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Autophagy-Enhanced Hybrid Pyroptotic Vesicles as Personalized Vaccines for Cancer Immunotherapy

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Abstract

Tumor vaccines represent a promising strategy for mobilizing the immune system against malignancies, however, their efficacy is limited by poor antigen immunogenicity, inefficient lymph nodes (LNs) accumulation, and inadequate antigen presentation by antigen-presenting cells (APCs), particularly dendritic cells (DCs). Pyroptosis, a pro-inflammatory form of cell death known to enhance immunogenicity, and autophagy, a cellular pathway that promotes MHC class I and II antigen presentation in APCs, have emerged as two compelling mechanisms to address these limitations. Here, we developed a biomimetic hybrid nano-vaccine (hDMVac) by repeatedly extruding pyroptotic B16 and DC2.4, followed by loading with beclin-1. The results show that hDMVac exhibited a particle size of approximately 160 nm and showed enhanced accumulation in LNs after subcutaneous injection, with time-dependent distribution within the lymphatic system. The incorporation of pyroptotic tumor cells significantly enhanced antigen immunogenicity. Moreover, beclin-1 loading robustly induced autophagy in DCs, facilitating DCs maturation and markedly enhancing cross-presentation of antigens within LNs. The synergistic effect significantly amplified antigen-specific T cell activation. Consequently, hDMVac effectively suppressed tumor growth in both prophylactic and therapeutic melanoma models. This dual strategy represents a synergistic approach to overcoming the limitations of conventional tumor vaccines, representing a clinically translatable platform compatible with existing immunotherapies for improved tumor treatment.

Keywords: Tumor vaccine, Pyroptosis, Autophagy, Biomimetic vesicle, Lymph node accumulation

1. Introduction

Tumor vaccine has emerged as a promising strategy to combat tumors by activating potent and durable antitumor immune responses[1]. Whole tumor cell vaccines (WTCVs) have attracted increasing attention in cancer immunotherapy owing to their broad antigenic spectrum and ability to stimulate both innate and adaptive immune responses[2-5]. Unlike peptide-based or single-antigen vaccines, WTCVs provide the entire repertoire of tumor-associated antigens, thereby minimizing the risk of immune escape and inducing long-lasting immunological memory. These features make WTCVs highly appealing as a universal and individualized vaccination strategy against heterogeneous tumors[6, 7]. However, the clinical translation of WTCVs has been limited by several major bottlenecks. First, tumor cells possess intrinsically low immunogenicity, resulting in inefficient immune priming and suboptimal activation of cytotoxic T lymphocytes (CTLs)[5, 8, 9]. Second, WTCVs exhibit inefficient antigen presentation, as cross-presentation of exogenous tumor antigens by antigen-presenting cells (APCs) is inherently limited and further hindered by excessive tumor cell debris, leading to inadequate CD8⁺ CTLs activation[10, 11]. Third, effective vaccine activity relies heavily on the delivery of antigens to secondary lymphoid organs, particularly lymph nodes (LNs), which serve as central sites for T-cell priming. Unfortunately, conventional WTCVs display poor LN accumulation and rapid clearance[12-14]. These shortcomings represent the critical obstacles that must be overcome for WTCVs to achieve robust clinical efficacy.

Pyroptosis, a highly inflammatory form of programmed cell death mediated by gasdermin family proteins, has recently gained attention in the context of cancer immunotherapy[15, 16]. Unlike apoptosis, which is largely immunologically silent, pyroptosis results in membrane pore formation and subsequent cell lysis, leading to the release of tumor-associated antigens together with large amounts of danger-associated molecular patterns (DAMPs),

such as HMGB1, ATP, and pro-inflammatory cytokines including IL-1 β and IL-18. These factors not only amplify local inflammation but also recruit and activate dendritic cells (DCs), thereby promoting efficient antigen uptake and presentation[17-20]. Therefore, the induction of pyroptosis in tumor cells *ex vivo* represents a promising strategy for developing WTCVs with enhanced immunogenicity and superior capacity to prime robust antitumor T-cell responses[21].

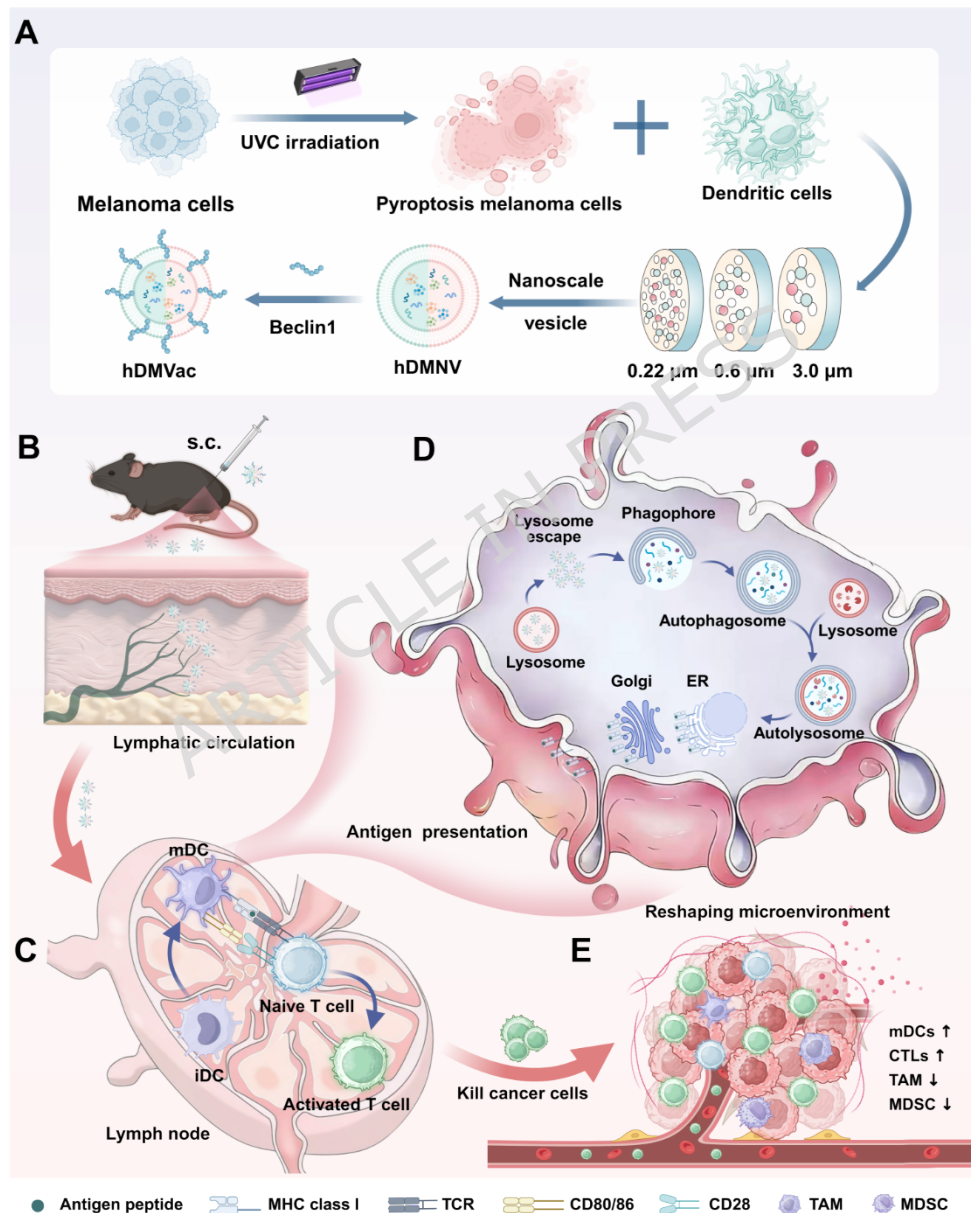
Autophagy is an evolutionarily conserved intracellular process responsible for degrading and recycling cellular components. This fundamental function enables it to play a pivotal role in antigen presentation, where it facilitates the processing and loading of tumor antigens onto both MHC class I and II molecules. For MHC class II presentation, autophagy captures intracellular proteins, including tumor antigens and viral components, and delivers them to endosomal compartments for loading onto MHC-II molecules. This process significantly expands the repertoire of antigens presented to CD4⁺ T helper cells. Furthermore, autophagy actively contributes to cross-presentation, the crucial pathway where exogenous antigens are presented on MHC class I molecules to activate CD8⁺ cytotoxic T cells[22, 23]. Beyond supplying antigens, autophagy also contributes to DCs maturation and the optimal expression of co-stimulatory molecules. This ensures that DCs not only present antigens effectively but also provide the necessary secondary signals for robust T cell activation[24, 25]. As an essential initiator of autophagosome formation, beclin-1, particularly its 267-284 amino acid sequence, governs the formation of autophagosomes through the activation of the class III PI3K complex. This activation enhances the cross-presentation capacity of DCs, and ultimately augments antigen-specific CD8⁺ T cell responses[26, 27]. Harnessing this property of autophagy thus provides a rational strategy to amplify autophagic activity and improve anti-tumor immunity, especially in vaccines based on exogenous tumor antigens.

In addition to improving immunogenicity and antigen presentation,

efficient LN accumulation is critical for vaccine efficacy[28]. LNs serve as central hubs of immune activation, containing dense populations of DCs, T cells, and B cells. Accumulation of vaccine components in LNs increases the probability of antigen uptake by APCs and strengthens interactions with lymphocytes[29, 30]. Particle size is a key determinant of LN drainage kinetics; particles within the 10-200 nm range can rapidly reach LNs through lymphatic vessels, facilitating early and efficient immune priming[31, 32]. Separately, extensive studies on DCs biology have revealed that DC2.4 cells, a widely used DC model, possess the ability to migrate to draining LNs following subcutaneous injection. This process is mediated by the high expression of CC-chemokine receptor 7 (CCR7), which guides mature DCs to draining LNs through interactions with its ligands CCL21 and CCL19 on lymphatic endothelial cells[33-36]. While such active migration is characteristic of intact DCs, the biomimetic nanovesicles used in this study lack intracellular signaling machinery and cytoskeletal support. Therefore, their accumulation in LNs is more likely governed by passive lymphatic drainage and size-dependent transport, potentially supplemented by interactions between membrane-associated molecules and the lymphatic microenvironment. Together, these factors provide a conceptual framework for the rational design of next-generation vaccine delivery systems with enhanced LNs accumulation.

Herein, we developed a biomimetic hybrid dendritic cell-melanoma cell nanovaccine (hDMVac) by fusing pyroptotic B16 cells with DC2.4 cells through serial extrusion across polycarbonate membranes of different pore sizes, followed by loading with the beclin-1 peptide. This design integrates the high immunogenicity and pro-inflammatory properties of pyroptotic tumor cells with the LNs accumulation capability associated with DC-derived membranes, further augmented by autophagy induction (Scheme 1). The resulting nanoparticles (~160 nm) exhibited enhanced accumulation in LNs after subcutaneous injection and showed time-dependent distribution within the

lymphatic system. By promoting DC maturation, antigen cross-presentation, and CTLs activation, hDMVac effectively inhibited melanoma growth and progression in both prophylactic and therapeutic settings. This dual-modality biomimetic vaccine platform provides a promising strategy for overcoming the limitations of conventional tumor vaccines and offers translational potential for combination with other immunotherapies.



Scheme 1. Construction of hybrid nanovaccine and the mechanism of enhancing antitumor immune response through autophagy. (A) Diagram of the hDMVac fabrication process, in which UVC-induced pyroptotic B16 cells are

co-processed with DC2.4 cells, followed by gradient mechanical extrusion and Beclin1 loading to generate the hybrid nanovaccine hDMVac. (B) Lymphatic trafficking of hDMVac after subcutaneous (s.c.) administration, enabling efficient delivery to draining lymph nodes. (C) DCs maturation and T-cell priming in lymph nodes, characterized by enhanced antigen presentation and costimulatory signaling. (D) Autophagy-mediated antigen processing and presentation in DCs, facilitating lysosomal escape and MHC-I cross-presentation. (E) Antitumor immune activation in the tumor microenvironment, resulting in increased cytotoxic T lymphocyte infiltration and reduced immunosuppressive cell populations.

