

Anti-Inflammatory Lipid Nanoparticles Derived from Kaempferol: A Dual- Functional mRNA Delivery Platform for Lung Cancer Therapy

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Abstract

To address pulmonary inflammation and poor endosomal escape of nebulized lipid nanoparticles (LNPs) for mRNA delivery, we developed LNPs incorporating the natural anti-inflammatory compound kaempferol (Kae). Kae-doped LNPs ("Kaesomes") showed potent anti-inflammatory effects in mice, significantly reducing neutrophil and lymphocyte infiltration after nebulization. They exhibited a unique "budding" morphology. Replacing DSPC with DOPE yielded optimized 5% Kae-DLNP, which increased lysosomal escape 2.3-fold and EGFP-mRNA transfection 9.5-fold in A549 cells versus standard LNPs. It delivered therapeutic p53-mRNA, potentially killing cancer cells, while reducing inflammatory toxicity 17-fold. In a lung metastasis model, Kae-DLNP achieved significant therapeutic efficacy without observable systemic toxicity in major organs. This strategy concurrently mitigates nebulization-induced inflammation and enhances cytoplasmic mRNA delivery by integrating a natural immunomodulator into LNPs, offering a promising avenue for pulmonary mRNA therapeutics.

1. Introduction

The rise of mRNA therapeutics, exemplified by the rapid clinical deployment of COVID-19 vaccines, has marked a paradigm shift in modern medicine. The clinical success of this modality is fundamentally underpinned by lipid nanoparticle (LNP) technology, which effectively protects and delivers fragile mRNA cargo to cells^[1–3]. Building on this foundation, pulmonary delivery via nebulization presents a highly attractive route for treating a wide range of respiratory diseases, including genetic disorders, infections, and cancers, offering direct targeting of the lung tissue while bypassing systemic first-pass metabolism^[4–6]. However, the translation of nebulized LNP-mRNA formulations faces two formidable and interconnected biological barriers that severely limit their efficacy and safety profile. First, the cationic lipids integral to LNP formulation and endosomal escape often provoke significant pulmonary inflammation. This innate immune activation, characterized by neutrophil infiltration and cytokine release, can lead to acute lung injury, reduce therapeutic tolerability, and potentially obscure the intended therapeutic effect^[7–9]. Second, even upon successful cellular uptake, the vast majority of internalized LNPs (> 96%) are sequestered within endosomal pathways and ultimately degraded in lysosomes, resulting in an abysmally low cytoplasmic delivery efficiency (< 4%)^[10–11]. This "endosomal trap" drastically limits the bioavailability of the mRNA payload, necessitating high and potentially toxic doses to achieve a therapeutic effect^[12–14]. Therefore, developing a next-generation platform that concurrently mitigates immunotoxicity and enhances endosomal escape is a critical unmet need for pulmonary mRNA therapeutics.

Inspired by nature's own sophisticated mechanisms for navigating biological barriers, we turned to botanical sources for a biocompatible solution^[15–17]. Many plants have evolved to produce compounds that modulate immune responses in their environment. Kaempferol (Kae), a natural flavonoid polyphenol abundantly found in various fruits and vegetables and known for its role in mitigating pollen-associated immune reactions, is a prime candidate. It possesses well-documented, potent anti-inflammatory,

antioxidant, and membrane-stabilizing properties^[18–20]. We postulated that strategic incorporation of Kae into the LNP matrix could serve a dual purpose. Primarily, it could act as a built-in anti-inflammatory agent, directly quenching the reactive oxygen species (ROS) and inhibiting the NLRP3 inflammasome activation often triggered by cationic lipid components, thereby addressing the toxicity issue at its source^[21–23]. Secondly, its amphiphilic nature and ability to modulate lipid packing could influence the biophysical properties of the LNP bilayer. This modulation might enhance membrane fluidity and promote destabilization of the endosomal membrane, a mechanism akin to that of some viral fusion peptides, thereby facilitating more efficient endosomal escape and cytoplasmic release of mRNA^[24–27]. This bio-inspired, dual-function strategy seeks to break the traditional efficacy-toxicity trade-off by making the carrier itself a part of the therapeutic solution.

Herein, we report the rational design, comprehensive characterization, and biological validation of Kaempferol-incorporated LNPs (Kaesomes) as an advanced platform for safe and efficient pulmonary mRNA delivery. We systematically doped LNPs with Kae at two concentrations (2% and 5% w/w) and evaluated their performance. Our studies demonstrate that nebulized Kae-LNPs significantly attenuate acute pulmonary inflammation in mice, showing a dose-dependent reduction in neutrophil, T cell, B cell, DC, and NK cell infiltration. To further amplify the endosomal escape capability, we engineered an optimized formulation (5% Kae-DLNP) by replacing the helper lipid DSPC with the fusion-prone lipid DOPE^[28–29]. This final construct exhibits a unique "budding vesicle" morphology, excellent nebulization stability, and enhanced lung retention. Mechanistic in vitro studies reveal that 5% Kae-DLNP achieves a 2.3-fold increase in lysosomal escape efficiency and potently suppresses NLRP3 inflammasome activation and ROS production in macrophages. Most importantly, it mediates outstanding mRNA delivery, boosting EGFP-mRNA transfection efficiency by 9.5-fold compared to standard LNPs in A549 lung epithelial cells. This high delivery efficiency translates into potent therapeutic outcomes, as 5% Kae-DLNP effectively delivers tumor-suppressive p53-mRNA, inducing potent cancer cell death, and shows compelling efficacy in a murine lung metastasis model. Crucially, this suite of enhanced functionalities is achieved with a dramatic 17-fold reduction in inflammatory toxicity and no observable systemic damage in major organs. This work presents a nature-inspired, integrative strategy that concurrently resolves the key challenges of toxicity and inefficient intracellular trafficking, paving the way for more effective and safer inhalable mRNA therapeutics.

2. Results

2.1 Kae-LNPs Significantly Attenuate Nebulization-Induced Pulmonary Inflammation In Vivo

To evaluate the potential of kaempferol (Kae) to mitigate the acute pulmonary inflammation commonly associated with inhaled LNPs, we doped LNPs with different concentrations of kaempferol (2% and 5%) and administered them to mice via nebulization. Twelve hours later, bronchoalveolar lavage (BAL) was performed to collect lung cells, and the positive rates of immune cells were analyzed to evaluate

inflammatory responses. Compared with plain LNPs, the positive rates of inflammation-related neutrophils, T cells, B cells, DCs, and NK cells were significantly decreased after Kae doping, and the inflammatory response further decreased with increasing Kae proportion, indicating that Kae-LNPs possess potent anti-inflammatory effects. Notably, nebulization of the nanomaterials did not significantly alter the proportion of macrophages, possibly because macrophages respond more slowly to inflammation (Fig. 3b-d, Figure S1, Supporting Information). In summary, kaempferol-doped LNPs exhibit marked anti-inflammatory effects and reduce the inflammation caused by nebulized materials.

2.2 Physicochemical Characterization and Enhanced Lung Retention of Kae-LNPs

Based on the excellent anti-inflammatory results, we next characterized 5% Kae-LNP and LNP. 5% Kae-LNP was synthesized by microfluidic mixing (Fig. 2a). Cryo-EM results showed that both LNP and 5% Kae-LNP had a size centered around 150 nm. Compared with the onion-like LNP, 5% Kae-LNP exhibited a budding vesicle structure (Fig. 2b). DLS measurements showed a hydrodynamic diameter of approximately 200 nm (LNP: 181.6 nm; 5% Kae-LNP: 225.7 nm), indicating a slight increase in size after Kae doping. After nebulization, both LNP and 5% Kae-LNP showed increased particle size and decreased PDI, indicating good nebulization stability (Fig. 2c,d, Figure S3, Supporting Information). The zeta potential decreased slightly after Kae doping (from 18.7 to 13.9 mV), possibly because Kae forms weak hydrogen bonds with the amine groups of cationic lipid headgroups, partially shielding some positive charge sites and reducing the effective positive charge in the aqueous phase (Fig. 2e).

Based on the favorable nebulization properties of 5% Kae-LNP, we administered LNP and 5% Kae-LNP via nebulization and analyzed pulmonary transfection and cellular uptake (Fig. 2f). First, IR820-loaded LNP and 5% Kae-LNP were delivered to mouse lungs by nebulization and imaged with a small animal NIR-II imaging system. Both in vivo and ex vivo results showed that the pulmonary accumulation of 5% Kae-LNP was significantly higher than that of LNP, indicating that Kae doping not only reduces toxicity but also effectively enhances pulmonary accumulation (Fig. 2g, h). Subsequently, Cy5-labeled LNP and Luc-mRNA-loaded LNP were administered via nebulization. Six hours later, mice were dissected, and major organs were collected for IVIS imaging. The results showed that the pulmonary accumulation of 5% Kae-LNP was 7.5-fold higher than that of LNP, and the Luc transfection efficiency was 11-fold higher, greatly improving pulmonary transfection efficiency (Fig. 2i, j). Further analysis of cellular uptake efficiency revealed that 5% Kae-LNP was significantly higher than LNP in pulmonary endothelial cells, epithelial cells, and macrophages (Fig. 2k-m, Figure S4, Supporting Information). These exciting data indicate that Kae incorporation can effectively enhance the pulmonary transfection efficiency of LNPs, with particularly significant uptake efficiency in epithelial cells.

2.3 Enhanced Cellular Uptake and Lysosomal Escape with DOPE-Modified Kae-DLNP

For the purpose of further enhancing endosomal escape efficiency, we replaced the helper lipid DSPC with the fusogenic lipid DOPE to formulate 5% Kae-DLNP, and systematically investigated its cellular uptake, intracellular trafficking, membrane fusion activity, and endocytic pathways in A549 lung epithelial cells using Cy5-labeled formulations (DMG-PEG-Cy5). We first evaluated lysosomal escape efficiency by co-localization studies with lysosomal markers (LysoTracker Green), and Pearson's correlation coefficients were calculated for quantitative assessment. 5% Kae-DLNP showed significantly reduced co-localization with lysosomes, supported by a low Pearson's correlation coefficient (decreased from 0.38 for standard LNP to - 0.23 for 5% Kae-DLNP), confirming that the combination of Kae and DOPE synergistically enhances lysosomal escape, thereby facilitating greater cytoplasmic delivery (Fig. 4a, b). We then assessed cellular uptake by confocal microscopy and quantitative flow cytometry. 5% Kae-DLNP exhibited the strongest red fluorescence signal, indicating superior cellular uptake compared to LNP, 2% Kae-LNP, and 5% Kae-LNP (Fig. 4c, Figure S5, Supporting Information). Notably, the enhanced cellular uptake, together with the reduced lysosomal entrapment, synergistically contributes to the superior cytoplasmic delivery of 5% Kae-DLNP.

