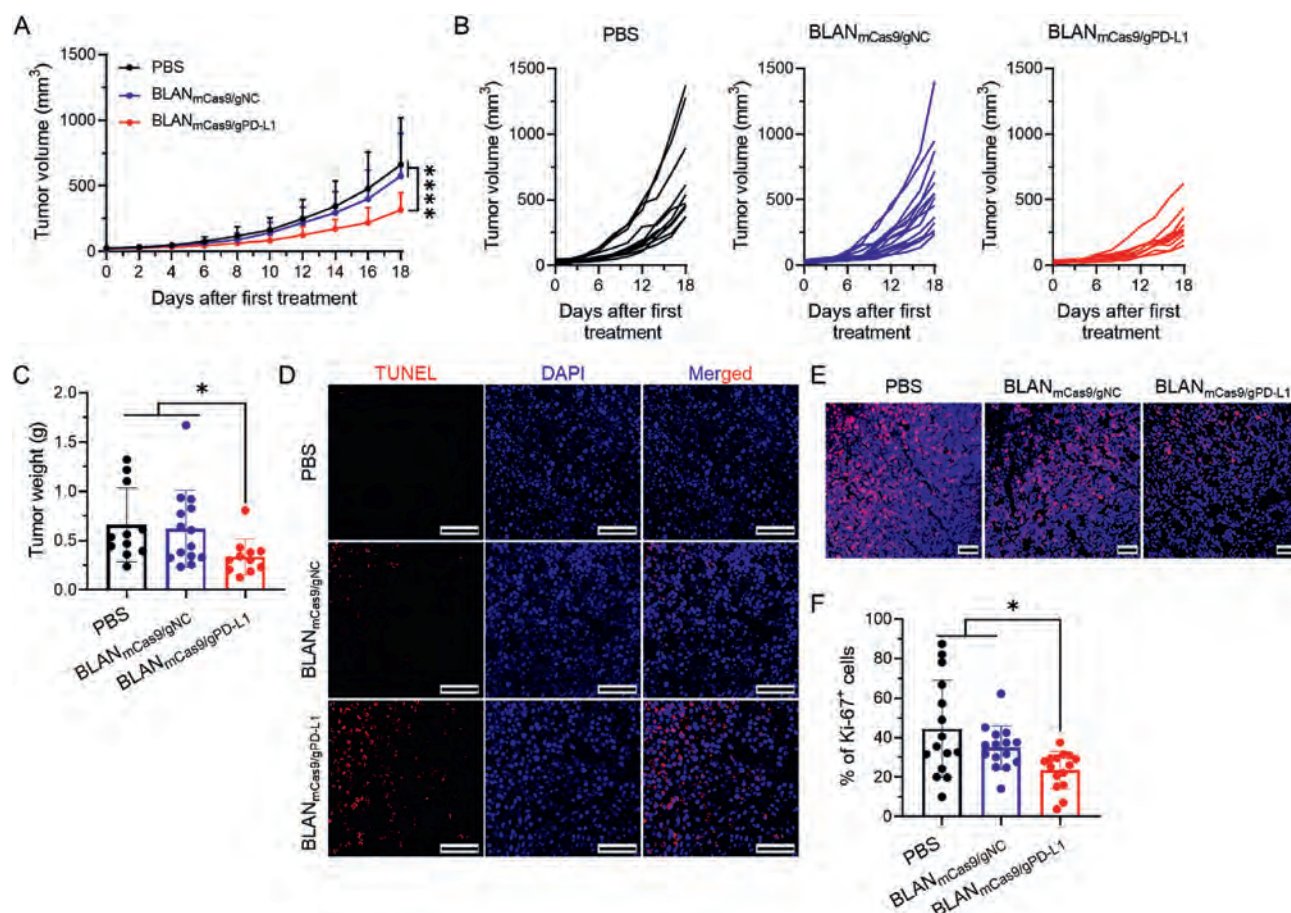


Figure 5 BLAN_{mCas9/gPD-L1} significantly inhibits PD-L1 KO CT26 tumor growth. (A–C) PD-L1 KO CT26 tumor-bearing mice were treated with PBS, BLAN_{mCas9/gNC}, or BLAN_{mCas9/gPD-L1} through intratumoral injection every other day for a total of 10 injections. Tumor growth curves (A), tumor-growth curves presented individually (B), and tumor weight on Day 19 (C) ($n = 11$ for PBS or BLAN_{mCas9/gPD-L1} group, and $n = 14$ for BLAN_{mCas9/gNC} group). (D) Representative images of apoptotic cells (red) in tumor tissues on Day 19 through TUNEL assay. Nuclei were stained with DAPI (blue). Scale bar = 50 μ m. (E, F) Representative immunofluorescence images of Ki-67 (red) (E) and statistical analysis of Ki-67⁺ cells (F) in tumor tissues at the end of therapeutic experiment. Nuclei were stained with DAPI (blue). Scale bar = 50 μ m. Data are presented as the means $\pm$ SD. * $P < 0.05$; *** $P < 0.0001$.