2. Materials and methods

2.1 Materials and reagents

Modified Beclin-1 peptide (DSPE-PEG2000-CG-TNVFNATFEIWHDGEFGT) was procured from Nanjing TGpeptide Biotechnology Co., Ltd. Recombinant Murine Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Cat: 315-03-20UG) was procured from PeproTech Co., Ltd. LysoTracker green (Cat: C1047S), Golgi-Tracker green (Cat: C1045S), DiO (Cat: C1038), DiI (Cat: C1036) cell membrane fluorescent probe, DAPI Staining Solution (Cat: C1005), PI staining solution (Cat: C1734), Coomassie Blue Staining Solution (Cat: ST030) and BCA Protein Assay Kit (Cat: P0011) were procured from Beyotime Co., Ltd. DiR cell membrane fluorescent probe (Cat: BN14006) was procured from Biorigin Co., Ltd. ER-Tracker blue (Cat:40761ES50) were procured from Yeasen Biotechnology Co., Ltd. Anti-mouse CD45 (Cat:35-0451-82), Anti-mouse CD11c (Cat: 45-0114-82), Anti-mouse MHC-II (Cat:63-5321-82), Anti-mouse CD80 (Cat:12-0801-82), Anti-mouse CD86 (Cat:11-0862-82), Anti-mouse CD3 (Cat:17-0032-82), Anti-mouse CD4 (Cat:46-0042-82), Anti-mouse CD8a (Cat:12-0081-82), Anti-mouse clek9a (Cat:2383284) were supplied by Thermo Fisher Scientific. Anti-mouse xCR1 (Cat:148205) was supplied by Biolegend. Anti-mouse MHC-I (Cat: AB95572) was supplied by Abcam. Anti-

LC3B antibody (Cat:12741T) was supplied by Cell Signaling Technology. Anti- β -tubulin antibody (Cat: M30109S) was supplied by Abmart. Mouse TNF- α ELISA Kit (Cat: RK04875), Mouse IFN- γ ELISA Kit (Cat: RK00019), and Mouse IL-6 ELISA Kit (Cat: RK00008) were purchased from ABclonal Co., Ltd.

2.2 Cells and animals

DC 2.4 (RRID: CVCL_J409) and B16 cells (RRID: CVCL_F936) were obtained from the National Collection of Authenticated Cell Cultures. mCherry-GFP-LC3B-expressing DC 2.4 cells were obtained from Obio Biotechnology Co., Ltd. DC 2.4 and B16 cells were cultured in the RPMI-1640 medium containing 10 % FBS and 1 % penicillin and streptomycin. mCherry-GFP-LC3B-expressing DC 2.4 cells were cultured in the RPMI-1640 medium containing 10 % FBS and 2 μ g/ml puromycin. All cell lines used in this study were tested and found to be free of mycoplasma contamination. Female C57BL/6 mice (6-8 weeks) were purchased from the SPF Biotechnology Co., Ltd. All animal experiments were conducted following the guidelines of the Animal Ethics Committee of Shanghai Skin Disease Hospital and by the Guide for the Care and Use of Laboratory Animals with the ID number: 2023-108 (Animal).

2.3 Cell death assays

To assess membrane lysis and UVC-induced pyroptosis, culture supernatants were collected and LDH release was quantified using the CytoTox 96 Cytotoxicity Assay (Beyotime) according to the manufacturer's instructions. B16 cells were irradiated with UVC for 30, 60, 90, or 120 min and subsequently incubated for 4 h before LDH measurement.

2.4 CCK8 assay

The cytotoxicity of beclin-1 at different concentrations was evaluated in vitro using the CCK-8 assay. DC2.4 cells were incubated with various concentrations of beclin-1 for 24 h. Following the addition of CCK-8 solution, absorbance was measured at 450 nm. Cell viability was calculated according to the formula: Viability (%) = (A_{sample} – A_{blank}) / (A_{control} – A_{blank}) $\times$ 100%

and used to evaluate the cytotoxic effects of beclin-1 on DC2.4 cells.

2.5 Preparation and characterization of hDMVac

B16 cells were irradiated with UVC using a ZF-1 ultraviolet analyzer (Shanghai Jinpeng, China; 254 nm) for 1 h at a distance of approximately 10 cm, followed by a 4 h incubation. Based on the reported irradiation intensity and distance-dependent estimation, the UVC dose was approximately 280–300 mJ/cm² under our experimental conditions. The selected irradiation condition corresponds to a high-dose UVC exposure, which has been reported to induce pyroptosis. The pyroptotic B16 cells were then mixed with DC 2.4 cells at a 1:1 ratio and processed under high pressure using a membrane extruder (NanoAble-150, pH Technology LLC, USA) through polycarbonate membranes (3.0 µm, 0.6 µm, and 0.2 µm; Merck, Cat: TSTP04700, DTTP04700, GTBP04700) to prepare the hybrid DC 2.4-pyroptotic B16 cells biomimetic nanovesicles (hDMNV). The final vaccine, hDMVac, was prepared by incorporating a DSPE-PEG2000-modified beclin-1 peptide into the hybrid vesicles. Specifically, the amphiphilic DSPE-PEG2000 moiety enables spontaneous insertion of the peptide conjugate into the lipid bilayer of the vesicles through hydrophobic interactions, thereby displaying beclin-1 on the vesicle surface. The solution was gently stirred with a magnetic stirrer for 4 h to ensure uniform peptide anchoring and stable presentation.

The size, PDI, and Zeta potential of hDMVac were measured using dynamic light scattering (DLS) and electrophoretic light scattering (ELS) with a Zetasizer Nano ZS (Malvern). The morphology of hDMVac was examined by transmission electron microscopy (TEM) (HT7800, Hitachi). Negative staining was performed with 3% uranyl acetate, and TEM images were captured. Protein concentration was determined using a BCA assay, and SDS-PAGE was conducted to assess the homology between hDMVac and DC2.4 or B16 cells proteins, with Coomassie Brilliant Blue staining.

2.6 TEM Analysis of Autophagy

DC2.4 cells (4×10^5 per well) were cultured in a 6-well culture dish, then

treated with different formulations, including Control, MNV, pMNV, hDMNV, and hDMVac, respectively. After 24 h of incubation, cells were washed twice with PBS and harvested into 1.5 mL EP tube, added with 2.5% glutaraldehyde solution, and stored at 4 °C. TEM images were captured using a Hitachi H-600(Japan) at 200 kV.

2.7 Subcellular localization of hDMVac in DC2.4 cells

The hDMVac was first stained with DiI for 30 min and then incubated with DC2.4 cells. Then the cells were labeled with LysoTracker green, ERTracker Blue, and Golgi-Tracker green, respectively. Subsequently, the working solution was removed, washed with PBS, and fixed with paraformaldehyde for 30 min, and finally washed by adding DAPI for 5 min of incubation. Confocal laser scanning microscope (CLSM) was used to investigate co-localization situations.

2.8 Western Blot Analysis

Protein samples were extracted from cells using RIPA lysis buffer (with protease and phosphatase inhibitors). The protein concentration was determined using the BCA assay. Equal amounts of protein (10-20 µg) were separated by SDS-PAGE on a 12.5% polyacrylamide gel and transferred onto a PVDF membrane. After blocking with blocking solution for 1 h at room temperature, the membrane was incubated overnight at 4°C with primary antibodies specific for the target proteins. After washing with TBST, the membrane was incubated with the appropriate HRP-conjugated secondary antibody for 1 h at room temperature. Protein bands were visualized using the ECL detection system, and the relative protein expression levels were quantified using ImageJ software.

2.9 Bone-Marrow Derived Dendritic Cells Isolation and Induction

Bone marrow-derived dendritic cells (BMDCs) were generated from C57BL/6 mice (>5 weeks old). After sacrifice, femurs were harvested, and bone marrow cells were flushed into centrifuge tubes using ice-cold RPMI-1640 medium. Red blood cells were lysed, and the suspension was centrifuged

at 800 g for 5 min. The remaining cells were cultured in RPMI-1640 medium supplemented with GM-CSF (20 ng/mL) and IL-4 (10 ng/mL). On days 3 and 5, half of the culture medium was replaced with fresh cytokine-containing medium. On day 6, BMDCs were co-incubated with PBS, MNV, pMNV, hDMNV, and hDMVac for subsequent analysis. A PBS-treated group was used as a negative control.

2.10 Evaluation of hDMVac on BMDCs maturation

BMDCs (1×10^6 cells/well) were seeded in 6-well plates and co-incubated with vaccines from each group for 24 h under standard conditions (37 °C, 5% CO₂). After treatment, cells were harvested, washed with PBS containing 2% FBS, and stained with fluorescence-conjugated antibodies against CD11c, CD80, and CD86 for 30 min at 4 °C in the dark. Flow cytometric analysis was performed on a Navios cytometer (Beckman Coulter), and data were analyzed using FlowJo software.

2.11 Evaluation of T cell activation induced by hDMVac-treated BMDCs

BMDCs were first treated with vaccines from each group for 24 h as described above. After washing with PBS to remove excess vaccines, BMDCs were co-cultured with splenic T cells at a ratio of 1:10 (BMDCs : T cells) in 6-well plates. T cells were isolated from mouse spleens and resuspended in complete culture medium. The co-culture was maintained for 24 h under standard conditions (37 °C, 5% CO₂).

After incubation, cells were harvested and washed with PBS containing 2% FBS. Cells were then stained with fluorescence-conjugated antibodies against CD3, CD4, CD8, and CD69 for 30 min at 4 °C in the dark. Flow cytometric analysis was performed and data were analyzed with FlowJo software. CD3⁺CD8⁺ T cells were gated to evaluate the expression of the activation marker CD69.

2.12 Lymph nodes distribution

Vaccines from each group were labeled with DiI for 15 min before use. Mice were subcutaneously injected with the vaccines - DiI, and 24 h post-

injection, the dLNs were excised, cryosectioned, and subjected to high-resolution scanning. For in vivo biodistribution studies, vaccines were labeled with DiR. Whole-body fluorescence imaging was performed at 24 h after subcutaneous administration to evaluate vaccine distribution. At 72 h post-injection, major organs (heart, liver, spleen, lung, and kidney) were collected to assess vaccine biodistribution and accumulation in different tissues.

2.13 Evaluation of Antigen Cross-Presentation by cDC1 in Lymph Nodes

C57BL/6 mice were randomly divided into five groups: Control, MNV, pMNV, hDMNV, and hDMVac (n = 3 per group). Mice were subcutaneously immunized every three days for a total of four doses. Two days after the final immunization, dLNs were collected and mechanically dissociated into single-cell suspensions. Cells were stained with fluorophore-conjugated antibodies against CD45, CD11c, MHC-II, XCR1, CLEC9A, and MHC-I to identify cDC1 populations and evaluate their antigen presentation capacity. Flow cytometric analysis was performed using a Navios cytometer (Beckman Colter), and data were analyzed with FlowJo software.

2.14 Therapeutic Effect of Nanovaccines on Melanoma

Female C57BL/6 mice were randomly divided into five groups (n=5). B16 cells were cultured and digested when approximately 80% confluent, and on day 0, 1×10^6 B16 cells were injected subcutaneously into the right dorsal region. Mice were treated with Control, MNV, pMNV, hDMNV, or hDMVac on days 1, 4, 7, and 10. Starting on day 9, body weight and tumor dimensions were measured every three days to calculate tumor volume. Tumor volume was calculated using the formula: Tumor volume = $0.5 \times \text{length} \times \text{width}^2$. At the end of the experiment, tumor tissues were homogenized, and LNs were harvested for cell collection. Flow cytometry was performed using antibodies against CD45, CD11c, CD80, CD86, CD3, CD4, and CD8a. Tumor tissues were processed for TUNEL and Ki67 staining, and immunofluorescence was performed for CD11B/Gr-1 and F4/80 /CD163 to evaluate immune cell

infiltration.

2.15 Preventive Effect of Nanovaccines on Melanoma

Female C57BL/6 mice were randomly assigned to five groups (n=5). On day -12, mice were immunized with Control, MNV, pMNV, hDMNV, and hDMVac. Booster immunizations were administered on days -9, -6, and -3. On day 0, 1×10^6 B16 cells were injected subcutaneously into the right flank of C57BL/6 mice. Starting from day 9, body weight and tumor dimensions (length and width) were measured every three days. Tumor volume and survival were recorded throughout the experiment. At the study endpoint, tumor tissues and LNs were fixed in 4% paraformaldehyde, and immunofluorescence staining was performed for CD8 and CD11c/CD80. Spleens were harvested and homogenized to collect cells, which were subsequently stained with antibodies against CD45, CD4, and CD8. Flow cytometric analysis was performed to assess immune cell populations.

2.16 ELISA assay

Peripheral blood from treated mice was allowed to clot at room temperature for 1 h and subsequently centrifuged at 2000 rpm for 10 min at 4 °C. The resulting supernatant was collected as serum and transferred into fresh microcentrifuge tubes. Serum levels of inflammatory cytokines (TNF- α , IFN- γ , and IL-6) were determined following the manufacturer's instructions.