The membrane fusion/destruction activity of the LNPs with endosomal membranes was directly quantified using a Förster resonance energy transfer (FRET)-based assay with endosome-mimicking liposomes co-loaded with NBD-PE (donor) and Rho-PE (acceptor). These liposomes (250 μ M lipid) were incubated with each LNP formulation (250 μ g/mL) in PBS (pH 5.5) at 37°C for 2 h, and fluorescence emission was recorded at 535 nm (NBD) and 565 nm (Rho) with excitation at 480 nm (Fig. 4d). The negative control (F_0) consisted of FRET liposomes alone, and the positive control (F_{100}) was obtained by adding 0.5% (v/v) Triton X-100. 5% Kae-DLNP exhibited the highest membrane fusion efficiency (19.2%), representing a 7.8-fold improvement over unmodified LNP (2.2%). This substantial enhancement is likely attributable to the synergistic effect of Kae's membrane-fluidity modulation and DOPE's propensity to form non-bilayer hexagonal phases, which together lower the energy barrier for endosomal membrane disruption (Fig. 4e, f).

We next sought to elucidate the internalization pathways through endocytic inhibition studies using specific pharmacological inhibitors. A549 cells were pre-incubated with Genistein (GEN, 100 μ M, caveolae-mediated endocytosis inhibitor), Chlorpromazine (CPZ, 10 μ g/mL, clathrin-mediated endocytosis inhibitor), or 5-(N-ethyl-N-isopropyl) amiloride hydrochloride (EIPA, 25 μ M, macropinocytosis inhibitor) for 30 min, followed by co-incubation with Cy5-labeled LNPs for 2 h. Cells treated with LNPs in the absence of inhibitors served as the positive control. Flow cytometric analysis of the mean fluorescence intensity revealed that as Kae incorporation increased, the predominant uptake pathway progressively shifted from clathrin-mediated endocytosis toward caveolae-mediated endocytosis. Notably, for the 5% Kae-DLNP formulation, the addition of DOPE further drove the internalization almost exclusively toward the caveolae-mediated pathway, which is known to facilitate more efficient cytosolic release by bypassing lysosomal degradation (Fig. 4g, Figure S6, Supporting Information). These results demonstrate that the combined modulation of lipid composition with Kae and DOPE not only enhances membrane fusion activity but also actively redirects the endocytic route toward a more favorable pathway for gene delivery and transfection.

2.4 In Vitro Anti-inflammatory Efficacy and Biocompatibility

We next evaluated the anti-inflammatory efficacy of the formulations in vitro using RAW 264.7 macrophages. NLRP3 inflammasome and the inflammatory marker p65 were visibly reduced in cells treated with Kae-doped LNPs, whereas standard LNP exhibited the highest expression levels of both markers, as visualized by immunofluorescence staining (Fig. 5a, Figure S7, Supporting Information). In an LPS-induced inflammation model, macrophages were stimulated with LPS (10 μ g/mL) for 12 h, followed by treatment with LNP, 2% Kae-LNP, 5% Kae-LNP, or 5% Kae-DLNP for an additional 12 h. Intracellular reactive oxygen species (ROS) levels were then assessed using a fluorescent ROS probe. Fluorescence microscopy and flow cytometric quantification demonstrated that 5% Kae-DLNP resulted in the most significant reduction in ROS levels among all treatment groups, confirming its potent antioxidant activity (Fig. 5b–d). MTT assays further verified the biocompatibility of the nanoparticles; RAW 264.7 macrophages were treated with 0.5 mg/mL of LNP, 2% Kae-LNP, 5% Kae-LNP, or 5% Kae-DLNP for 24 h, and none of the formulations exhibited significant cytotoxicity, with cell viability remaining above 90% across all groups (Fig. 5e). These results collectively demonstrate that Kae incorporation effectively suppresses NLRP3 inflammasome activation and ROS production without compromising macrophage viability, underscoring the excellent safety profile of the Kae-LNP platform.

2.5 Superior mRNA Delivery and Therapeutic Efficacy In Vitro

Having established the enhanced cellular uptake and endosomal escape of DOPE-modified Kae-DLNP, we next evaluated its mRNA delivery performance. 5% Kae-DLNP mediated the most potent EGFP expression, which increased progressively with higher Kae doping levels, and achieved a 9.5-fold increase in GFP-positive cells compared to standard LNPs (Fig. 5a–c). Using p53-mRNA as a therapeutic gene model, 5% Kae-DLNP induced the highest p53 protein expression and the most potent tumor cell killing, reducing A549 cell viability to approximately 8% (Fig. 5d–e). Furthermore, Acridine Orange (AO) staining, a metachromatic fluorescent assay in which AO emits red fluorescence in acidic lysosomes and green fluorescence in neutral cytosol, was used to distinguish lysosomally trapped mRNA from cytosolic mRNA. 5% Kae-DLNP exhibited predominantly green fluorescence with minimal red signal, indicating efficient cytosolic mRNA release, whereas the LNP group showed extensive lysosomal co-localization (Fig. 5f). Quantitative analysis showed that 5% Kae-DLNP achieved a cytosolic release efficiency of 62.3%, a 2.6-fold improvement over LNP (23.9%) (Fig. 5g). These results collectively demonstrate that 5% Kae-DLNP possesses exceptional gene delivery capability and potent p53-mediated antitumor activity.

2.6 In Vivo Biocompatibility and Therapeutic Outcome in a Lung Metastasis Model

Having established the superior in vitro delivery performance of 5% Kae-DLNP, we next evaluated its in vivo antitumor efficacy using LLC (Lewis lung carcinoma) tumor model in C57BL/6 mice. Tumor-bearing mice were intravenously administered PBS, LNP, 5% Kae-LNP, or 5% Kae-DLNP encapsulating p53-mRNA at 3-day intervals for a total of six doses (Fig. 6a). The 5% Kae-DLNP group exhibited significantly superior antitumor efficacy compared to all other treatment groups, as evidenced by the smallest tumor volumes. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and hematoxylin and eosin (H&E) staining of tumor tissues revealed that 5% Kae-DLNP treatment induced substantially more apoptosis and necrosis of tumor cells compared to the other groups, further validating its potent capacity to deliver functional p53-mRNA for tumor suppression (Fig. 6b–c). Immunofluorescence staining and Western blot (WB) analysis of p53 protein expression in tumor tissues confirmed that the 5% Kae-DLNP group exhibited markedly higher p53 protein levels than all other groups, demonstrating its superior mRNA delivery efficiency in vivo (Fig. 6d). No significant body weight changes were observed during the treatment period, confirming the favorable biosafety profile of the nebulized LNP (Figure S8, Supporting Information).

p53 protein is known to elicit antitumor immune responses, and combining p53 mRNA nanotherapy with immune checkpoint blockade can reprogram the tumor immune microenvironment for effective cancer therapy. We therefore investigated the immune activation effects of our formulations. Tumor-infiltrating CD8⁺ T cells were characterized by flow cytometry using specific antibodies. The results revealed that LNP and 5% Kae-LNP recruited moderate numbers of CD8⁺ T cells, while 5% Kae-DLNP recruited the highest number of CD8⁺ T cells among all groups (Fig. 6e and Figure S9, Supporting Information). Furthermore, analysis of memory T cell formation in the spleen (CD44⁺/CD62L⁺ double-positive T cells) demonstrated that 5% Kae-DLNP treatment generated significantly more memory T cells compared to other treatments (Fig. 6f and Figure S10, Supporting Information). Cytokine levels in serum were quantified by ELISA, revealing that the 5% Kae-DLNP group exhibited significantly elevated levels of interferon- γ (IFN- γ), interleukin-12 (IL-12), tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6) compared to the PBS group, indicating a robust tumor-killing immune response (Fig. 6g–j). Collectively, these results demonstrate that 5% Kae-DLNP not only achieves effective tumor suppression through precise p53-mRNA delivery but also potentially activates antitumor immunity.

Finally, we assessed the in vivo biosafety of the nebulized formulations. Mice were euthanized 24 h after pulmonary administration of LNP, 2% Kae-LNP, 5% Kae-LNP, or 5% Kae-DLNP, followed by comprehensive histopathological analysis of major organs (heart, liver, spleen, and kidneys). H&E staining revealed no apparent pathological alterations or signs of damage in any treatment group, confirming the excellent systemic biocompatibility of all formulations, including the optimized 5% Kae-DLNP (Figure S11, Supporting Information). Critically, examination of lung tissue sections showed pronounced immune cell infiltration in the LNP group, indicative of the acute inflammatory response commonly triggered by cationic lipid-based carriers. In stark contrast, lung sections from animals treated with Kae-incorporated formulations, particularly 5% Kae-LNP and 5% Kae-DLNP, exhibited markedly reduced immune cell infiltration, confirming that kaempferol incorporation effectively mitigates LNP-induced pulmonary

immunotoxicity. These findings collectively demonstrate that the 5% Kae-DLNP platform successfully combines potent therapeutic delivery with a significantly improved safety profile in vivo.

3. Discussion

Pulmonary nebulization of mRNA therapeutics holds immense promise for treating a wide spectrum of respiratory pathologies. Yet, its clinical translation is hampered by the inherent toxicity of cationic lipid nanoparticles (LNPs) and their inefficient endosomal escape, creating a significant efficacy-safety trade-off. Traditional approaches often treat inflammation as a side effect to be managed separately. Our work introduces a paradigm shift by integrating the natural immunomodulator kaempferol (Kae) directly into the LNP architecture, creating a multifunctional “Kaesomes” platform that concurrently addresses both critical barriers. This bio-inspired, dual-function strategy stems from the rationale that Kae, a flavonoid known for mitigating pollen-associated immune responses, could serve as a built-in anti-inflammatory agent while potentially modulating the biophysical properties of the lipid bilayer to enhance membrane fusion and destabilization. The anti-inflammatory efficacy of Kae-LNPs was unequivocally demonstrated both in vivo and in vitro. Nebulization triggered a pronounced, dose-dependent reduction in pulmonary infiltration of key pro-inflammatory cells—neutrophils, T cells, B cells, DCs, and NK cells—without affecting alveolar macrophage populations. This specificity suggests Kae effectively quenches the acute neutrophilic cascade initiated by cationic lipids. Mechanistically, this is supported by the potent suppression of NLRP3 inflammasome activation and ROS production in macrophages, hallmarks of LNP-induced immunotoxicity. Remarkably, this dramatic reduction in inflammation (quantified as a 17-fold decrease in toxicity) did not come at the cost of delivery performance but rather enhanced it. The enhanced delivery performance of the optimized 5% Kae-DLNP formulation can be attributed to a synergistic multi-factor mechanism. First, the incorporation of Kae induced a distinct “budding vesicle” morphology, contrasting with the conventional multilamellar structure of standard LNPs. This structural rearrangement, likely due to Kae’s amphiphilic nature intercalating into the lipid bilayer, may contribute to improved membrane fluidity and interfacial properties. Second, replacing the helper lipid DSPC with the fusogenic lipid DOPE created a powerful combinatorial effect. The resulting 5% Kae-DLNP exhibited a 2.3-fold increase in lysosomal escape efficiency, as evidenced by significantly reduced co-localization with lysosomal markers. This synergism suggests that Kae not only reduces bilayer stiffness but may also work in concert with DOPE to lower the energy barrier for endosomal membrane disruption, a process analogous to viral fusion peptides. Consequently, this led to a dramatic 9.5-fold enhancement in EGFP-mRNA transfection efficiency in lung epithelial cells. The successful translation of this delivery prowess into therapy was confirmed by the efficient cytoplasmic delivery of p53-mRNA, resulting in potent tumor cell killing and significant efficacy in a lung metastasis model.