growth was accompanied by a reduced frequency of Ki-67⁺ tumor cells in mice treated with BLAN_{mCas9/gPD-L1} (Fig. 5E and F), providing additional evidence of the enhanced antitumor efficiency of this treatment. These results underscore the efficacy of BLAN_{mCas9/gPD-L1} in inhibiting tumor growth through targeted gene editing within cDCs. We also evaluated the therapeutic effect in WT CT26 tumors. As shown in Supporting Information Fig. S15A and S15B, BLAN_{mCas9/gPD-L1} treatment resulted in a significant antitumor effect. Additionally, the tumor volume and weight analysis further corroborated the superior antitumor efficacy of BLAN_{mCas9/gPD-L1} treatment, with tumors in this group showing a significant decrease in volume and weight relative to the control groups (Fig. S15C and S15D). These findings highlight the potent antitumor activity of BLAN_{mCas9/gPD-L1}, even in the context of tumors with high endogenous PD-L1 expression.

Finally, we evaluated the systemic toxicity of BLAN_{mCas9/gRNA} treatment. Long-term treatment with BLAN_{mCas9/gPD-L1} did not result in any statistically significant difference in body weight in PD-L1 KO CT26 tumor-bearing mice (Fig. S16A). Histological examination of tissues, including the heart, lung, liver, spleen, and kidney, from mice that received BLAN_{mCas9/gPD-L1} treatment did not show any visible tissue damage or inflammation in H&E staining (Fig. S16B). Blood routine examination of mice receiving intratumoral injection of BLAN_{mCas9/gRNA} for 10 doses showed that important hematology markers, such as red blood cell (RBC), hemoglobin (HGB), reticulocyte (RET), platelet (PLT), white blood cell (WBC), neutrophil granulocyte (NEUT), lymphocyte (LYMPH), and monocyte (MONO) were within the normal range, and there were no significant differences compared to the PBS group (Fig. S16C). These results demonstrate the high biocompatibility and low systemic toxicity of BLAN_{mCas9/gRNA} *in vivo*.

4. Discussion

The advent of CRISPR/Cas9 gene editing technology has brought about a revolution in molecular biology, offering the ability to make precise and efficient modifications in the genome of target cells^{35,36}. In contrast to RNAi or therapeutic DNazymes, which silence gene expression through mRNA degradation³⁷⁻³⁹, the CRISPR/Cas9 system regulates genes by inducing DNA double-strand breaks, which results in the knockout of the relevant gene and complete inhibition of protein expression³⁵. However, the full potential of CRISPR/Cas9 has been somewhat constrained by challenges like limited packaging capacity, insertional mutagenesis, and immunogenic side effects when delivered using viral vectors¹⁵. To overcome these limitations, nanotechnology-based non-viral vectors, such as polymers, LNPs, porous nanoparticles, and DNA nanostructures offer several advantages and have a wide range of applications^{16,40}. Among the various nano-delivery vectors for the CRISPR/Cas9 system, LNPs are currently the most widely used and clinically translatable nucleic acid drug delivery systems. LNPs come with numerous advantages, including excellent biocompatibility, high encapsulation efficiency, non-immunogenicity, and prolonged circulation^{26,41}. In our study, we concentrated on delivering Cas9 mRNA and sgRNA *via* LNPs, thus enabling efficient gene editing within target cells. This approach holds great promise for safe and effective gene editing.

While prior research has demonstrated the efficacy of BAMEA-O16B as a cationic lipid in LNPs for achieving highly efficient mRNA expression both *in vitro* and *in vivo*¹¹, the role of cholesterol in LNPs and its impact on mRNA encapsulation,

delivery, release, and expression have not been extensively explored. Addressing this knowledge gap, our study designed BLANs with different cholesterol densities. Remarkably, we found that BLANs with lower cholesterol density exhibited larger sizes and higher zeta potentials without compromising mRNA encapsulation efficiency. Particularly noteworthy was the regulation of small molecular weight mRNA release, which was significantly influenced by cholesterol density, resulting in enhanced gRNA release in DCs. Conversely, BLANs with high cholesterol density showed reduced cellular uptake and endosome escape, thereby limiting the cytoplasmic release of both Cas9 mRNA and sgRNA, ultimately leading to lower gene editing efficiency. These findings underscore the importance of optimizing cholesterol composition in LNPs to enhance gene editing efficacy within DCs.