2.17 Safety evaluation of nanovaccines

Peripheral blood was collected from mice in anticoagulant-treated tubes for routine hematological profiling. Major organs, including the heart, liver, spleen, lungs, and kidneys, were excised, fixed in 4% paraformaldehyde, and subsequently processed for paraffin embedding. Hematoxylin and eosin (H&E) staining was performed to evaluate potential histopathological alterations and tissue integrity.

2.18 Statistical analysis

All results are expressed as mean $\pm$ SD. Statistical analyses were carried out using GraphPad Prism 8. Comparisons between two groups were

conducted with a paired t-test, while differences among three or more groups were assessed using one-way ANOVA followed by Tukey's post hoc test. A P value < 0.05 was considered statistically significant.

3. Results

3.1 Preparation and characterization of hDMVac

Studies have shown that the full-length GSDME induces pyroptosis in cells exposed to strong UVC, and this process does not require protease cutting[37]. B16 cells inherently express GSDME, so UVC was used to induce their pyroptosis. To determine the intensity of UVC irradiation that would induce tumor cell pyroptosis, we first observed the morphological changes of cells under different UVC irradiation times by CLSM and SEM. Following UVC irradiation, PI staining revealed a time-dependent increase in B16 cells death, with initial positivity observed at 20 min and complete cell death by 60 min (Fig. 1A). Consistently, SEM images displayed early membrane alterations at 60 min and characteristic morphological changes indicative of pyroptosis including cellular swelling, bubble-like protrusions, and membrane perforation were observed in B16 cells (Fig. 1B). Based on initial microscopic observations, 30, 60, 90, and 120 min were selected for the LDH release assay to assess prolonged UVC irradiation. LDH release markedly increased between 30 and 60 min, then rose only slightly and plateaued at 90 and 120 min (Fig. S1), indicating that 60 min represented a near-maximal level of cell rupture. Together, these results demonstrate that 60min of UVC exposure is sufficient to induce pyroptotic cell death in the majority of the cell population. Hybrid vesicle was prepared by fusing DC2.4 cell membranes with pyroptotic B16 cells membranes through co-extrusion. To confirm successful fusion, the DC2.4 and B16 membranes were pre-labeled with DiO (green) and DiI (red), respectively. CLSM validated the integration of dual-source membranes. The homogeneous co-localization of differentially labeled membranes confirmed

efficient membrane hybridization rather than mere physical mixing (Fig. 1C). Next, the hybrid vesicle was used to deliver the autophagy-related protein beclin-1, an autophagy-inducing component. To determine the safe concentration of delivered beclin-1 for inducing autophagy in DCs, cytotoxicity of beclin-1 towards DC 2.4 cells was assessed using the CCK-8 assay. As shown in Figure S2, beclin-1 (7.5 μ M) exhibited negligible cytotoxicity against DC 2.4 cells, a concentration deemed suitable for subsequent studies. Additionally, the encapsulation efficiency of the beclin-1 peptide reached approximately 62.8%, confirming sufficient loading of the therapeutic agent for downstream immune activation. Protein composition of nanovesicles and their parental cells was assessed using SDS-PAGE to determine whether nanovesicles retained the characteristic protein profiles of their cellular sources. The results showed that hDMVac largely retained protein bands from both parent cell membranes, suggesting effective preservation of native membrane proteins throughout the fusion process (Fig. 1D). Besides, western blot analysis further demonstrated that hDMVac exhibited significantly enriched expression of CCR7-a key LN-homing chemokine receptor-compared to single-source membrane vesicles (Fig. S3). This indicates that the hybrid vesicles inherited key biofunctional proteins from both pyroptotic tumor cells and DC2.4 cells. To more rigorously validate the retention of protein components in hDMVac, we further performed fast data-independent acquisition (fast-DIA) proteomic analysis comparing hDMVac with its parental cells (B16 and DC2.4). As shown in Fig. 1E and S4, the Venn diagram revealed a substantial overlap of identified proteins among the three groups, with hDMVac retaining a large proportion of proteins derived from both B16 and DC2.4 cells. Quantitative analysis further demonstrated that the majority of proteins detected in hDMVac were shared with parental cells, accounting for over 70% overlap with each source, indicating efficient preservation of the parental proteome during vesicle fabrication. Importantly, heatmap analysis of representative functional proteins demonstrated that hDMVac simultaneously preserved

tumor-associated antigens (such as GP100, TYRP1, DCT, and TYR) derived from B16 cells, as well as antigen processing and presentation-related molecules (including TAP1, TAP2, H2-K1, PSMB8, and PSMB9) and adhesion/homing-associated proteins (e.g. CD44) originating from DC2.4 cells (Figure. 1F). Collectively, these results provide comprehensive proteomic evidence that hDMVac not only retains a broad spectrum of tumor-specific antigens but also inherits key immune-functional molecules from dendritic cells. The prepared vaccine was then characterized by TEM and DLS. TEM revealed that hDMVac exhibited a uniform spherical morphology, indicating favorable morphological stability and consistency (Fig. 1G). DLS further confirmed an average hydrodynamic diameter of 163.9 nm for hDMVac (Fig. 1H), with a narrow size distribution (polydispersity index, PDI < 0.1). This monodispersity highlights excellent size uniformity and colloidal stability, a dimension optimal for LNs drainage and cellular uptake. Zeta potential measurements demonstrated a surface charge of approximately -10 mV (Fig. 1I), suggesting that this moderate negative charge contributes to physical stability in physiological environments by minimizing non-specific aggregation.

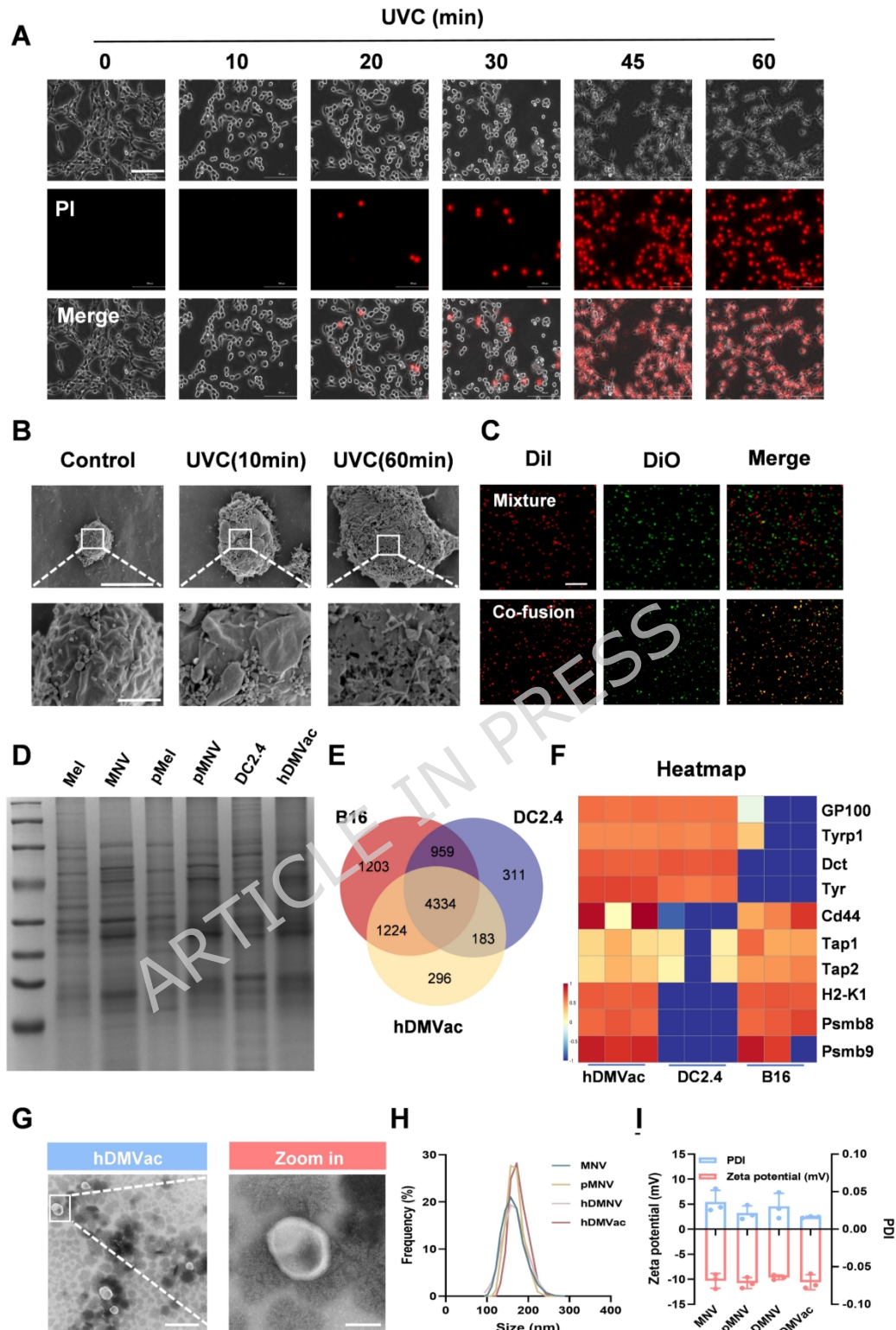


Figure 1 □ Fabrication and characterization of the dendritic cell-melanoma hybrid biomimetic nanovaccine (hDMVac). (A) Morphological changes and cell death in B16 cells following UVC irradiation at the indicated doses. Scale bar: 100 μ m. (B) SEM images revealing pyroptosis-associated pore formation on

the membranes of B16 cells following UVC irradiation. Scale bars: 10 μ m (upper), 2 μ m (lower). (C) CLSM validation of successful membrane fusion using differentially labeled membranes. Scale bar: 10 μ m. (D) SDS-PAGE protein analysis of different cells and nano-vesicles derived from their sources by Coomassie blue staining. (E) Proteomic characterization of hDMVac and parental cells. (F) Heatmap analysis of representative tumor antigens and immune-related proteins. (G) Morphology of TEM hDMVac. Scale bar: 500 nm, left side; 100 nm, right side. (H) Biomimetic nanovesicles size distribution. (I) Zeta potential analysis and PDI of the biomimetic nanovesicles.

3.2 Autophagy induction by hDMVac

To validate whether hDMVac activates autophagy in DCs, we employed a mCherry-GFP-LC3B dual-labeling system in DC2.4 cells. In this assay, yellow puncta indicate autophagosomes, whereas red puncta represent autolysosomes. Thus, the relative distribution of yellow versus red puncta reflects autophagic flux under different treatments. As shown in Fig. 2A, the control group showed minimal yellow and red puncta formation, which indicated that autophagy activity was relatively low in DC2.4 cells. MNV group induced a slight increase in both yellow and red puncta, suggesting a modest enhancement in autophagic activity. Moreover, the pMNV and hDMNV groups exhibited a pronounced increase in the number of puncta, indicating that both groups more effectively promoted autophagy upregulation in DC2.4 cells. Notably, the hDMVac group demonstrated the highest density of puncta, with the majority fluorescing red. This pattern indicates that beclin-1 potentially stimulates autophagosome formation and drives highly efficient progression through the autophagic pathway to the autolysosome stage. Microtubule-associated protein light chain 3 (LC3) is a well-established marker for monitoring autophagy. Under basal conditions, LC3 exists in a cytosolic form (LC3-I). Upon autophagy induction, LC3-I underwent lipidation to generate LC3-II, which subsequently accumulated on autophagosomal membranes. To

further validate the degree of autophagy activation at the molecular level, we performed western blotting to detect LC3-II expression. The results revealed that the ratio of LC3-II to the loading control protein was significantly increased in the hDMVac group compared to other groups (Fig. 2B and C). This directly reflects enhanced LC3 lipidation and the accumulation of LC3-II on autophagic membrane structures, confirming robust autophagy activation at the molecular marker level. As the gold standard for autophagy detection, TEM further provided direct ultrastructural evidence. In hDMVac-treated DC2.4 cells, we observed a significantly increased number of autolysosome structures (Fig. 2D and E). These vesicular structures generally possessed a single membrane and enclosed cytoplasmic materials or organelles undergoing different degrees of degradation. Collectively, these results consistently demonstrate that hDMVac significantly upregulates the autophagic level in DC cells and efficiently drives the autophagic process through to the degradation stage.