A critical advantage of our platform is its self-reinforcing safety profile. The reduction in inflammatory toxicity is not merely an additive benefit but is intrinsically linked to the delivery mechanism. By mitigating the initial “danger signal” posed by cationic lipids, Kae-LNPs likely reduce nonspecific immune activation that can clear nanoparticles and damage tissue. Furthermore, the enhanced endosomal escape efficiency means less mRNA is degraded in lysosomes, potentially allowing for lower and safer

administered doses to achieve therapeutic effects. The excellent systemic biosafety, confirmed by histopathological analysis of major organs, underscores the platform's clinical potential. Looking forward, the principle of integrative material design demonstrated here—where a therapeutic agent is rationally incorporated to serve both pharmacological and carrier-enhancing functions—offers a versatile blueprint. While Kae was chosen for its anti-inflammatory and membrane-modulating properties, this strategy could be extended to other natural compounds or synthetic molecules with desired dual functionalities. Future work should explore the detailed biophysical interactions between Kae and the LNP lipid components using techniques like molecular dynamics simulations, investigate the pharmacokinetics and long-term safety of chronic nebulization, and validate the platform's efficacy in models of specific lung diseases such as pulmonary fibrosis, infection, or cystic fibrosis. Moreover, tuning the membrane properties through lipid composition, as shown with the DOPE substitution, opens avenues for further optimizing selectivity and escape for different target cell types within the complex lung environment. In conclusion, we have developed and validated Kae-incorporated LNPs as an advanced inhalable mRNA delivery platform that successfully breaks the traditional efficacy-toxicity compromise. By leveraging the innate properties of a natural immunomodulator to simultaneously dampen inflammation and enhance cytoplasmic delivery, this work provides a compelling and translatable strategy for the next generation of safe and effective pulmonary mRNA therapeutics.

4. Experimental Section

Materials

The ionizable lipid SM102, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), cholesterol, and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000) were purchased from Avanti Polar Lipids. Kaempferol (Kae), hyaluronidase, and collagenase type IV were purchased from Sigma-Aldrich. DMG-PEG2000 conjugated to Cy5 (DMG-PEG-Cy5) was purchased from Nanocs. The near-infrared dye IR820 was purchased from AAT Bioquest. Fluorescent antibodies for flow cytometry including PE anti-mouse CD19, PerCP anti-mouse CD45, FITC anti-mouse CD3, FITC anti-mouse CD45, APC anti-mouse NK-1.1, PE anti-mouse Ly-6G, PerCP anti-mouse CD11b, APC anti-mouse F4/80, APC anti-mouse CD4, PE anti-mouse CD44, and FITC anti-mouse CD62L were purchased from BioLegend. The NLRP3 primary antibody for immunofluorescence was purchased from Cell Signaling Technology. LysoTracker Green DND-26, DCFH-DA ROS assay kit, and the MTT cell proliferation assay kit were purchased from Beyotime. The mouse cytokine multiplex ELISA kit for IFN- γ , IL-6, IL-12, and TNF- α was purchased from Bio-Rad. The ECL detection kit for western blot was purchased from Epizyme. EGFP-mRNA (CleanCap® EGFP mRNA) and p53-mRNA (CleanCap® p53 mRNA) were purchased from APE×BIO. The murine macrophage cell line RAW 264.7 and the human lung adenocarcinoma cell line A549 were purchased from the American Type Culture Collection (ATCC). Female BALB/c mice were purchased from Charles River Laboratories. All other chemicals and reagents were of analytical grade.

The anti-inflammatory effect of kaempferol (Kae)-loaded LNPs: Mice received intranasal nebulization of plain LNP, 2% Kae-LNP, or 5% Kae-LNP (10 mg/kg in 200 μ L). Twelve hours post-treatment, immune cells were harvested via bronchoalveolar lavage (BAL) and processed into a single-cell suspension for flow cytometric analysis. Cells were stained with two antibody panels: Panel 1 (FITC-CD45, PE-CD19, PerCP-CD45, APC-NK1.1) for B cells and NK cells, and Panel 2 (FITC-CD45, PE-Ly6G, PerCP-CD11b, APC-F4/80) for neutrophils, dendritic cells (DCs), and macrophages. T cells (CD3+) were analyzed by separate staining. The percentages of each immune subset within the live CD45 + leukocyte population were quantified.

Formulation of Kaempferol-doped Lipid Nanoparticles (5% Kae-LNP): Lipid nanoparticles were formulated via a microfluidic-based method. For plain LNP, the ethanol phase contained SM102, DSPC, cholesterol, and DMG-PEG2000 at a molar ratio of 50:10:38.5:1.5. For 5% Kae-LNP, kaempferol was added to this ethanol phase at 5% (wt/wt, relative to total lipids) prior to mixing. The aqueous phase consisted of the cargo (e.g., mRNA in citrate buffer for physicochemical characterization, or IR820 dye for imaging studies) or buffer alone. The two phases were mixed in a microfluidic device at a fixed flow rate ratio (typically 3:1, aqueous-to-ethanol) to form nanoparticles.

Summary of the Nebulization Stability Test

3 mL of each formulation (plain LNP and 5% Kae-LNP) was subjected to nebulization using a jet nebulizer at a controlled airflow. The generated aerosol was collected by directing the output into a chilled vial. The particle size (Z-average diameter) and polydispersity index (PDI) of the samples were measured via Dynamic Light Scattering (DLS) before and after the nebulization process.

In Vivo and Ex Vivo Imaging Post-Nebulization

Mice were subjected to intranasal nebulization of IR820-loaded LNP or 5% Kae-LNP formulations (10 mg/kg in 200 μ L). Six hours post-administration, mice were anesthetized with isoflurane and whole-body fluorescence imaging was performed using a small animal imaging system (excitation/emission filters appropriate for IR820) to assess pulmonary distribution in vivo. Immediately following imaging, mice were euthanized by cervical dislocation under deep anesthesia. Major organs (heart, liver, spleen, lungs, and kidneys) were then carefully dissected, rinsed in PBS, and placed on the imaging stage for ex vivo fluorescence imaging to quantify the biodistribution and specific accumulation within the lungs. Fluorescence intensity was quantified using the system's software for both live and excised tissues.

In Vivo Imaging and Cellular Uptake Analysis of Nebulized Cy5-Labeled LNPs: To evaluate the pulmonary biodistribution and cell-type-specific uptake of Cy5-labeled LNP formulations (plain LNP and 5% Kae-LNP) encapsulating Luc-mRNA, C57BL/6 mice were administered a single dose (10 mg/kg total lipid, 200 μ L) via intratracheal nebulization. At 6 h post-administration, mice were anesthetized with isoflurane and subjected to whole-body IVIS imaging to detect both Cy5 fluorescence (excitation/emission: 640/680 nm) and luciferase bioluminescence (after intraperitoneal injection of D-luciferin at 150 mg/kg, imaging 10 min post-injection). After imaging, the mice were euthanized, and

the lungs were carefully excised for ex vivo imaging to quantify the total Cy5 fluorescence and luminescence signals, with region-of-interest (ROI) analysis performed using Living Image software to determine the mean radiant efficiency for both channels (quantitative analysis of panel g, data expressed as average radiance $\pm$ SD). For cell-type-specific uptake analysis, the excised lungs from separate groups of mice ($n = 3$ per formulation) at the same 6-h time point were minced and digested in RPMI-1640 medium containing 1.5 mg/mL collagenase type IV and 0.5 mg/mL DNase I at 37°C for 45 min with gentle agitation. The digested tissue was filtered through a 70- μ m cell strainer, and erythrocytes were lysed with ACK lysis buffer to obtain a single-cell suspension. Cells were then stained with the following fluorophore-conjugated antibodies for 30 min at 4°C in the dark: FITC anti-mouse CD45 (clone 30-F11), PE anti-mouse CD11b (clone M1/70) to identify macrophages (CD45⁺/CD11b⁺); PE anti-mouse CD31 (clone MEC13.3) for endothelial cells (CD45⁻/CD31⁺); and PE-Cy7 anti-mouse CD326 (EpCAM) for epithelial cells (CD45⁻/CD326⁺). After washing, the Cy5 signal (representing LNP uptake) was analyzed by flow cytometry (BD LSRFortessa), gating on live single cells. For each cell population, the percentage of Cy5-positive cells and the mean fluorescence intensity (MFI) of Cy5 were recorded.

Cellular Uptake and Lysosomal Escape Evaluation: A549 cells were seeded into confocal dishes and 6-well plates. At 80% confluency, cells were treated for 4 hours with serum-free medium containing 50 μ g·mL⁻¹ of the different Cy5-labeled LNP formulations: plain LNP, 2% Kae-DLNP (DOPE-LNP), 5% Kae-DLNP, and 5% Kae-LNP (DSPC-LNP). Following treatment, cells were washed with PBS. For confocal imaging, cells in dishes were stained with a green fluorescent lysosomal dye (e.g., LysoTracker Green) for 15 minutes, washed, and then fixed. Images were acquired using a confocal laser scanning microscope to assess the co-localization of red (Cy5-LNP) and green (lysosome) signals, and the Pearson's correlation coefficient was calculated for quantitative analysis. For flow cytometry, cells in the 6-well plates were trypsinized, washed, and resuspended in PBS. The cellular uptake of Cy5-labeled LNPs was quantified by measuring the mean fluorescence intensity (MFI) of Cy5 using a flow cytometer.