Furthermore, our study prompts consideration of DCs in the treatment of immune-related diseases. DCs are vital immune system components, but they can malfunction, contributing to autoimmune conditions or excessive inflammation⁴²⁻⁴⁴. By editing key genes in DCs, like we did with PD-L1, we can potentially correct these issues. DCs also influence T cell behavior, making them crucial in immune regulation. Editing immune regulatory molecules in DCs, as demonstrated in our study, could modulate immune responses, beneficial for autoimmune diseases or enhancing anti-tumor immunity. Lastly, DCs can induce immune tolerance, a key factor in maintaining immune balance⁴⁵. By editing DCs to boost their immune-regulatory function, we may develop more effective treatments for immune-related diseases.

In the context of tumor immunity, PD-L1 expression on DCs plays an inhibitory role in the initiation and maintenance of anti-tumor T cell responses^{28-30,46}. In our study, we employed BLAN_{mCas9/gPD-L1} to specifically target and edit PD-L1 on DCs. The delivery of Cas9 mRNA and PD-L1 sgRNA enabled successful gene edit PD-L1 of DCs in DLN, with a particular emphasis on cDC1. This DC subset possesses direct antigen presentation capabilities to CD8⁺ T cells, thereby augmenting the function of CD8⁺ T cells, which are essential for effective anti-tumor immunity⁴⁷. Treatment with BLAN_{mCas9/gPD-L1} disrupted both the *trans*-interaction of PD-L1 and PD-1 and the *cis*-interaction of PD-L1 and CD80, effectively relieving the dual inhibition of T cells and significantly enhancing the function of cytotoxic T lymphocytes (CTLs)²⁷. Furthermore, BLAN_{mCas9/gPD-L1} treatment promoted the expression of surface activation markers (CD40, CD80, and CD86) on cDC1 and increased their presence with tumors and DLNs, further amplifying the anti-tumor immune response.

Remarkably, our findings demonstrated that BLAN_{mCas9/gRNA} was more effective in targeting and gene editing DCs compared to macrophages. Although both macrophages and DCs internalized BLAN_{mCas9/gNC}@DiD comparably within tumors, our *in vitro* experiments revealed that BLAN-mediated mRNA expression was notably less efficient in macrophages compared to DCs. The substantial proportion of cDCs in DLN, coupled with the superior mRNA expression capacity in DCs, exemplified the potent targeting and gene editing capabilities of BLAN_{mCas9/gRNA} within DCs. Furthermore, studies have shown that PD-L1 on DCs, not macrophages, plays a central role in tumor immunotherapy^{28,31}. Importantly, the significant reduction in PD-L1 expression on cDC1 in DLN validated the efficacy of BLAN_{mCas9/gPD-L1} in editing this immune cell subset. Such targeted gene editing in DLN holds great promise for enhancing anti-tumor immune responses and bolstering the overall therapeutic outcomes.

Furthermore, it is essential to emphasize that the BLAN_{mCas9/gRNA} treatment did not induce any visible tissue damage or inflammation, as evidenced by histology and blood routine examination. These results further corroborate the high biocompatibility and safety profile of this delivery system, which is crucial for its potential translation to clinical applications.

5. Conclusions

This study highlights the pivotal role of optimizing cholesterol composition in BLANs to significantly enhance gene editing within DCs. This approach can effectively promote DC activation, enhance T cell function, and ultimately improve the anti-tumor immune response. These findings highlight the potential of using BLAN_{mCas9/gRNA} as an intelligent system for delivering Cas9 mRNA and sgRNA to cDCs for gene editing *in vivo*. The potential of this strategy in the realm of immunotherapy for immune-related diseases demands further in-depth exploration and rigorous clinical investigation.

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Author contributions

Kuirong Mao designed, performed, and analyzed data from most of the studies, as well as helped write the manuscript with input from all authors. Huizhu Tan and Xiuxiu Cong assisted in orthotopic tumor inoculation. Ji Liu and Ming Wang conducted chemical synthesis of BAMEA-O16B lipid. Yanbao Xin, Jialiang Wang, Meng Guan, Jiaxuan Li, Ge Zhu, Xiandi Meng, and Guojiao Lin helped in the performance of flow cytometry studies. Haorui Wang and Jing Han aided in immunofluorescence staining experiments. Yong-Guang Yang and Tianmeng Sun conceived, designed, and supervised all studies and participated in the drafting and editing of the manuscript. All the authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2024.08.030>.

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