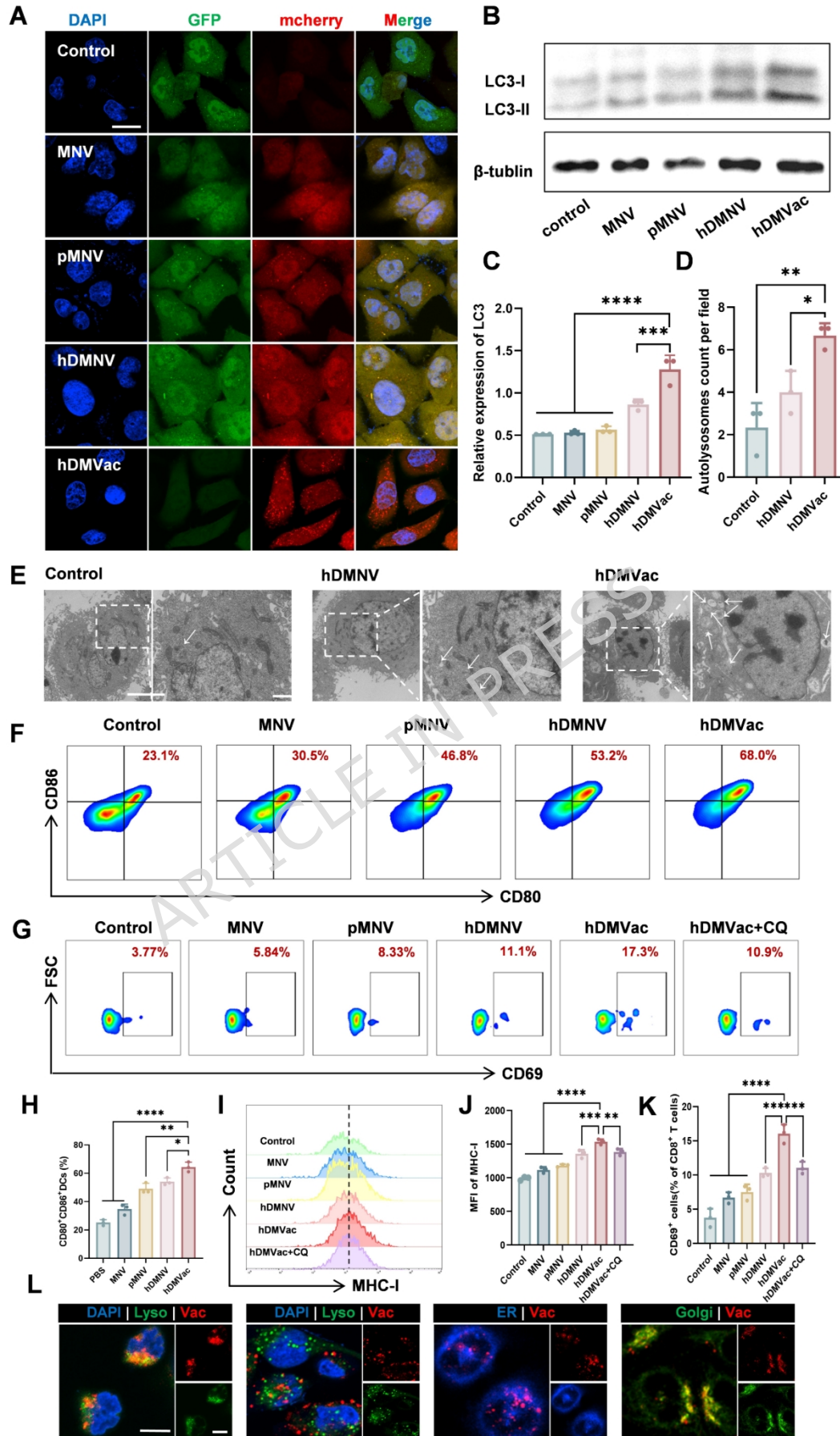


Figure 2. The autophagy and antigen presentation of hDMVac in DCs. (A) CLSM images of mCherry-GFP-LC3 DC2.4 cells after exposure to various formulations. Scale bar: 20 μ m. (B) Relative expression of LC3-II in DC2.4 treated with various formulations. (C) Quantification of LC3-II expression in DC2.4 cells under different treatments ($n = 3$). (D) Quantitative analysis of autolysosome formation in DC2.4 cells after incubation with different formulations ($n = 3$). (E) TEM images of the autolysosome in DC2.4 cells after incubation with various formulations. White arrows aim at a typical autolysosome. Scale bar: Left, 5 μ m; right, 2 μ m. (F) Flow cytometry analysis of the percentage of CD80⁺ CD86⁺ BMDCs. (G) Flow cytometry analysis of CD69 expression in CD8⁺ T cells. (H) Quantification of CD80⁺CD86⁺ BMDCs as assessed by flow cytometry ($n = 3$). (I) Representative flow cytometry histograms showing MHC-I expression on BMDCs after treatment with different formulations. (J) Quantification of MHC-I expression on BMDCs shown as MFI ($n = 3$). (K) Quantification of CD69⁺ cells among CD8⁺ T cells after co-culture with BMDCs treated with different formulations ($n = 3$). (L) Co-localization of organelle Lysosomal (Lyso), Endoplasmic Reticulum (ER), and Golgi apparatus (Golgi) with hDMVac in DC2.4 cells after incubation for 2 h, 3 h, 5 h, and 8 h, respectively. Scale bar: 10 μ m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

3.3 hDMVac promoted DCs maturation and antigen presentation

Having established the potent induction of autophagy, we next evaluated the impact of hDMVac on DCs maturation and antigen-presenting capacity. Flow cytometry analysis of BMDCs revealed that surface expression of the co-stimulatory molecules CD80 and CD86 was low in the control and MNV groups. In contrast, pMNV markedly elevated CD80/CD86 expression to 46.8%, which was further increased by hDMNV. Notably, hDMVac induced the highest level, showing a 2.9-fold increase compared with the control group (Fig. 2F and G). hDMVac markedly enhanced antigen-presenting capacity, as evidenced by a

1.5-fold increase in the mean fluorescence intensity (MFI) of MHC-I compared with the control group (Fig. 2H and I), suggesting an enhanced potential for antigen cross-presentation. To determine whether autophagy is required for this effect, BMDCs were treated with the autophagy inhibitor chloroquine (CQ) during stimulation. CQ treatment significantly attenuated hDMVac-induced MHC-I upregulation, as reflected by a marked reduction in MHC-I MFI compared with hDMVac alone (Fig. I). Although MHC-I levels in the hDMVac + CQ group remained higher than those in the control group, this partial reversal indicates that autophagy plays a critical role in mediating hDMVac-enhanced antigen presentation. Similarly, hDMVac treatment led to a substantial increase in MHC-II expression (Fig. S5), indicating a coordinated enhancement of the antigen-presenting machinery in DCs. In a BMDC-T cell co-culture system, hDMVac-treated BMDCs markedly promoted CD8⁺ T-cell activation, as indicated by a significantly increased proportion of CD69⁺ cells compared with the control and other vaccine groups (Fig. J). Consistently, inhibition of autophagy by CQ partially suppressed hDMVac-induced CD8⁺ T-cell activation, further supporting the involvement of autophagy in DC-mediated T-cell priming.

To elucidate the intracellular trafficking route of hDMVac and its link to enhanced antigen presentation, we tracked its localization relative to key cellular organelles in DC2.4 cells over time using CLSM. As shown in Fig. 2J, early after internalization (2 h), hDMVac (labeled with DiR) showed significant co-localization with lysosomes (stained with LysoTracker green), indicating that it is taken up into the endosome-lysosome system. Strikingly, at later time points (3 h), a pronounced reduction in co-localization with lysosomal markers was observed, demonstrating that it escaped from lysosomes to the cytoplasm successfully. Following escape, hDMVac exhibited significant co-localization with the endoplasmic reticulum and the Golgi apparatus (5-8h). This trafficking pattern is consistent with the canonical pathway of antigen cross-presentation.

3.4 Biodistribution and biosafety evaluation of hDMVac in vivo

To assess the lymphatic distribution of the hDMVac vaccine, small animal imaging and frozen tissue section analysis were performed to track the in vivo distribution. At 24 h post-injection, DiR fluorescence was detected in the dLNs in all groups. The hDMNV and hDMVac group exhibited significantly higher fluorescence intensity compared to the control group, suggesting a stronger accumulation in LNs (Fig.3A and C). At 72 h post-injection, fluorescence signals in the hDMNV and hDMVac group were not only retained in the initial LNs but also observed in the contralateral inguinal lymph nodes (ILNs), indicating a broader lymphatic distribution (Fig.3B and D). This prolonged retention and expanded distribution within the lymphatic system may be attributed to passive lymphatic drainage and vesicle size effects, and suggest that the hybrid membrane nanovaccine facilitates efficient accumulation and retention in LNs. Frozen tissue section imaging further validated the in vivo findings. Consistent with the live imaging results, the hDMNV and hDMVac groups displayed a more pronounced DiI signal accumulation within the LNs compared to the control group (Fig.3E). This result confirms the effective lymphatic uptake and retention of the hybrid nanovaccines, which may contribute to prolonged antigen presentation and enhanced immune activation. These findings collectively suggest that the hDMVac vaccine exhibits superior lymphatic accumulation and sustained retention, allowing for prolonged immune modulation within the lymphatic microenvironment.

Subsequently, the safety profile of the hDMVac was further evaluated through H&E staining of major organs and comprehensive hematological and serum biochemical analyses (Fig. S6). No significant histopathological changes were observed in any of the major organs, indicating that hDMVac treatment did not cause detectable tissue damage. Additionally, hematological and biochemical parameters remained within normal ranges, further confirming the safety of hDMVac treatment.

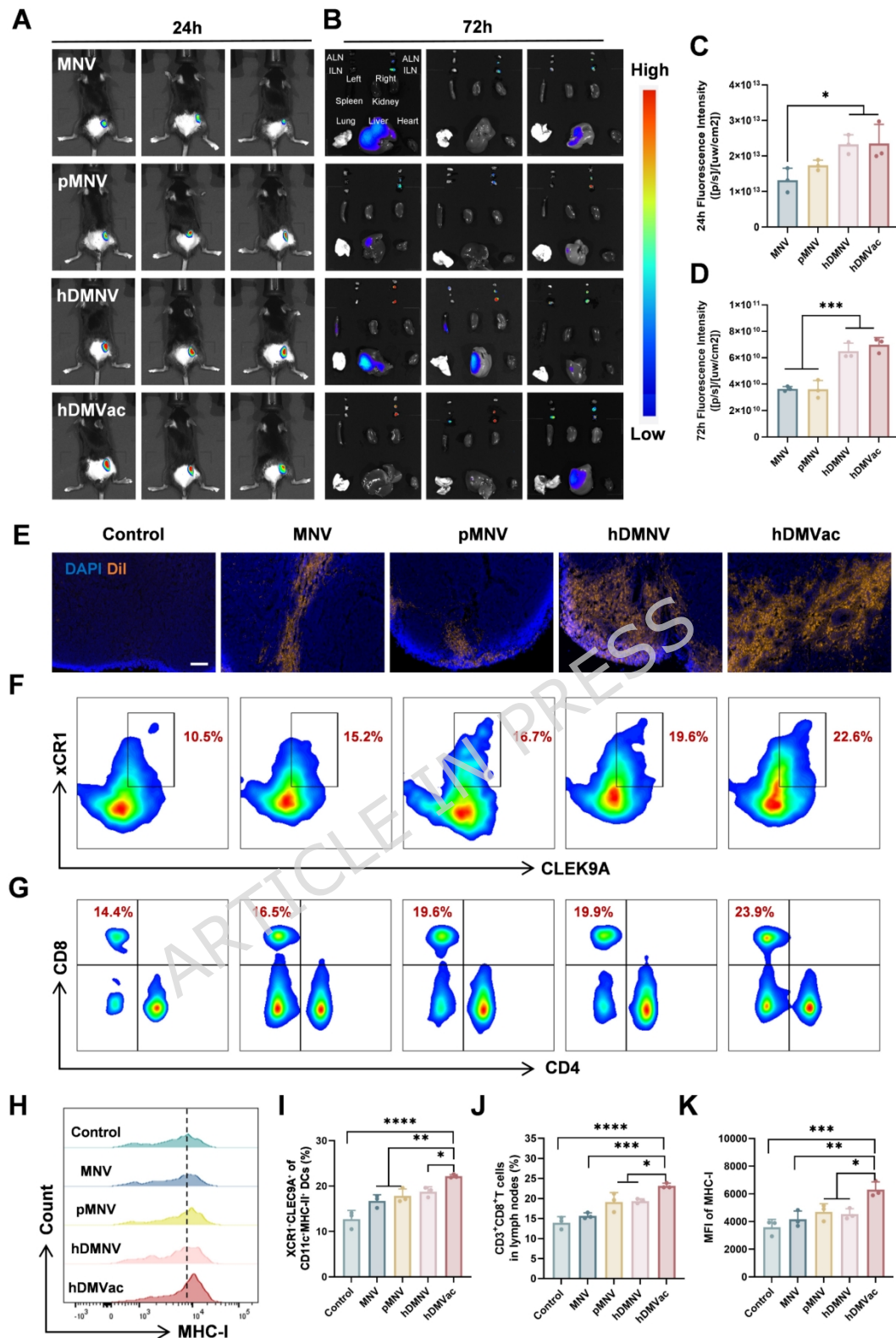


Figure 3. hDMVac accumulates in LNs and boosts cDC1 function and T cell immunity in vivo. (A) Biodistribution of nanovesicles in mice at 24 h post-administration. (B) Biodistribution of nanovesicles in the heart, liver, spleen, lung, and kidney at 72 h post-administration. (C) Quantification of DiR

fluorescence in whole mice at 24 h post-administration. (D) Quantification of DiR fluorescence in isolated heart, liver, spleen, lung, and kidney at 72 h post-administration. (E) Fluorescence of frozen sections of each group of LNs. Scale bars: 100 μ m. (F) Flow cytometry analysis of XCR1⁺ CLEC9A⁺ cDC1 among CD11c⁺ MHC-II⁺ DCs in LNs tissues (n = 3). (G) Flow cytometry analysis of CD8⁺ T cells in LNs tissues. (H) Flow cytometry analysis of MHC-I⁺ cells in XCR1⁺ CLEC9A⁺ cDC1. (I) Quantification of XCR1⁺CLEC9A⁺ cDC1 among CD11c⁺ MHC-II⁺ DCs in LNs tissues (n = 3). (J) Quantification of CD8⁺ T cells in LNs tissues (n = 3). (K) Quantification of MHC-I expression on XCR1⁺ CLEC9A⁺ cDC1 (geometric mean fluorescence intensity, n = 3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