In Vitro Endosomal Membrane Fusion Assay for Kae-LNPs: To assess the fusogenic activity of Kae-doped LNPs (plain LNP, 2% Kae-LNP, 5% Kae-LNP, and 5% Kae-DLNP) with endosomal membranes, we prepared endosome-mimicking liposomes composed of DOPC/DOPS/cholesterol (60:20:20 mol%) incorporating 0.8 mol% NBD-PE (donor) and 0.8 mol% Rho-PE (acceptor) as a FRET pair. These liposomes (final lipid concentration 250 μ M) were incubated with each LNP formulation (final lipid concentration $\sim$ 250 μ g/mL) in 10 mM citrate-phosphate buffer (pH 5.5) at 37°C for 2 h in a black 96-well plate. After incubation, fluorescence emission spectra were recorded from 500 to 600 nm with excitation at 460 nm, and the intensities at 535 nm (NBD) and 565 nm (Rho) were measured. A negative control (F_0) consisted of FRET liposomes alone (0% fusion), and a positive control (F_{100}) was obtained by adding 0.5% (v/v) Triton X-100 to completely disrupt the liposomes (100% fusion). The membrane fusion/destruction efficiency (η) was calculated using the formula: η (%) = $[(F_{\text{sample}} - F_0) / (F_{100} - F_0)] \times 100\%$, where F_{sample} is the NBD fluorescence of the LNP-treated sample.

Endocytic Inhibition Assay for Cellular Uptake of Kae-LNPs

To investigate the internalization pathways of Cy5-labeled LNP formulations (plain LNP, 2% Kae-LNP, 5% Kae-LNP, and 5% Kae-DLNP) in A549 cells, we performed endocytic inhibition studies using three specific pharmacological inhibitors. A549 cells were seeded in 12-well plates and cultured overnight to reach ~80% confluence. Prior to nanoparticle treatment, cells were pre-incubated for 30 min at 37°C with optimized concentrations of Genistein (GEN, 100 μ M, caveolae-mediated endocytosis inhibitor), Chlorpromazine (CPZ, 10 μ g/mL, clathrin-mediated endocytosis inhibitor), or 5-(N-ethyl-N-isopropyl) amiloride hydrochloride (EIPA, 25 μ M, macropinocytosis inhibitor). Subsequently, the Cy5-labeled LNP formulations were added to each well at a final concentration of 50 μ g/mL and co-incubated with the respective inhibitors for an additional 2 h at 37°C in the dark. Cells treated with the Cy5-labeled LNPs in the absence of any inhibitors served as the positive control (representing 100% uptake). After the 2-h incubation, cells were washed three times with ice-cold PBS to remove unbound nanoparticles and inhibitors, trypsinized, harvested by centrifugation, and resuspended in PBS containing 2% FBS. The cellular uptake was quantified by measuring the mean fluorescence intensity (MFI) of the Cy5 signal for each sample using a flow cytometer (BD Accuri C6) with excitation at 635 nm and emission at 670 nm. For each formulation, the relative uptake efficiency was calculated by normalizing the MFI of inhibitor-treated groups to that of the corresponding positive control group (without inhibitors) set as 100%, allowing determination of the contribution of each endocytic pathway to the internalization of the different Kae-LNP formulations.

NLRP3 Inflammasome Immunofluorescence Staining

RAW264.7 macrophages were seeded into confocal dishes and cultured to approximately 80% confluence. The cells were then treated with different LNP formulations (plain LNP, 2% Kae-LNP, 5% Kae-LNP, 5% Kae-DLNP) for 6 hours. After treatment, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 minutes at room temperature, and permeabilized with 0.1% Triton X-100 for 10 minutes. Following blocking with 5% bovine serum albumin (BSA) for 1 hour, cells were incubated overnight at 4°C with a primary antibody against NLRP3. After washing, cells were incubated with a fluorophore-conjugated secondary antibody (e.g., Alexa Fluor 488, green) for 2 hours at room temperature in the dark. Nuclei were counterstained with DAPI (blue). Cells were washed and imaged using a confocal laser scanning microscope to visualize NLRP3 and P65 expression and cellular localization.

LPS-induced ROS Scavenging Assay: RAW264.7 macrophages were seeded in culture plates. To induce inflammation, cells were first stimulated with LPS (10 μ g/mL) for 12 hours. Subsequently, the culture medium was replaced with fresh medium containing the different LNP formulations (LNP, 2% Kae-LNP, 5% Kae-LNP, 5% Kae-DLNP) for an additional 12 hours. Cells were then washed and incubated with a fluorescent ROS indicator dye (e.g., DCFH-DA, 10 μ M) in serum-free medium for 30 minutes at 37°C in the dark. After washing, intracellular ROS levels were immediately analyzed using two methods: qualitative observation under a fluorescence microscope and quantitative measurement of mean fluorescence intensity (MFI) via flow cytometry.

MTT Cell Viability Assay

RAW264.7 macrophages were seeded into a 96-well plate and allowed to adhere overnight. Cells were then treated with various concentrations of the different LNP formulations (LNP, 2% Kae-LNP, 5% Kae-LNP, 5% Kae-DLNP) for a predetermined period (e.g., 24 hours). Following treatment, the medium was replaced with fresh medium containing 0.5 mg/mL MTT reagent, and cells were incubated for 4 hours at 37°C. The MTT-containing medium was carefully removed, and the resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance of each well was measured at a wavelength of 560 nm using a microplate reader. Cell viability was calculated as a percentage relative to the untreated control wells.

Experimental Procedure for EGFP-mRNA Transfection and Analysis

A549 cells were seeded in 6-well plates and allowed to adhere overnight. Cells were then treated with different LNP formulations (LNP, 2% Kae-DLNP, 5% Kae-LNP, 5% Kae-DLNP) encapsulating EGFP-mRNA (1 µg per well) for 6 hours. After treatment, the medium was replaced with fresh complete medium, and cells were cultured for an additional 24 hours. EGFP expression was first visualized using a confocal fluorescence microscope. For quantitative analysis, cells were trypsinized, washed with PBS, and resuspended. The percentage of EGFP-positive cells and the mean fluorescence intensity (MFI) were measured via flow cytometry.

p53 Protein Detection by Western Blot

A549 cells were treated with LNP formulations encapsulating p53-mRNA (2 µg per well) for 6 hours, followed by replacement with fresh medium and further incubation for 48 hours. Total protein was then extracted using RIPA lysis buffer containing protease inhibitors, and protein concentration was determined with a BCA assay. Equal amounts of protein (20–30 µg per lane) were separated by SDS-PAGE and transferred to a PVDF membrane. After blocking with 5% skim milk in TBST, the membrane was incubated overnight at 4°C with a primary antibody against p53. Following TBST washes, the membrane was incubated with an HRP-conjugated secondary antibody for 2 hours at room temperature. Protein bands were visualized using an ECL detection kit and imaged with a chemiluminescence system. Densitometric analysis was performed using ImageJ software, with GAPDH serving as the loading control.

p53-mRNA Delivery and Cytotoxicity Assessment via MTT Assay: A549 cells were seeded in a 96-well plate and treated with different LNP formulations encapsulating p53-mRNA (3 µg per well) for 6 hours. The medium was then replaced with fresh complete medium, and cells were cultured for an additional 72 hours. Cell viability was assessed using the MTT assay: 20 µL of MTT solution (5 mg/mL) was added to each well, followed by incubation for 4 hours at 37°C. The formed formazan crystals were dissolved in DMSO, and the absorbance at 570 nm was measured using a microplate reader. Cell viability was calculated as a percentage relative to untreated control wells.

Acridine Orange Lysosomal Escape Assay for EGFP-mRNA-Loaded LNPs: A549 cells were seeded in confocal culture dishes at a density of 2×10^5 cells per dish and cultured overnight to ~ 80% confluence. The cells were then treated with LNP formulations (plain LNP, 2% Kae-DLNP, 5% Kae-LNP, and 5% Kae-DLNP) encapsulating EGFP-mRNA at a final mRNA concentration of 1 $\mu\text{g/mL}$ (equivalent to ~ 50 $\mu\text{g/mL}$ nanoparticles) in serum-free medium for 6 h at 37°C. Untreated cells served as the control group. After the 6-h incubation, the medium was removed, and cells were washed twice with PBS, followed by staining with Acridine Orange (AO) at a final concentration of 5 $\mu\text{g/mL}$ in serum-free medium for 15 min at 37°C in the dark. AO exhibits metachromatic fluorescence: it emits green fluorescence (510–530 nm) when bound to nucleic acids in the cytoplasm, indicating mRNA released from lysosomes, and red fluorescence (> 600 nm) when concentrated in acidic compartments (lysosomes), indicating trapped mRNA. After staining, cells were washed three times with PBS and immediately imaged using a confocal laser scanning microscope (Olympus FV3000) with excitation at 488 nm; green emission was collected at 510–530 nm and red emission at 600–650 nm. For each sample, at least five random fields were captured with identical acquisition parameters. Quantitative analysis of lysosomal escape efficiency was performed using ImageJ software by calculating the ratio of green fluorescent area to total cellular area (green + red) within the cytoplasm: $\text{Escape efficiency (\%)} = [\text{Green area} / (\text{Green area} + \text{Red area})] \times 100\%$, where Green area represents the cytosolic mRNA signal and Red area represents the lysosome-trapped mRNA.

Histopathological Analysis via H&E Staining

Following euthanasia at 24 hours post-nebulization, major organs (heart, liver, spleen, lungs, and kidneys) were harvested from mice. Tissues were immediately fixed in 4% paraformaldehyde for 48 hours at room temperature. Subsequently, fixed tissues were dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin wax. Sections were cut at a thickness of 5 μm using a microtome and mounted onto glass slides. For staining, sections were deparaffinized in xylene and rehydrated through a descending ethanol series to distilled water. Nuclei were stained with Harris's hematoxylin for 5 minutes, followed by rinsing in running tap water. Cytoplasm was counterstained with eosin for 2 minutes. After a final dehydration and clearing steps, stained sections were mounted with a coverslip using a resinous mounting medium. Histopathological evaluation was performed by examining the stained sections under a light microscope to assess tissue morphology and potential signs of toxicity.

Declarations

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Competing interests

The authors declare that they have no competing interests.

Institutional review board statement

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Anyang Tumor Hospital. The experiments were conducted in strict accordance with the approved guidelines.

AI usage statement

The authors acknowledge the use of Deepseek in the revision of this manuscript. AI assistance was used for structural revision and language editing of the manuscript text. All scientific content, data interpretation, experimental design, and conclusions reviewed, revised, and approved by the authors. No AI tools were used for data collection, statistical analysis, or image generation.

Author Contribution

#R.L., R.C., and Z. C. contributed equally to this work. The manuscript was written based on the contributions of all authors. Conceptualization: Z.C., Y.L., S.G.; Methodology: R.L., R.C., Z.C.; Data collection: R.L., R.C., Z.C., K.C.; Visualization: R.L., R.C., Z.C., Y.L.; Funding acquisition: R.C., Y.L., X.L., K.C.; Project management: R.C., B.L., X.L.; Supervision: B.L., X.L., Y.L.; Writing-original draft: R.L., Z.C., Y.L., S.G.; Writing-review and editing: Z.C., Y.L., S.G.; All authors have approved the final version of the manuscript.

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Data availability

The main data supporting the results in this study are available within the paper and its Supplementary Information. All the raw and analyzed data generated during the study are available from the corresponding authors on reasonable request. Source data are provided with this paper.

References

1. Shi Y, Shi M, Wang Y, et al. Progress and prospects of mRNA-based drugs in pre-clinical and clinical applications. *Sig Transduct Target Ther*. 2024;9:322.
2. Batisani K. The role of mRNA vaccines in infectious diseases: a new era of immunization. *Trop Dis Travel Med Vaccines*. 2025;11:12.
3. Leong KY, Tham SK, Poh CL. Revolutionizing immunization: a comprehensive review of mRNA vaccine technology and applications. *Viol J*. 2025;22:71.
4. Horejs C. Nebulized lipid nanoparticles. *Nat Rev Mater*. 2021;6:1077.
5. Jiang AY, Witten J, Raji IO, et al. Combinatorial development of nebulized mRNA delivery formulations for the lungs. *Nat Nanotechnol*. 2024;19:364–75.
6. Reynard O, Gonzalez C, Dumont C, et al. Nebulized fusion inhibitory peptide protects cynomolgus macaques from measles virus infection. *Nat Commun*. 2022;13:6439.
7. Omo-Lamai S, Wang Y, Patel MN, et al. Limiting endosomal damage sensing reduces inflammation triggered by lipid nanoparticle endosomal escape. *Nat Nanotechnol*. 2025;20:1285–97.
8. Zhao Y, Li R, Liu P et al. Low reactogenicity and high tumour antigen expression from mRNA-LNPs with membrane-destabilizing zwitterionic lipids. *Nat Biomed Eng* (2025).
9. Zhao Z, Shan X, Ding J, et al. Boosting RNA nanotherapeutics with V-ATPase activating non-inflammatory lipid nanoparticles to treat chronic lung injury. *Nat Commun*. 2025;16:6477.
10. Gilleron J, Querbes W, Zeigerer A, et al. Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. *Nat Biotechnol*. 2013;31:638.
11. Maugeri M, Nawaz M, Papadimitriou A, et al. Linkage between endosomal escape of LNP-mRNA and loading into EVs for transport to other cells. *Nat Commun*. 2019;10:4333.
12. Simon B, Ceglédi A, Dolgos János et al. L Combined immunosuppression for acquired hemophilia A: CyDRi is a highly effective low-toxicity regimen *Blood* 140, 18 (2022).
13. Maugeri M, Nawaz M, Papadimitriou A, et al. Dual Intratumoral Redox/Enzyme-Responsive NO-Releasing Nanomedicine for the Specific, High-Efficacy, and Low-Toxic Cancer Therapy. *Adv Mater*. 2018;30:1704490.
14. Xia Y, Chen Z, Zhang X, Yang P, Wang Z. Phosphatidylcholine Liposome Accelerated Platinum Nanomachines (PLANEs) With Enhanced Penetration Capability for Thrombus Mechanotherapy. *Adv Mater*. 2025;37:2418590.
15. Schone HR, Udeozor M, Moninghoff M, et al. Biomimetic versus arbitrary motor control strategies for bionic hand skill learning. *Nat Hum Behav*. 2024;8:1108–23.
16. Song H, Hsieh TH, Yeon SH, et al. Continuous neural control of a bionic limb restores biomimetic gait after amputation. *Nat Med*. 2024;30:2010–9.
17. Jakob A, Faber, et al. Bioinspired spring origami *Science*. 2018;359:1386.

18. Imran M, Salehi B, Sharifi-Rad J, Aslam Gondal T et al. Kaempferol: A Key Emphasis to Its Anticancer Potential *Molecules* 24, 2277 (2019).
19. Rho HS, Ghimeray AK, Yoo DS, Ahn SM, et al. Kaempferol and Kaempferol Rhamnosides with Depigmenting and Anti-Inflammatory Properties. *Molecules*. 2011;16:3338.
20. Kim E-N, Lee H-S, Trang NM. *Silico* Target Discovery of Kaempferol: Therapeutic Effect of Kaempferol on Atopic Dermatitis through Regulation of Aryl Hydrocarbon. Receptor Biomater Res. 2025;29:0270.
21. Patel MN, Tiwari S, Wang Y et al. Safer non-viral DNA delivery using lipid nanoparticles loaded with endogenous anti-inflammatory lipids. *Nat Biotechnol* (2025).
22. Zhao Z, Shan X, Ding J, et al. Boosting RNA nanotherapeutics with V-ATPase activating non-inflammatory lipid nanoparticles to treat chronic lung injury. *Nat Commun*. 2025;16:6477.
23. Bian X, Guo Q, Yau LF, et al. Berberine-inspired ionizable lipid for self-structure stabilization and brain targeting delivery of nucleic acid therapeutics. *Nat Commun*. 2025;16:2368.
24. Zhang Y, Li M, Li L, et al. β -arrestin 2 as an activator of cGAS-STING signaling and target of viral immune evasion. *Nat Commun*. 2020;11:6000.
25. Khan K, Lustig G, Römer C, et al. Evolution and neutralization escape of the SARS-CoV-2 BA.2.86 subvariant. *Nat Commun*. 2023;14:8078.
26. Ku Z, Xie X, Davidson E, et al. Molecular determinants and mechanism for antibody cocktail preventing SARS-CoV-2 escape. *Nat Commun*. 2021;12:469.
27. Mangolini M, Maiques-Diaz A, Charalampopoulou S, et al. Viral transduction of primary human lymphoma B cells reveals mechanisms of NOTCH-mediated immune escape. *Nat Commun*. 2022;13:6220.
28. Inge S, Zuhorn U, Bakowsky E, Polushkin et al. Nonbilayer phase of lipoplex–membrane mixture determines endosomal escape of genetic cargo and transfection efficiency *Molecular Therapy* 11, 5 (2005).
29. Iwakawa K, Sato R, Konaka M, Yamada Y, Harashima H, et al. Cubic Phase-Inducible Zwitterionic Phospholipids Improve the Functional Delivery of mRNA. *Adv Sci*. 2025;12:2413016.

Figures

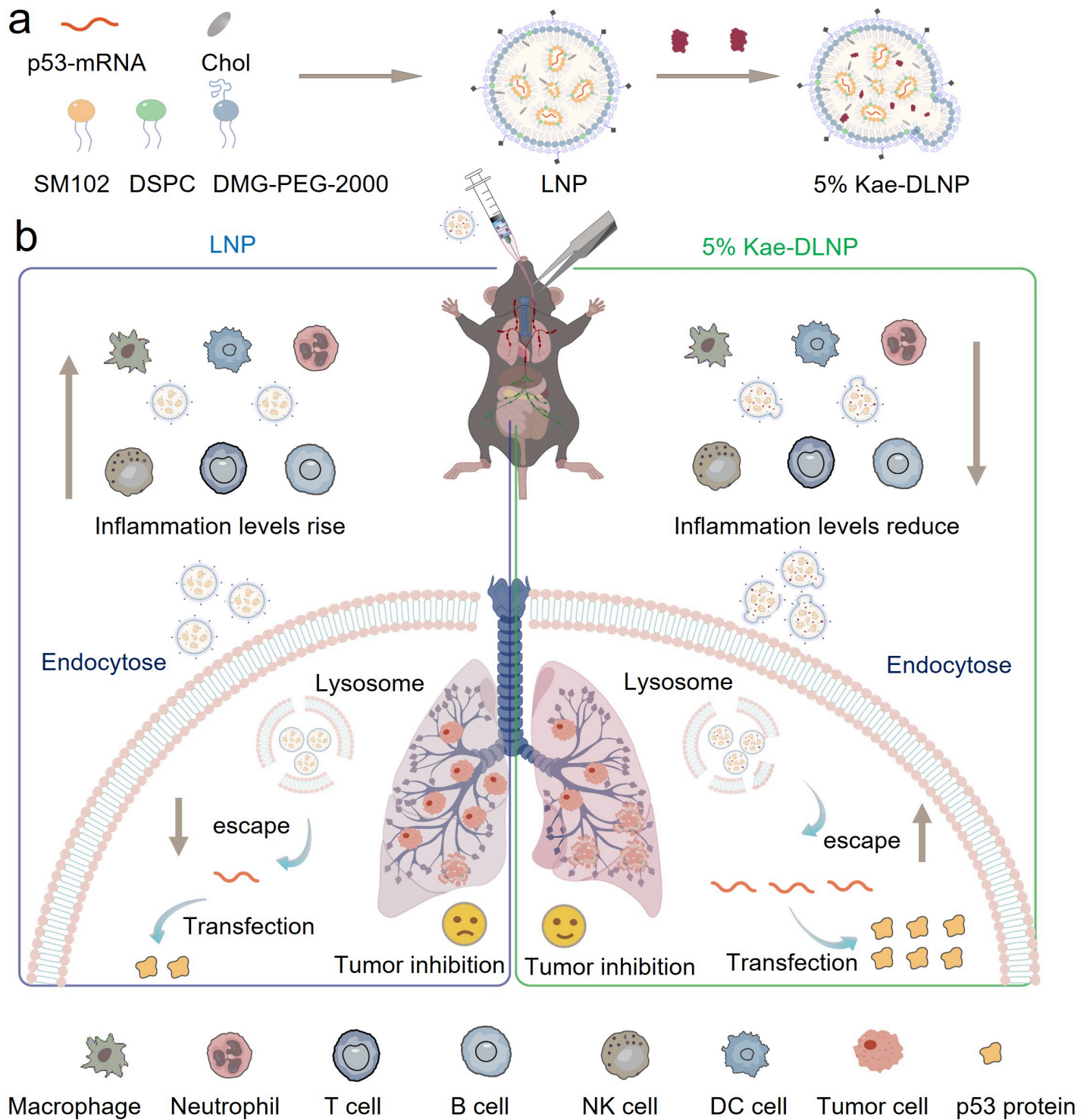


Figure 1

Schematic illustration of Kaempferol-doped LNP (Kae-LNP) formulation and its dual-function mechanism. (a) Formulation of Kaempferol-loaded LNPs (Kae-LNPs). Conventional LNPs are composed of ionizable lipid SM102, DSPC, cholesterol, and DMG-PEG2000. Kae-LNPs are synthesized by incorporating kaempferol (Kae) into the lipid mixture via a microfluidic process. The resulting particles exhibit a distinct "budding vesicle" morphology compared to the multilamellar "onion-like" structure of

standard LNPs, indicating successful Kae integration and structural modulation. (b) Dual-function mechanism of Kae-LNPs for enhanced safety and efficacy in pulmonary mRNA delivery. Upon nebulization, standard LNPs provoke acute pulmonary inflammation, characterized by infiltration of neutrophils, T cells, B cells, DCs, and NK cells. In contrast, Kae-LNPs significantly attenuate this inflammatory response through Kae's intrinsic anti-inflammatory and antioxidant activity, which suppresses NLRP3 inflammasome activation and ROS production. Concurrently, Kae modulates the lipid bilayer properties, and when combined with the fusogenic lipid DOPE (in 5% Kae-DLNP), synergistically enhances endosomal escape. This leads to efficient cytoplasmic release of mRNA (e.g., EGFP-mRNA or therapeutic p53-mRNA), resulting in high transfection efficiency and potent tumor-cell killing. The platform thus achieves a dual benefit: reduced immunotoxicity and boosted cytoplasmic delivery, enabling effective and safe inhalable mRNA therapy.