3.5 hDMVac enhances cDC1 cross-presentation in lymph nodes

Having demonstrated that hDMVac effectively induces autophagy, promotes DC maturation, and facilitates antigen processing in vitro, we further evaluated its functional impact on antigen cross-presentation within LNs in vivo, a crucial step for initiating cytotoxic T cell responses. We first performed immunophenotyping of LNs cells by flow cytometry. Conventional type 1 dendritic cells (cDC1), defined as CD45⁺CD11c⁺Clec9A⁺/XCR1⁺ populations, are specialized APCs with a unique ability to mediate cross-presentation and prime cytotoxic CD8⁺ T lymphocytes. Notably, the proportion of cDC1 among total DCs was significantly increased in the hDMVac-treated group, showing a 2-fold elevation compared with the control (Fig. 3F, I, and S7), suggesting enhanced recruitment or differentiation of this key cross-presenting subset. We then examined the expression of antigen-presenting molecules on cDC1 and found that vaccination markedly upregulated MHC-I expression, with a 1.52-fold increase over the control (Fig. 3H and K), suggesting a preferential enhancement of cross-presentation pathways. Consistently, analysis of T cell subsets revealed a pronounced expansion of CD8⁺ T cells in the dLNs of vaccinated mice (Fig. 3G and J), demonstrating

successful priming of CTLs. These results demonstrate that hDMVac augments the frequency and cross-presentation capacity of cDC1, thereby promoting efficient CD8⁺ T cell priming in LNs.

3.6 Therapeutic Effect of hDMVac In Vivo

The therapeutic efficacy of hDMVac was evaluated in a B16 cells bearing mouse model. Mice received four subcutaneous injections at three-day intervals and were monitored for 24 days (Fig. 4A). Body weights remained stable across all groups, confirming the safety of treatment (Fig. 4B). Modest inhibitory effects were observed in the MNV, pMNV, whereas hDMVac treatment led to the most pronounced suppression of tumor growth (Fig. 4C-I). By day 18, tumor volumes in the hDMVac group were markedly smaller than those in the control and other treatment groups, and this divergence became progressively more evident as the study continued. Histological analyses further corroborated these findings. Tumor tissues from the hDMVac-treated group showed markedly reduced proliferative activity, as indicated by a substantial decrease in Ki67-positive cells, together with a robust induction of apoptosis, evidenced by abundant TUNEL-positive signals. (Fig. 4J).

To elucidate the underlying immune mechanisms, we next examined local immune responses in mice treated with vaccines. Flow cytometric analysis of dLNs revealed that hDMVac markedly promoted DC maturation, as evidenced by increased CD80 and CD86 expression (Fig. 5A, F, and S8). We then investigated DCs within the tumor microenvironment (TME). hDMVac treatment induced favorable immune remodeling, characterized by enhanced CD80/CD86 expression on tumor-infiltrating myeloid cells, which increased from 18.3% in the control group to 46.6% (Fig. 5B and G), likely strengthening local T cell priming through co-stimulatory signaling. Meanwhile, immunofluorescence analysis showed a marked reduction in immunosuppressive myeloid populations, including F4/80⁺CD163⁺ tumor-associated macrophages (TAMs) and CD11b⁺Gr-1⁺ myeloid-derived

suppressor cells (MDSCs) (Fig. 5D and E), thereby alleviating local immunosuppression. Consistent with these observations, flow cytometry confirmed significantly increased infiltration of CD8⁺ T cells into tumor tissues, rising from 27.6% in the control group to 54.1% in hDMVac-treated mice (Fig. 5C, H, and S9). Furthermore, ELISA analysis of serum from hDMVac-treated mice revealed elevated levels of IL-6, TNF- α , and IFN- γ , indicative of robust systemic immune activation (Fig. 5K-M). Importantly, IFN- γ levels were particularly pronounced, showing a 4.18-fold increase relative to the control, reflecting strong activation of CTLs and Th1-type immune responses, both of which are essential for effective antitumor immunity. Collectively, these results demonstrate that hDMVac reprograms both local and systemic immunity, establishing a TME highly permissive to antitumor immune activity.

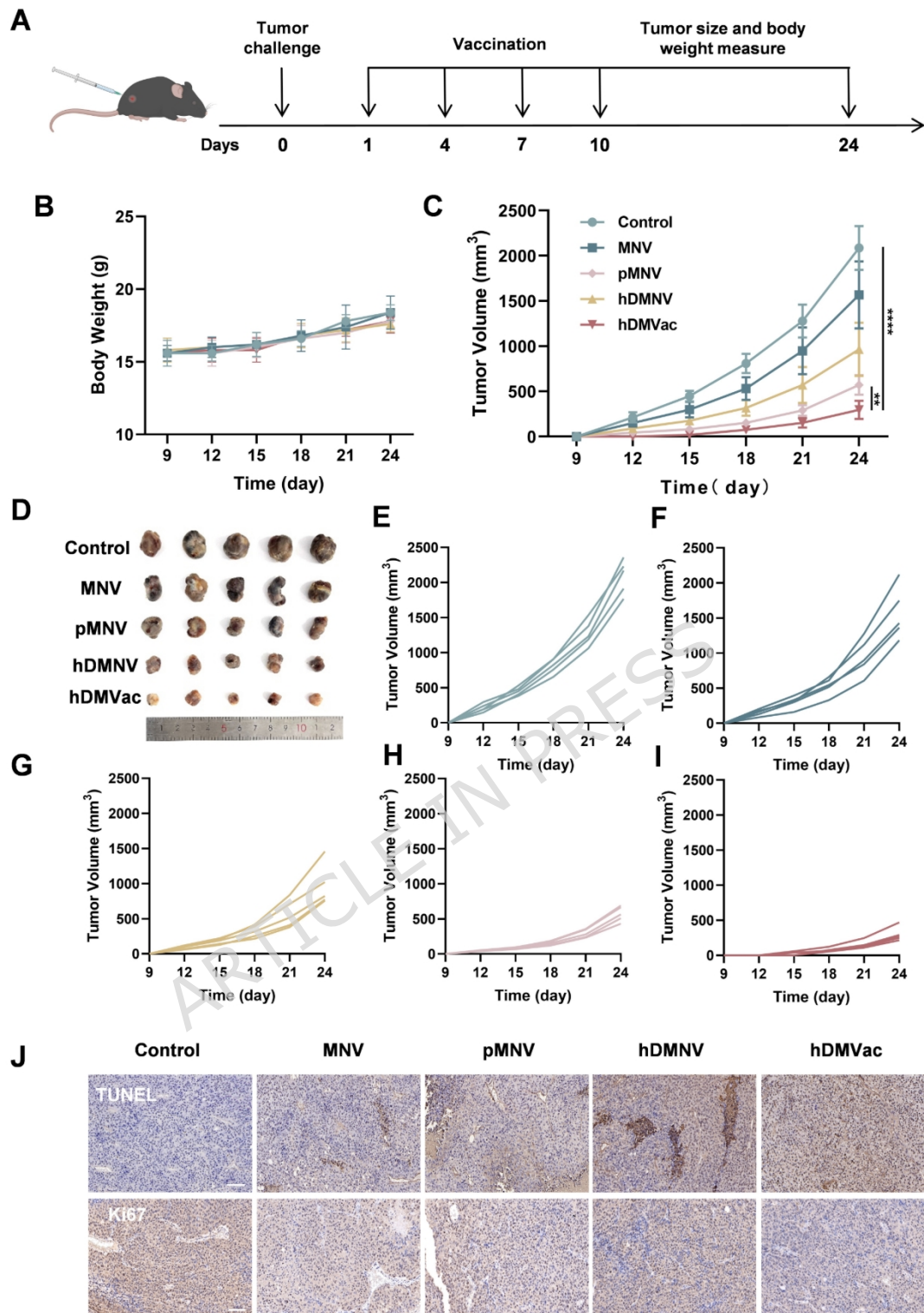


Figure 4. Antitumor effect of hDMVac in vivo. (A) Schematic of the experimental design for melanoma therapeutic vaccination. (B) Body weight changes of tumor-bearing mice ($n = 5$). (C) Tumor volume changes in each group ($n = 5$). (D) Representative images of tumors from mice at day 24 ($n = 5$). (E-I) Tumor volume changes in each group ($n = 5$). (J) TUNEL staining and

Ki67 staining of tumor tissues. Scale bar: 100 μ m.

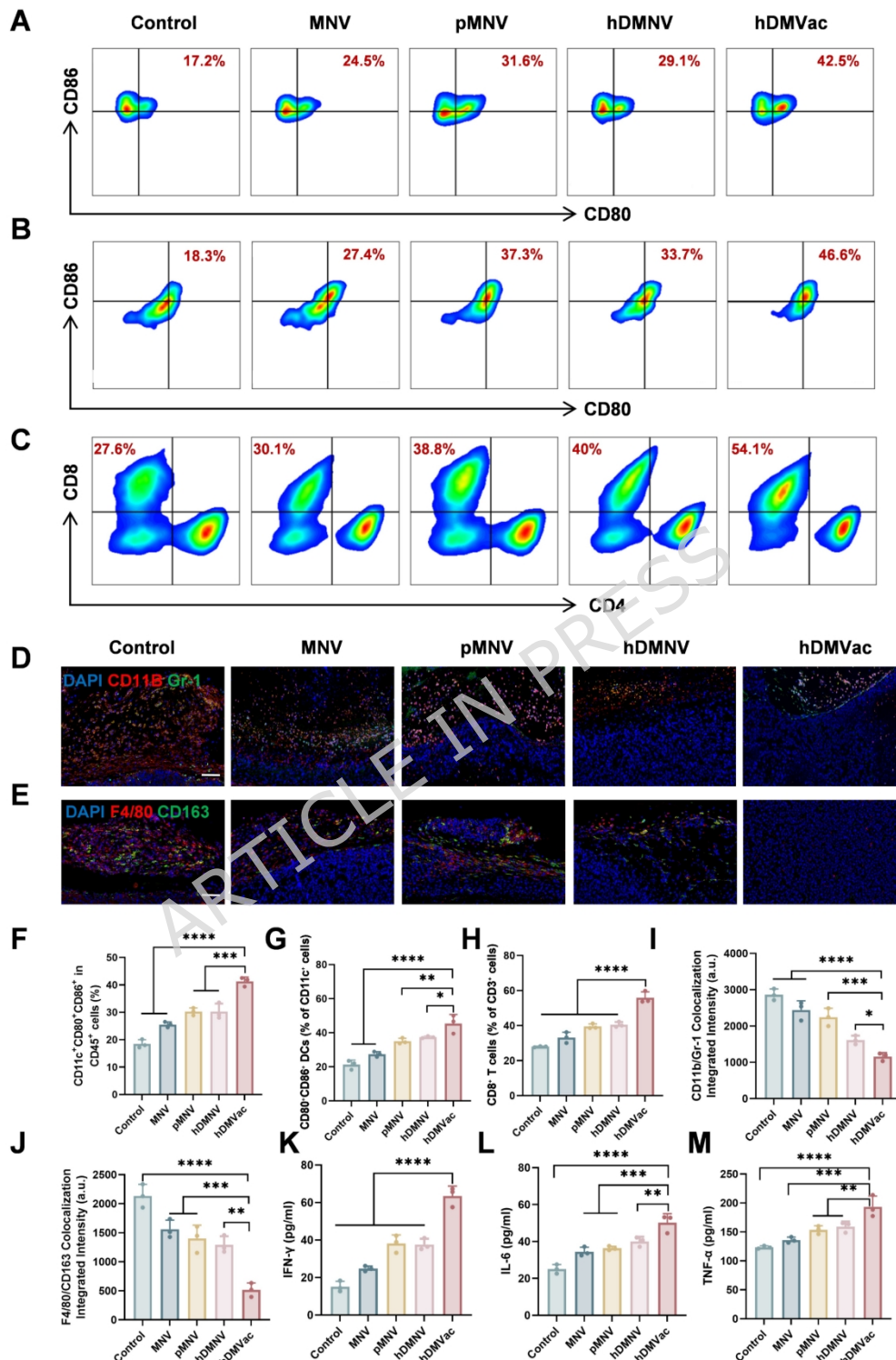


Figure 5. hDMVAc-Induced Tumor Immunity: Local Immune Remodeling and Systemic Immune Activation. (A) Flow cytometric analysis of CD80 and CD86 expression on LN DCs. (B) Flow cytometric analysis of CD80 and CD86