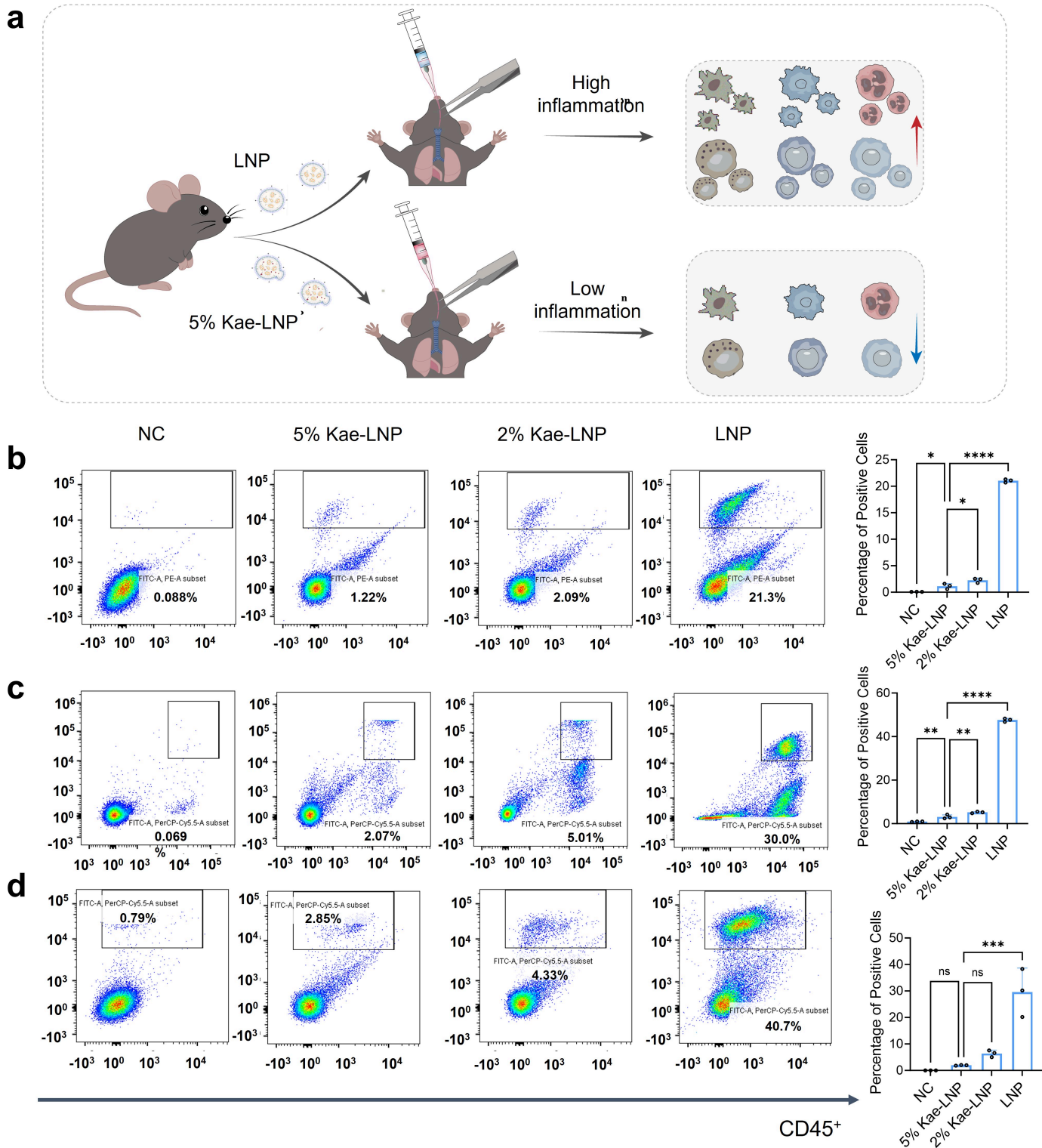


Figure 2

Kaempferol-incorporated LNPs attenuate pulmonary inflammation induced by nebulization in vivo. (a) Schematic illustration of immune cell changes after nebulization. Mice were administered LNP, 5% Kae-LNP, or 2% Kae-LNP (10 mg/kg, 100 μ L) via intranasal nebulization. Twelve hours later, immune cells were collected by bronchoalveolar lavage and analyzed by flow cytometry for positive rates. (b) Neutrophils, (c) DCs, (d) T cells, and the corresponding quantitative analysis of positive rates. Data are

presented as mean $\pm$ S.E.M ($n = 3$). one-way ANOVA with Tukey's post hoc test. All values are presented as mean $\pm$ S.D. $P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

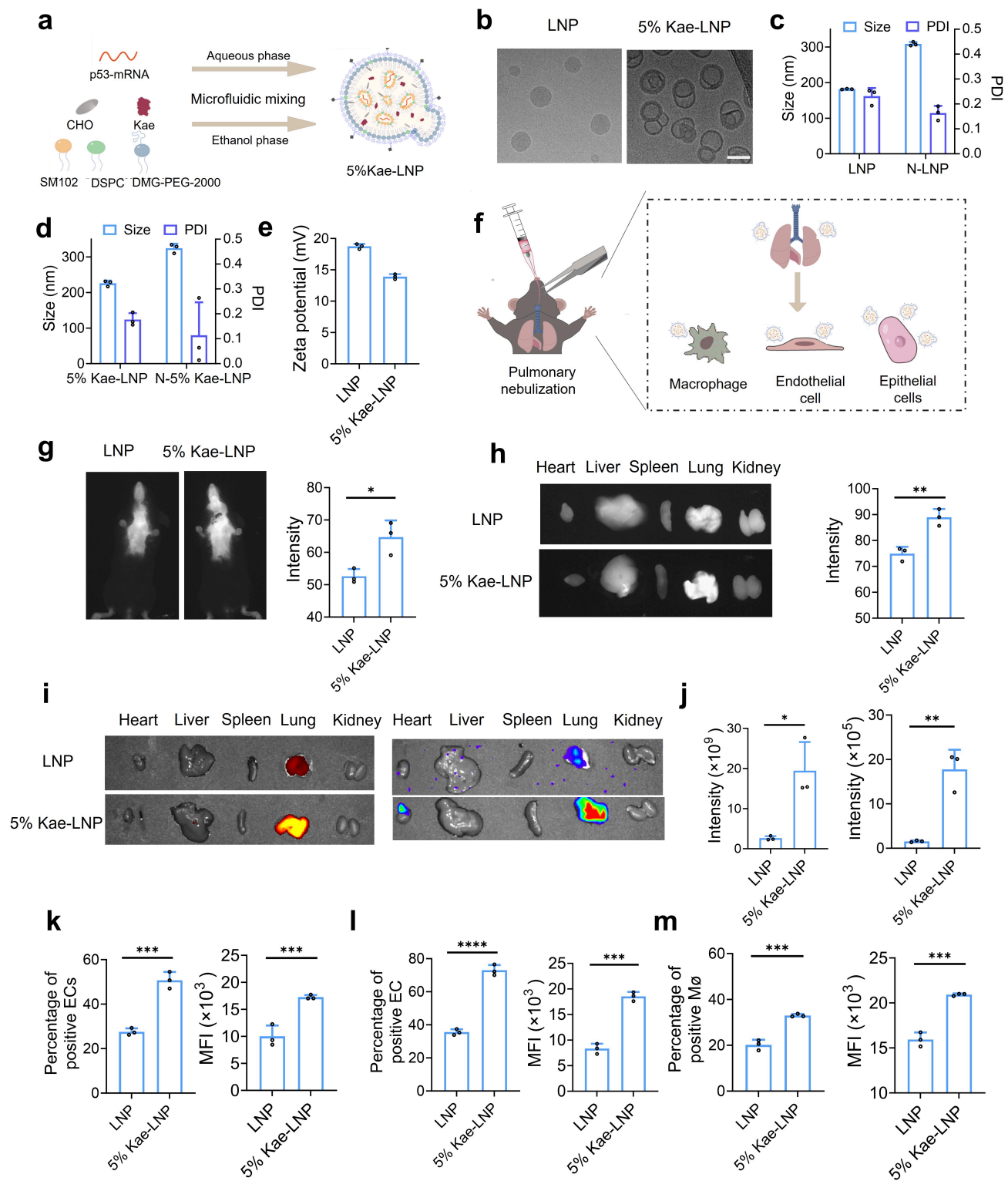


Figure 3

Physicochemical characterization and biodistribution of Kae-LNPs. (a) Schematic illustration of the synthesis of 5% Kae-LNP. (b) Cryo-EM images of LNP and 5% Kae-LNP. (c) Particle size and PDI of LNP

and nebulized LNP (N-LNP). (d) Particle size and PDI of 5% Kae-LNP and nebulized 5% Kae-LNP. (e) Zeta potential of LNP and 5% Kae-LNP. (f) Schematic illustration of pulmonary transfection and cellular uptake analysis after nebulization. (g) In vivo imaging and quantification of LNP and 5% Kae-LNP loaded with IR820 after pulmonary nebulization (10 mg/kg, 200 μ L). (h) Ex vivo NIR-II imaging and quantification of major organs (heart, liver, spleen, lung, and kidney) 6 h after nebulization. (i) IVIS imaging of C57 mice 6 h after pulmonary nebulization of Cy5-labeled LNP and 5% Kae-LNP loaded with Luc-mRNA, followed by dissection. (j) Quantitative analysis of (g). (k–m) Mice were nebulized for 6 h, then dissected and lung tissues were harvested, ground, and digested into single cells. Antibodies CD45[−]/CD31⁺ were used to label (k) endothelial cells, CD45[−]/CD326⁺ to label (l) epithelial cells, and CD45⁺/CD11b⁺ to label (m) macrophages. The positive percentage and fluorescence intensity of LNP-Cy5 in each cell type were analyzed. Data are presented as mean $\pm$ S.E.M (n = 3). One-way ANOVA with Tukey's post hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

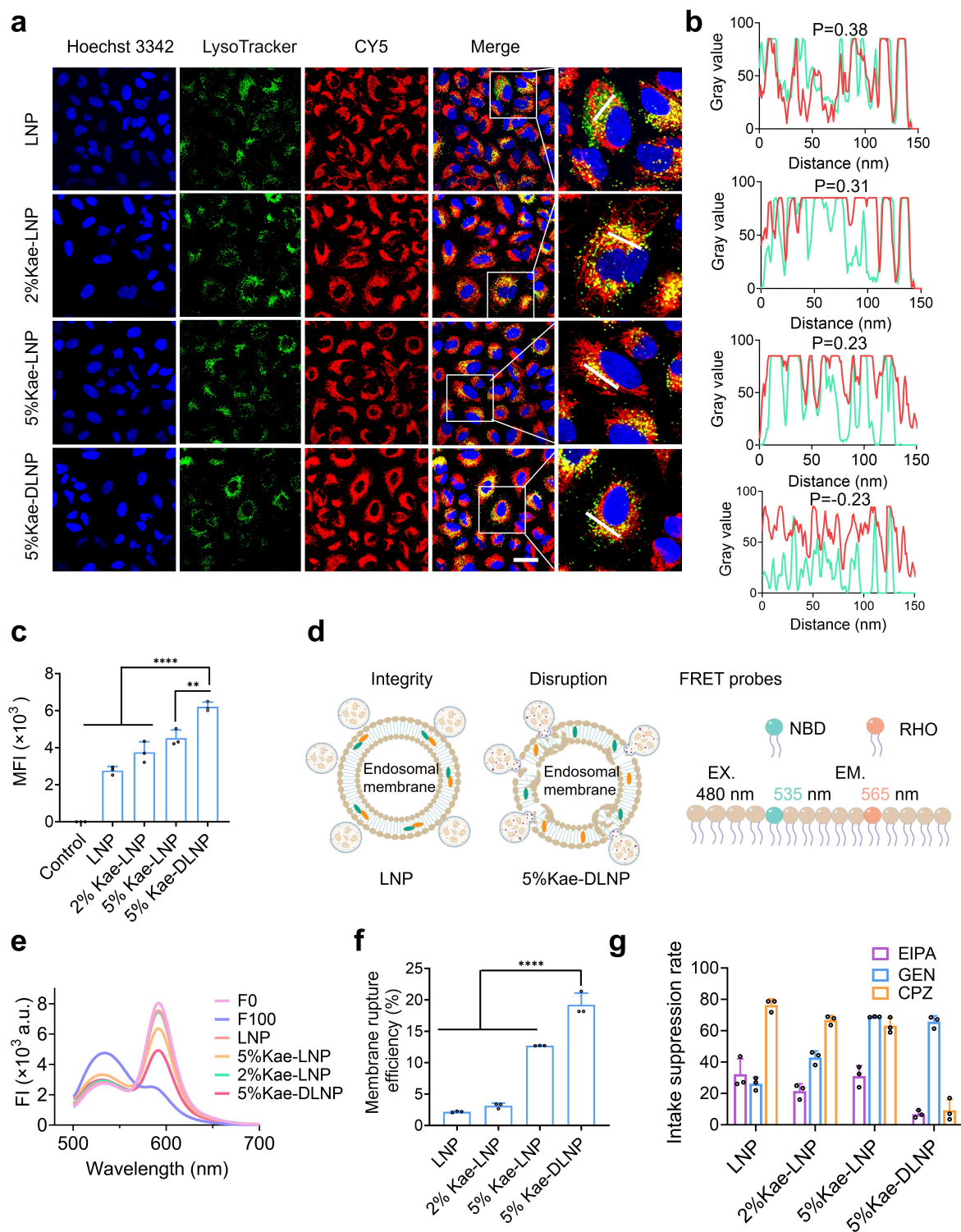


Figure 4

Enhanced cellular uptake, lysosomal escape, membrane fusion activity, and endocytic pathway modulation of DOPE-modified Kae-DLNP. A549 cells were treated with Cy5-labeled LNP, 2% Kae-LNP, 5% Kae-LNP, or 5% Kae-DLNP for 4 h. (a) Representative confocal laser scanning microscopy (CLSM) images showing cellular uptake (red, Cy5) and lysosomal co-localization (green, LysoTracker). Nuclei were stained with DAPI (blue). Scale bar: 20 μ m. The Pearson's correlation coefficient (P) for

Cy5/LysoTracker co-localization is indicated. (b) Intensity profile plots along the white lines in (a). (c) Quantitative mean fluorescence intensity (MFI) of Cy5 from flow cytometry. (d) Schematic representation of a FRET-based fluorescent tool used to verify the endosomal membrane disruption efficiency of 5% Kae-DLNP. (e) FRET-based membrane fusion assay: endosome-mimicking liposomes (NBD/Rho-labeled) were incubated with indicated LNP formulations at pH 5.5 for 2 h; fluorescence emission spectra (500–700 nm) were recorded with excitation at 460 nm; F_0 (negative control, liposomes alone) and F_{100} (positive control, 0.5% Triton X-100) are shown. (f) Quantitative analysis of membrane fusion efficiency ($\eta\%$) calculated from (e). (g) Endocytic inhibition assay: A549 cells were pre-incubated with Genistein (GEN, caveolae inhibitor), Chlorpromazine (CPZ, clathrin inhibitor), or EIPA (macropinocytosis inhibitor), followed by 2-h co-incubation with Cy5-labeled LNPs; flow cytometric MFI was measured and calculation of endocytic inhibition efficiency, normalized to the untreated positive control. Data in (d, f, g) are presented as mean $\pm$ SD ($n = 3$). One-way ANOVA with Tukey's post hoc test. $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$; ns, not significant.

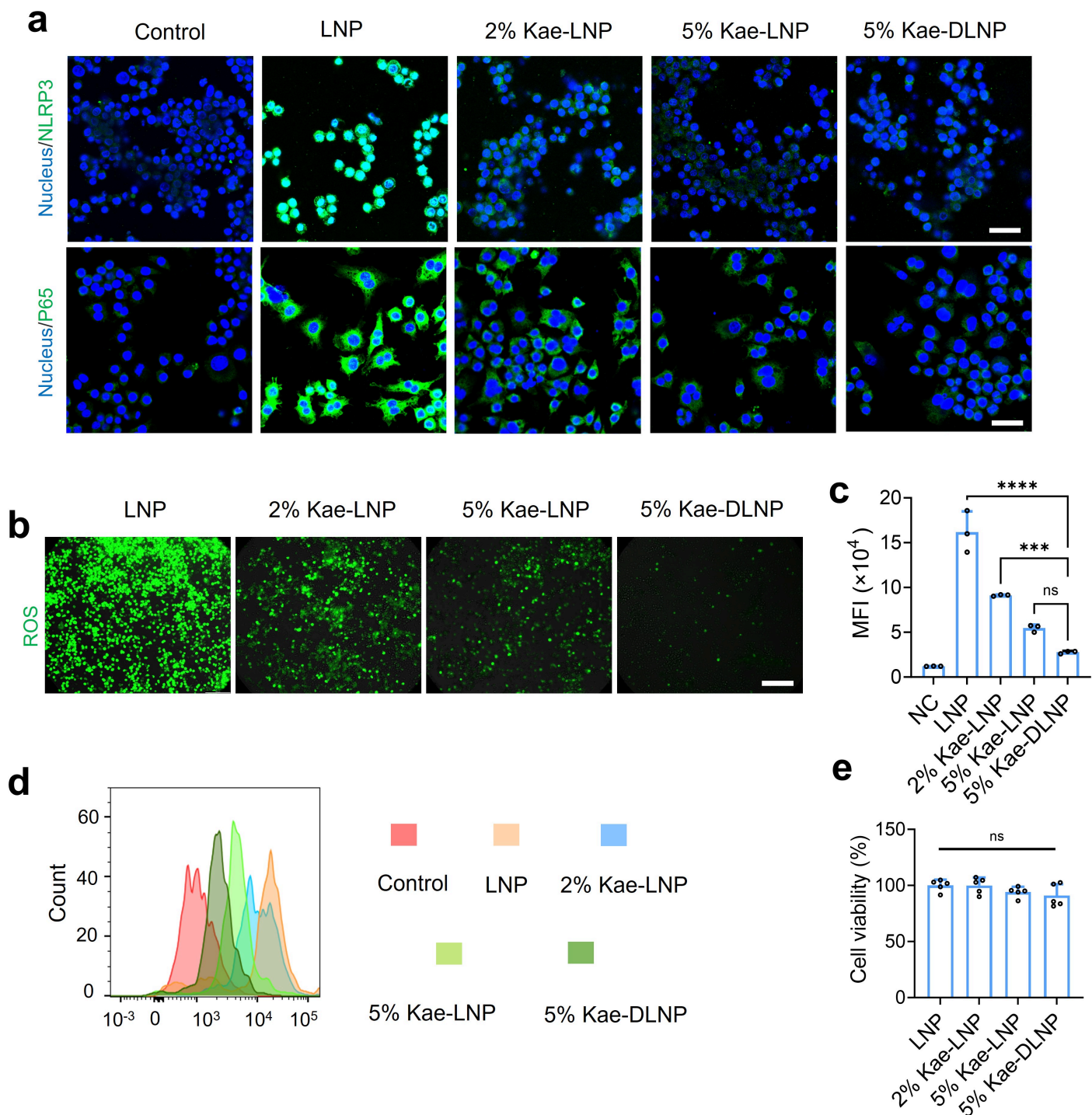


Figure 5

In vitro anti-inflammatory efficacy and biocompatibility of Kae-doped formulations. RAW 264.7 macrophages were used. (a) CLSM images showing NLRP3 inflammasome (green) and p65 (green) staining after 6-h treatment with indicated formulations; nuclei were stained with DAPI (blue). Scale bar: 100 μ m. (b) Fluorescence microscopy images of intracellular ROS levels (green) in LPS-primed (10 μ g/mL, 12 h) macrophages after 12-h treatment with indicated formulations. Scale bar: 200 μ m. (c) Representative flow cytometry histograms of DCFH-DA fluorescence for ROS detection. (d) Quantitative

analysis of ROS-positive cell percentage from flow cytometry. (e) Cell viability of RAW 264.7 macrophages assessed by MTT assay after 24-h treatment with different formulations (0.5 mg/mL). Data in (d, e) are presented as mean $\pm$ SD (n = 3). One-way ANOVA with Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