expression on tumor-infiltrating DCs. (C) Flow cytometric analysis of CD8⁺ T cells infiltrating tumor tissues. (D-E) Immunofluorescence analysis of CD11b/Gr-1 and F4/80 / CD163 in tumor tissues. (F) Quantification of CD80 and CD86 expression on LNs DCs (n = 3). (G) Quantification of CD80 and CD86 expression on tumor-infiltrating DCs (n = 3). (H) Quantification of CD8⁺ T cells infiltrating tumor tissues (n = 3). (I-J) Quantification of CD11b/Gr-1 and F4/80 / CD163 expression in tumor tissues (n = 3). (K-M) Serum IL-6/ TNF- α / IFN- γ levels after different treatments (n = 3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

3.7 Prophylactic Immunization with hDMVac Confers Robust Antitumor Immunity

Building on its promising therapeutic efficacy, we further assessed the prophylactic potential of hDMVac. Mice were immunized with four doses of the vaccine on days -12, -9, -6, and -3 before tumor cell inoculation (Fig. 6A). All groups maintained stable body weights during the study (Fig. 6B), indicating that hDMVac treatment did not cause systemic toxicity. Following the challenge with B16 cells, tumor growth kinetics were monitored over time. Compared with the control and other treatment groups, hDMVac vaccination markedly delayed tumor onset and significantly inhibited tumor progression (Fig. 6C-H). By day 15, all mice in the control and other treatment groups had developed tumors, whereas two mice in the hDMVac group remained tumor-free, and the tumor growth rate in this group was noticeably slower than in the control. Consistent with these findings, survival analysis revealed that hDMVac prolonged survival time to the greatest extent among all groups: by day 43, all mice in the control and other treatment groups had succumbed, while three mice in the hDMVac group were still alive (Fig. 6I), further confirming its potent antitumor efficacy.

To further validate the immune changes in the prophylactic group, we investigated the vaccine-induced tri-level immune response cascade. First, DC

activation was examined in dLNs. Immunofluorescence analysis revealed a marked increase in CD86 expression in the hDMVac group (Fig. 6J and N), indicating efficient DC maturation in situ and the provision of essential initiating signals for adaptive immunity. Similarly, co-staining of CD11c and MHC-I in LNs tissues showed enhanced MHC-I expression on CD11c⁺ DCs (Fig. 6K and O), further confirming that hDMVac promotes antigen-presenting capacity in the local microenvironment. Secondly, immunofluorescence of tumor tissues demonstrated a robust increase in CD8⁺ T cell infiltration in the hDMVac group (Fig. 6L and P), with immune cells prominently localized in the tumor core region. In agreement with this, flow cytometric analysis demonstrated a significantly elevated proportion of CD8⁺ T cells in the spleen of vaccinated mice (Fig. 6M and S10), suggesting that local immune activation in dLNs was effectively translated into a systemic antitumor response. This confirms that vaccine-induced effector T cells can be efficiently recruited to tumor sites during early development, thereby providing direct cytotoxic support.

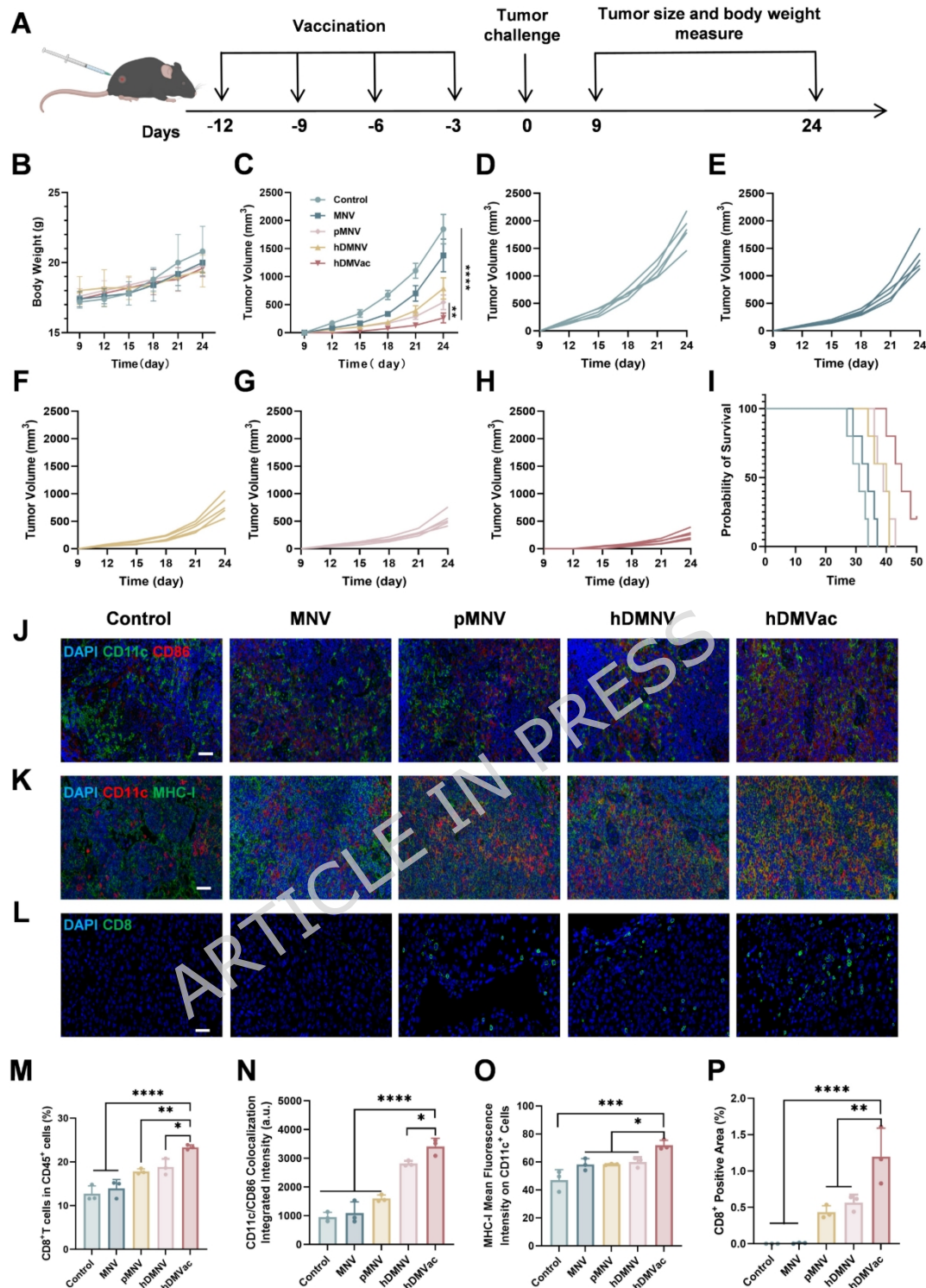


Figure 6. In vivo prophylactic immunotherapy effect of hDMVac. (A) Schematic illustration of the experimental design for melanoma prophylactic vaccination. (B) Tumor volume changes (n = 5). (C) Body weight changes of tumor-bearing mice (n = 5). (D) Survival curve of tumor-bearing mice (n = 5). (E-I) Tumor volume changes in each group (n = 5). (J-K) Immunofluorescence analysis of

CD11c/ CD86 and CD11c/ MHC-I in LNs (n = 3). Scale bar: 50 μ m. (L) Immunofluorescence analysis of CD8 in tumor tissues (n = 3). Scale bar: 50 μ m. (M) Flow cytometry analysis of CD8⁺ T cells in spleen tissues with quantification (n = 3). (N-O) Quantification of CD11c/ CD86 and CD11c/ MHC-I in LNs (n = 3). (P) Quantification of CD8 in tumor tissues (n = 3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

4. Discussion

In this study, we rationally designed and constructed a novel biomimetic hybrid nanovaccine that synergistically integrates the immune-priming capability of tumor cell pyroptosis with the antigen-presentation enhancement of autophagy, thereby establishing a highly efficient and comprehensive antitumor immune cascade [38,39]. Using a gradient extrusion approach, we co-extruded UVC-induced pyroptotic B16 cells with DCs while incorporating the autophagy-inducing peptide beclin-1, generating hDMVac with favorable physicochemical characteristics. Structural and antigenic analyses confirmed that hDMVac retained the complete repertoire of tumor antigens, thus capable of eliciting broad-spectrum T-cell responses and providing a robust biophysical and immunological foundation for its use as an effective nanovaccine. Compared with natural exosomes, biomimetic nanovesicles exhibit higher yield and superior scalability, supporting their feasibility for future large-scale clinical translation. Mechanistically, our design confers a triple synergistic advantage to hDMVac: (i) inheritance of the full set of tumor antigens and DAMPs from pyroptotic cells to effectively stimulate innate immunity; (ii) incorporation of DC2.4 - derived membrane components that naturally express LN-associated receptors, thereby contributing to enhanced LNs accumulation; and (iii) inclusion of beclin-1 to actively modulate autophagy within APCs, thereby promoting efficient cross-presentation of tumor antigens through the “autophagy-lysosome-MHC-I” axis.

In recent years, tumor cell pyroptosis has been widely utilized to enhance antitumor immunity. Unlike conventional strategies that employ pyroptotic derivatives as vaccines or use nanomaterials/viruses to induce pyroptosis in situ[40-42], our study generated pyroptotic B16 cells via 1 h of UVC irradiation. These cells exhibited characteristic morphological features of pyroptosis, including swelling, bubble-like protrusions, and plasma membrane pore formation, consistent with previously reported studies. Subsequently, nanovesicles were prepared from intact pyroptotic cells through gradient extrusion. SDS-PAGE analysis confirmed that these pyroptotic melanoma-derived nanovesicles (pMNVs) retained the complete antigen spectrum, which may support broader T cell activation. Both in vitro and in vivo experiments demonstrated that, compared with melanoma-derived nanovesicles (MNVs), pMNVs significantly promoted BMDC maturation and enhanced DC activation in dLNs. Moreover, pMNVs modestly increased cDC1 abundance and promoted CD8⁺ T cell activation, in line with the findings of Jordon et al., who reported that pyroptotic cells preferentially activate cDC1 and enhance tumor-specific CD8⁺ T cell responses[43]. These results highlight the immunostimulatory potential of pMNVs.

Beyond the intrinsic immunogenicity derived from pyroptosis, this study further sought to enhance cross-presentation efficiency by modulating autophagy. To this end, we incorporated beclin-1 into the vaccine design and observed that hDMVac indeed altered its intracellular trafficking pathway. Co-localization studies with ER and Golgi markers revealed that hDMVac synergized with the autophagy pathway. After escaping lysosomal degradation, hDMVac was sequestered by nascent autophagosomes, which subsequently fused with lysosomes to form autolysosomes. Within autolysosomes, tumor antigens were degraded into peptides, which were then translocated to the ER and Golgi-critical sites for peptide loading onto MHC-I molecules. In line with this mechanism, we observed elevated MHC-I expression in BMDCs in vitro, as well as enhanced MHC-I expression in cDC1 and increased CD8⁺ T cell

abundance in dLNs in vivo. These findings collectively support the conclusion that the introduction of beclin-1 enhanced antigen cross-presentation and T cell activation. Moreover, the synergistic effects of pyroptosis and autophagy further reinforced DC maturation and CD8⁺ T cell priming.