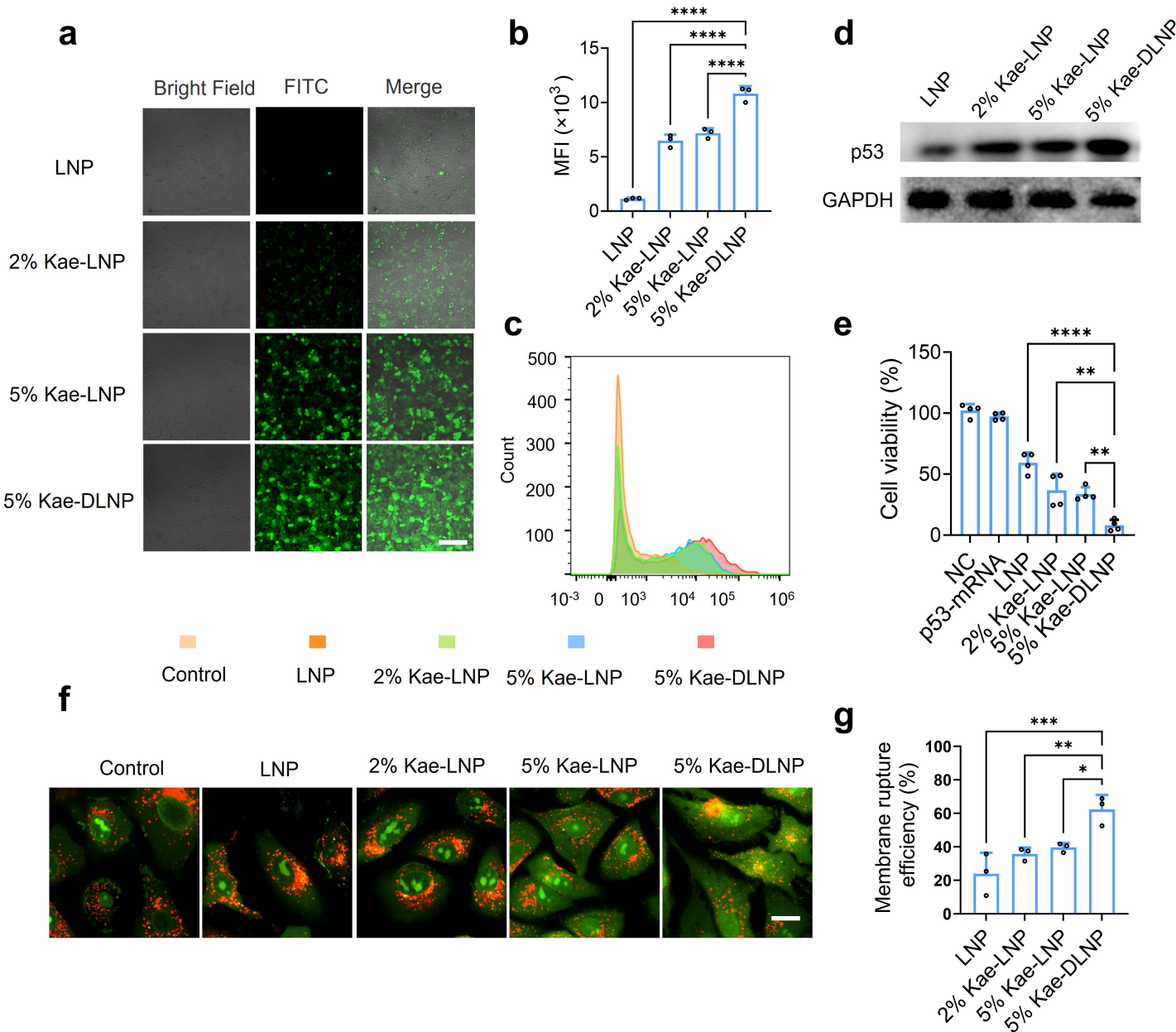


Figure 6

Superior mRNA delivery, therapeutic efficacy and cytosolic release efficiency in vitro. A549 cells were treated with LNP, 2% Kae-LNP, 5% Kae-LNP, or 5% Kae-DLNP encapsulating EGFP-mRNA (1 μ g/well) for 6 h, followed by 24 h culture. (a) CLSM images of EGFP expression (green). Nuclei were stained with DAPI (blue). Scale bar: 100 μ m. (b) Representative flow cytometry histograms of EGFP fluorescence. (c) Quantitative analysis of EGFP-positive cell percentage. (d) Western blot analysis of p53 protein expression 24 h after treatment with p53-mRNA-loaded formulations (2 μ g/well). GAPDH served as a

loading control. (e) Cell viability of A549 cells assessed by MTT assay 72 h after treatment with p53-mRNA-loaded formulations (3 $\mu\text{g}/\text{well}$). (f) AO staining for cytosolic release evaluation: A549 cells were treated with EGFP-mRNA-loaded formulations for 6 h, stained with AO (5 $\mu\text{g}/\text{mL}$, 15 min), and imaged by CLSM; red fluorescence indicates lysosomally trapped mRNA, while green fluorescence indicates cytosolic mRNA. Scale bar: 10 μm . (g) Quantitative analysis of cytosolic release efficiency. Data are presented as mean $\pm$ SD ($n = 3$). One-way ANOVA with Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

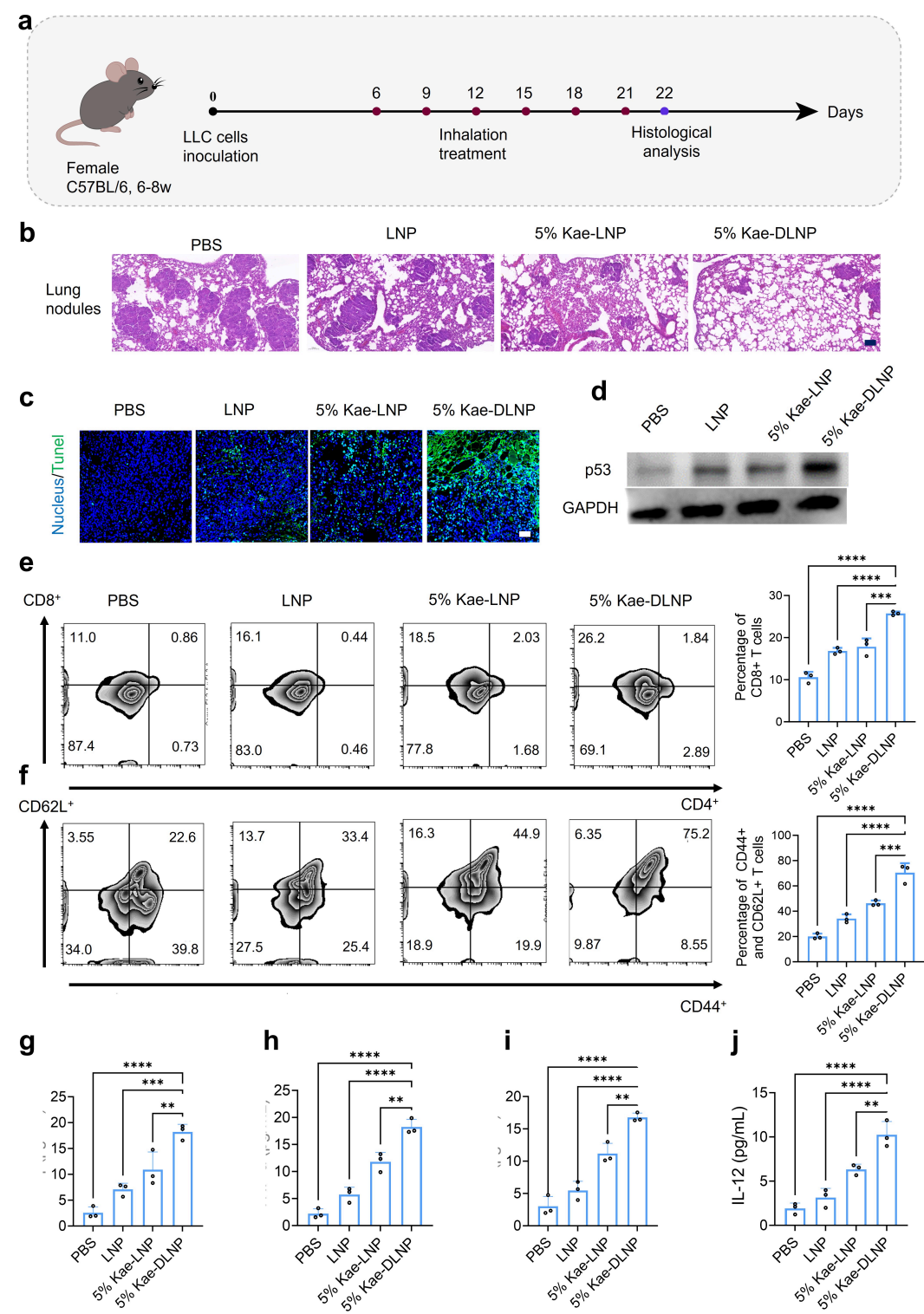


Figure 7

In vivo therapeutic efficacy, immune activation and biocompatibility in the subcutaneous LLC tumor model. (a) Treatment schedule: C57BL/6 mice bearing subcutaneous LLC tumors were intravenously injected with p53-mRNA-loaded formulations every 3 days for a total of six doses. (b) Representative H&E staining images of tumor sections after treatment (scale bar: 100 μ m). (c) Representative TUNEL fluorescence staining images of tumor sections (scale bar: 100 μ m). (d) Western blot analysis of p53 protein expression in tumor tissues after treatment. GAPDH served as a loading control. (e) Flow cytometric quantification of CD8⁺ T cell infiltration (percentage of CD8⁺ cells among CD45⁺ cells) in tumors after treatment (n = 3). (f) Flow cytometric quantification of memory T cells (CD44⁺/CD62L⁺ double-positive among CD4⁺ cells) in spleens after treatment (n = 3). (g–j) ELISA quantification of serum cytokine levels after treatment: (g) IFN- γ , (h) TNF- α , (i) IL-6, and (j) IL-12 (n = 3). All data are presented as mean $\pm$ SEM. One-way ANOVA with Tukey's post hoc test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Supplementary Files

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