CCR7 is a key receptor involved in the homing of mature DCs to LNs. In this study, we observed that MNV intrinsically expressed CCR7, and fusion with DCs further increased CCR7 expression in hDMVac. While CCR7 is known to mediate chemotactic migration in intact cells, the biomimetic vesicles used in this study lack intracellular signaling machinery and cytoskeletal support. Therefore, hDMVac is unlikely to actively migrate in a CCR7-dependent manner. Instead, we propose that the LNs accumulation of hDMVac is likely mediated by multiple factors, including passive lymphatic drainage, size-dependent transport, and potential interactions between membrane-associated molecules (such as CCR7) and the LNs microenvironment. Consistent with this interpretation, in vivo biodistribution experiments demonstrated that hDMVac exhibited enhanced accumulation in LNs compared with single-origin nanovesicles at both 24 h and 72 h post-injection. Notably, at 72 h, hDMVac was detected in both ipsilateral axillary lymph nodes (ALNs) and contralateral LNs, suggesting a broad distribution within the lymphatic system. In addition, hDMVac preferentially accumulated in LNs rather than in major metabolic organs such as the liver, indicating its favorable lymphatic distribution profile and prolonged retention in lymphoid tissues, which may provide advantageous conditions for immune activation. These findings highlight the advantage of the dual-membrane fusion strategy in integrating functional membrane components, thereby contributing to enhanced LNs accumulation and retention.

In the therapeutic setting, hDMVac markedly inhibited tumor growth and induced apoptosis, while in the prophylactic setting, it delayed tumor onset and prolonged survival. Mechanistically, hDMVac promoted DC maturation and MHC-I upregulation in dLNs, effectively driving local antigen cross-

presentation and CD8⁺ T cell infiltration into tumors. These effects were accompanied by elevated serum levels of IFN- γ and TNF- α , indicating that the vaccine orchestrated both local and systemic immune responses to establish a favorable antitumor immune microenvironment. Although effector molecules such as Granzyme B and Perforin were not directly measured, the significant intratumoral CD8⁺ T cell infiltration, upregulation of CD80/CD86 in the TME, reduction of immunosuppressive cells, and elevated serum cytokines collectively provide indirect evidence of potent effector T cell activity. Together, these results indicate that hDMVac establishes a coordinated immune network capable of suppressing tumor progression.

Despite the promising results of hDMVac in the B16 model, several limitations remain. First, we did not evaluate the combination of hDMVac with immune checkpoint inhibitors (e.g., anti-PD-1/PD-L1 antibodies), which represents a highly relevant direction for clinical translation. Mechanistically, hDMVac is designed to enhance antigen presentation and promote the priming and activation of tumor-specific T cells, whereas immune checkpoint blockade primarily functions to reinvigorate exhausted T cells within the tumor microenvironment. Therefore, the combination of these two strategies may exert complementary and potentially synergistic effects by integrating enhanced T cell priming with sustained effector function. However, several challenges need to be considered for such combinational strategies, including the optimization of dosing regimens and treatment schedules, as well as the potential risk of exacerbated immune-related adverse events. These factors warrant careful investigation in future studies.

Second, the present study was limited to a single tumor model, and its generalizability across diverse tumor types remains to be established. Finally, although biomimetic nanovesicles generated via gradient extrusion possess clear advantages in yield and scalability, systematic optimization and rigorous validation are still required to ensure quality control and industrial-scale

production suitable for clinical translation. Future work should expand upon these findings to further enhance the therapeutic efficacy of hDMVac.

5. Conclusion

In summary, the hDMVac vaccine demonstrates a novel and effective approach to enhancing antitumor immunity by combining pyroptosis-induced immunogenicity with autophagy-driven antigen presentation. The biomimetic hybrid nanovaccine exhibits enhanced accumulation in LNs, promoting the maturation and antigen presentation of DCs. This dual strategy not only strengthens antigen-specific cellular immune responses but also significantly inhibits melanoma growth and progression in both prophylactic and therapeutic models. The modular design of hDMVac, along with its potent immune activation profile, underscores its potential as a promising candidate for clinical application and its ability to complement existing immunotherapies.

CREDIT Author Statement

Han Yan: Writing-original draft, Validation. **Ling Wu:** Validation, Data curation. **Jun Liu:** Methodology, Investigation. **Ruyi Wang:** Methodology. **Jiaye Lu:** Conceptualization. **Fengze Miao:** Formal analysis. **Ying Li:** Investigation. **Zongguang Tai:** Writing-review & editing, Funding acquisition. **Quangang Zhu:** Resources, Funding acquisition. **Zhongjian Chen:** Conceptualization, Resources.

Ethics approval and consent to participate

All animal experiments were conducted following the guidelines of the Animal Ethics Committee of Shanghai Skin Disease Hospital and by the Guide for the Care and Use of Laboratory Animals with the ID number: 2023-108 (Animal).

Ethics, Consent to Participate, and Consent to Publish declarations: not applicable.

Consent for publication

All authors have approved the manuscript and agree with the submission.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

This article and its supplementary data file include all data generated or analyzed in this study. Raw data points are available from the corresponding author on a reasonable request.

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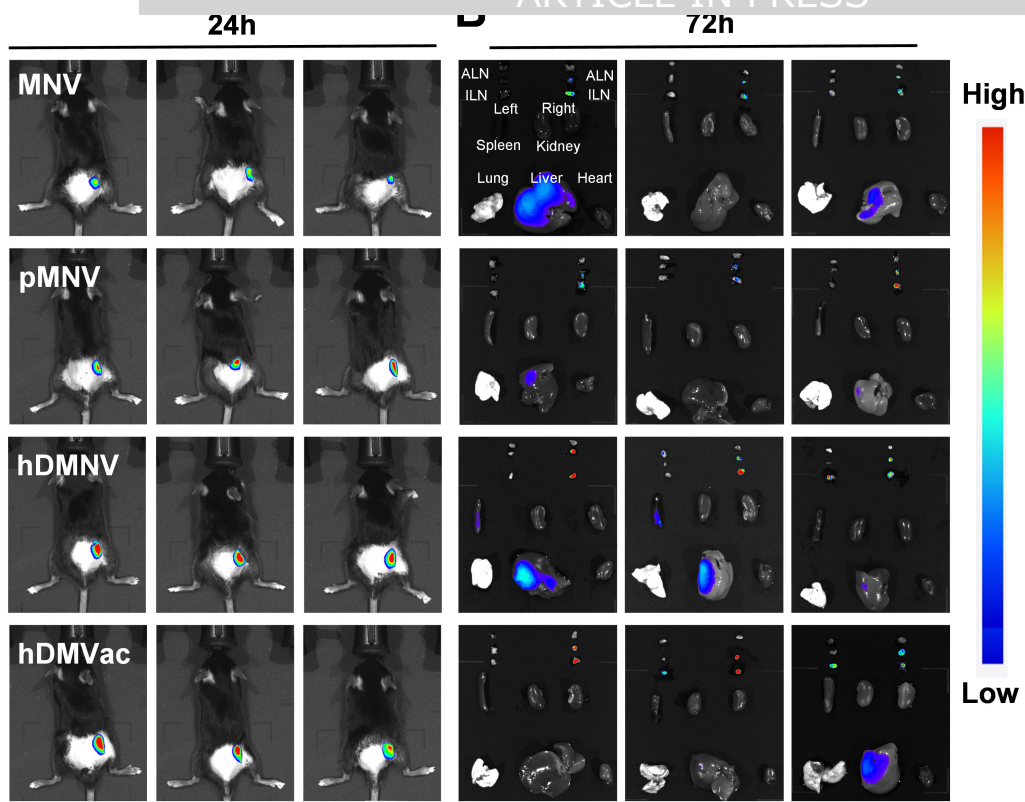
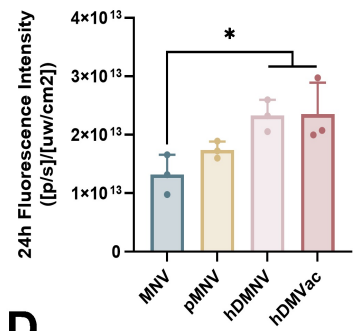
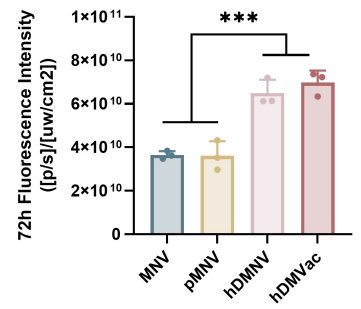
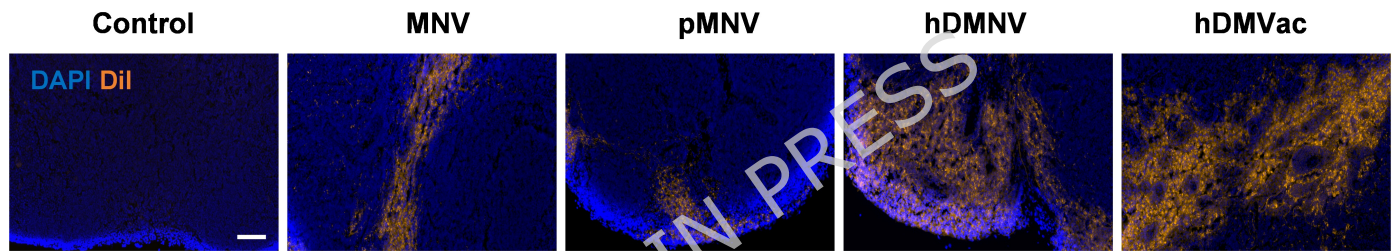
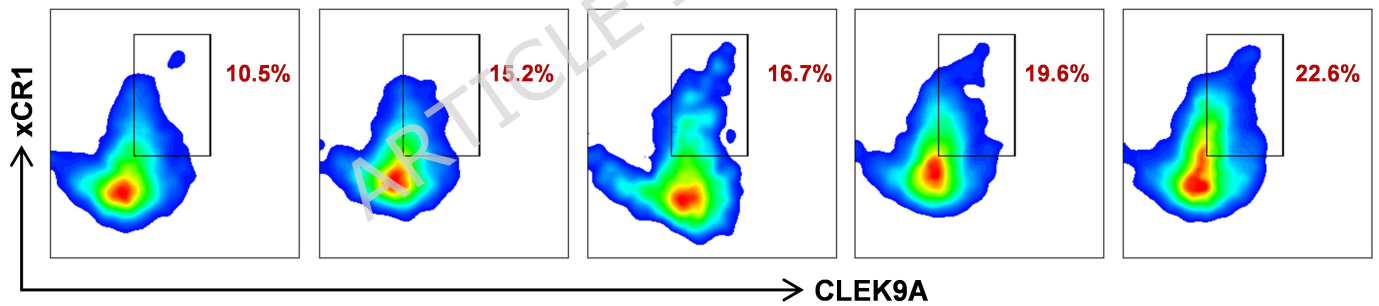
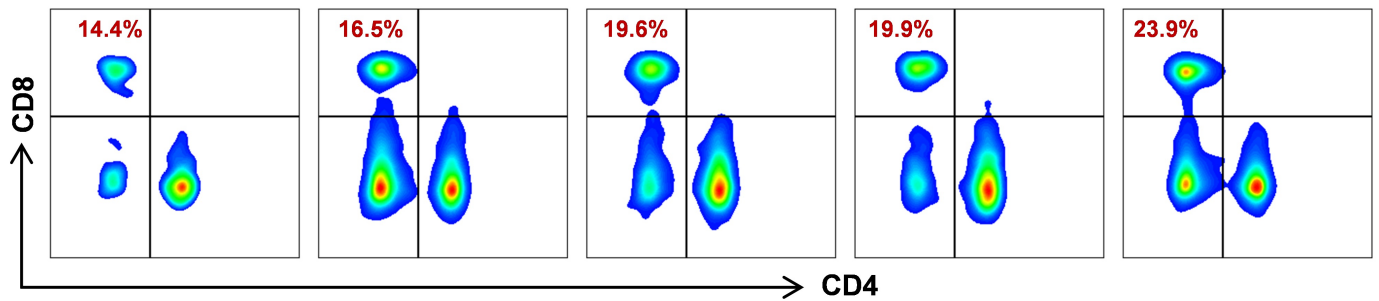
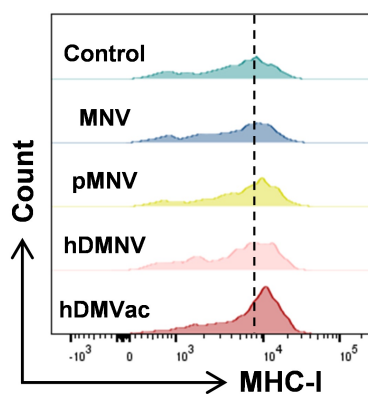
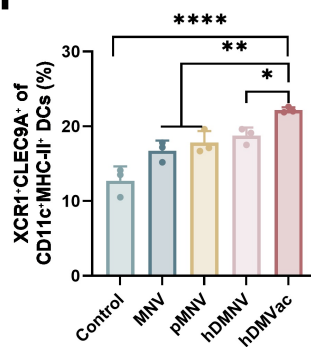
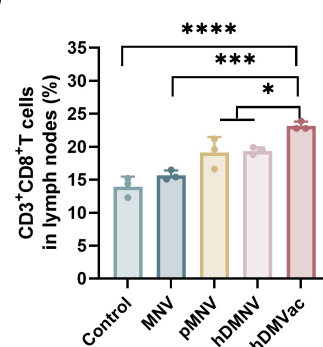
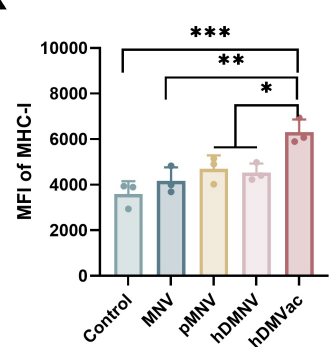
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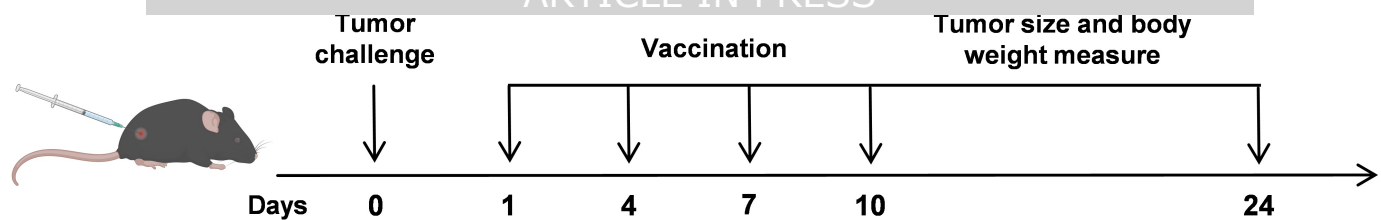
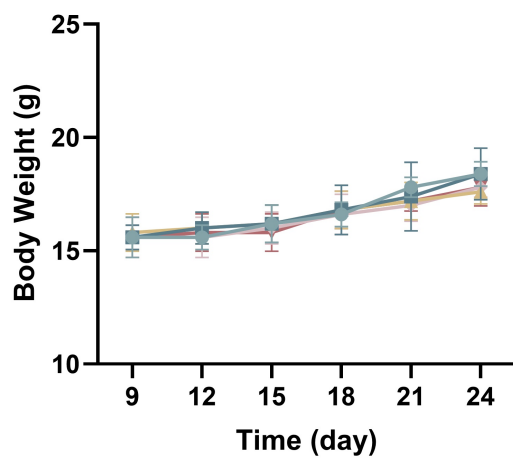
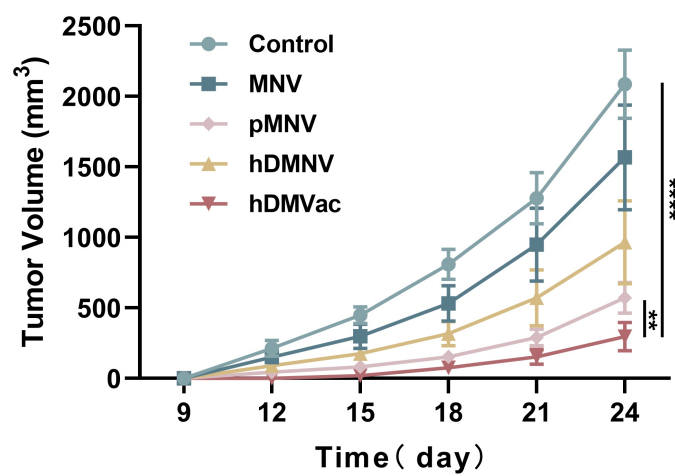
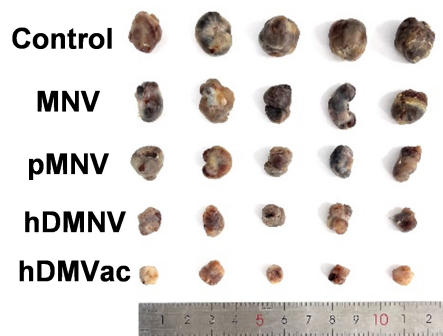
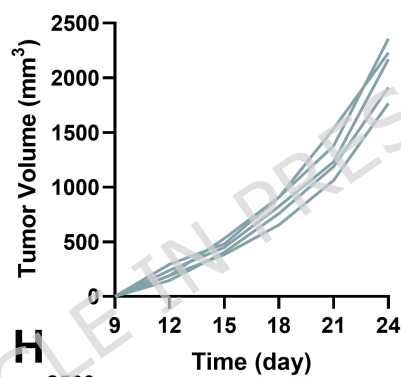
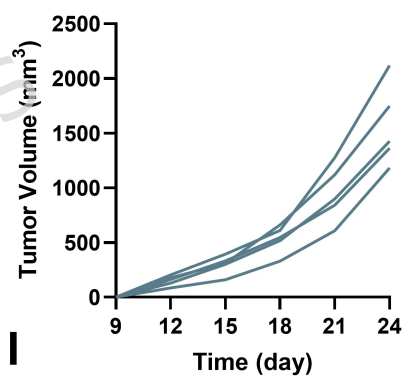
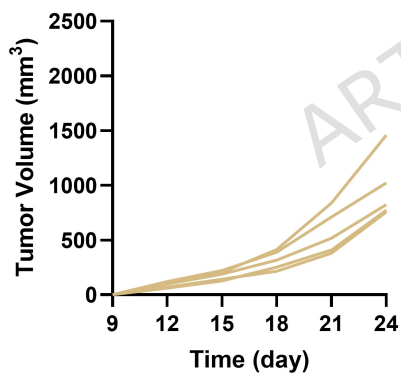
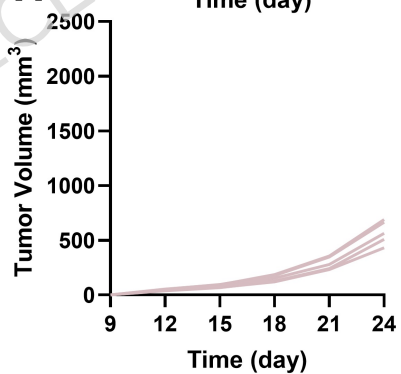
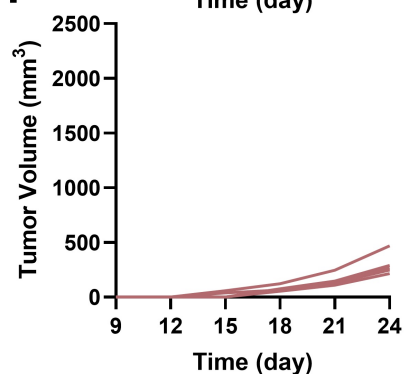
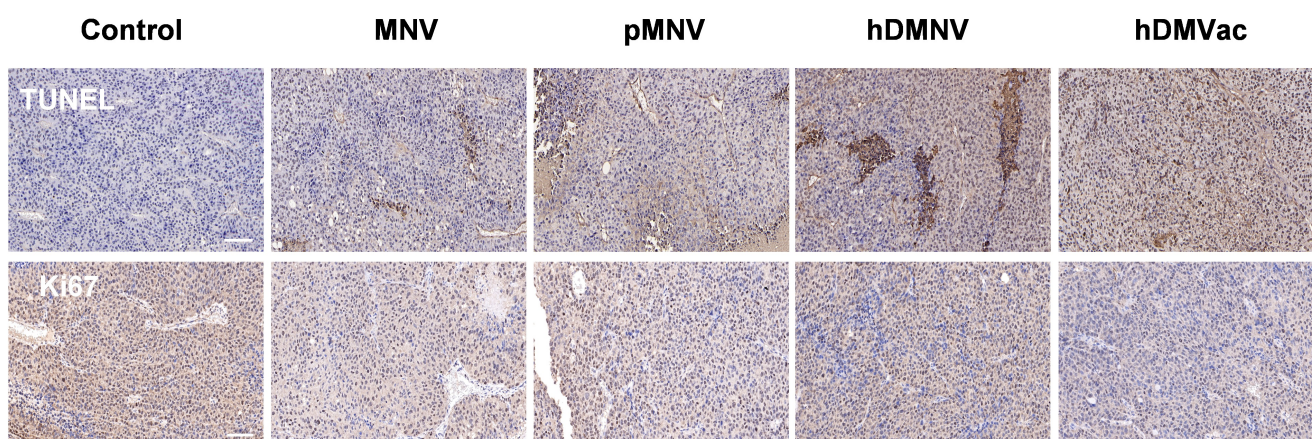
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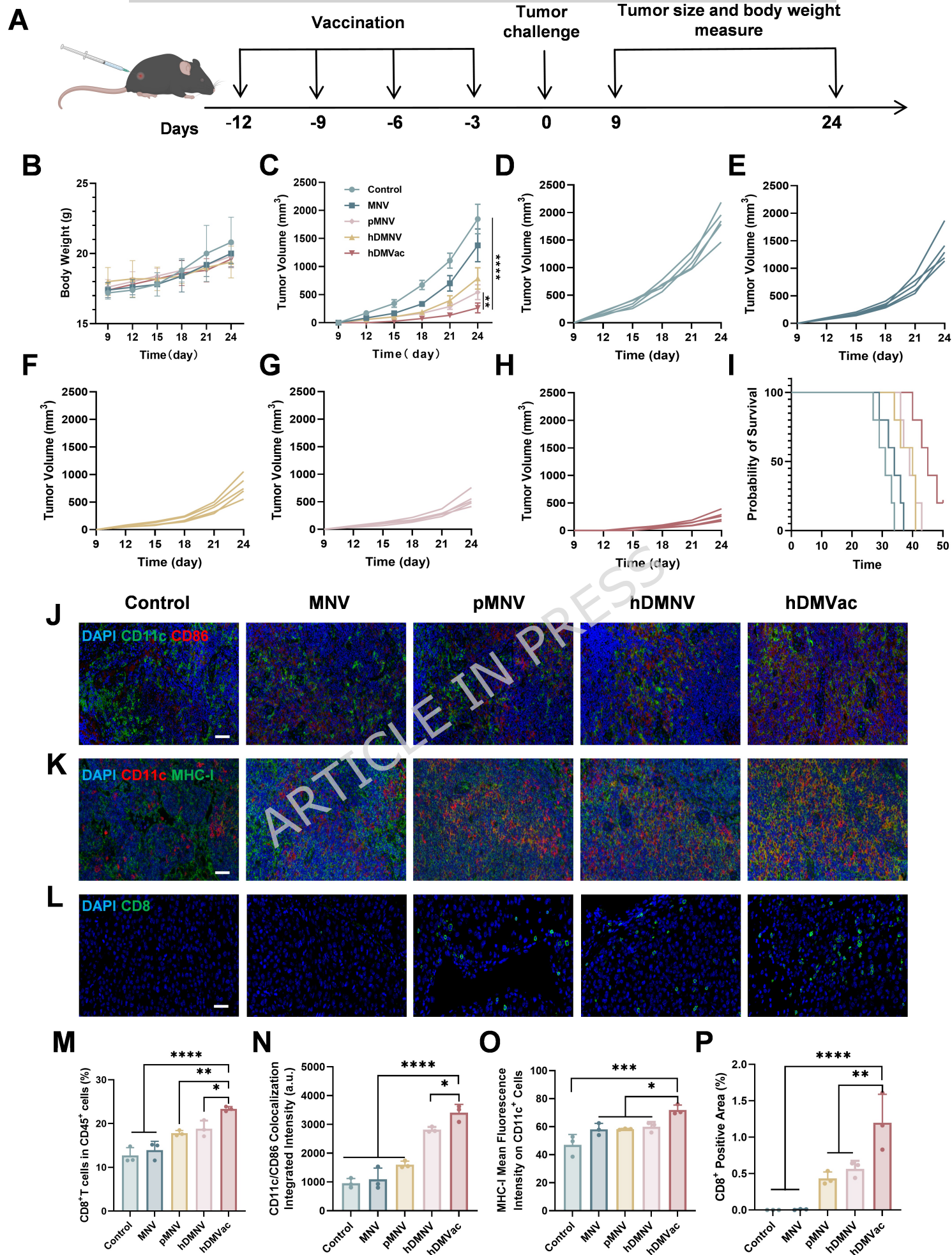
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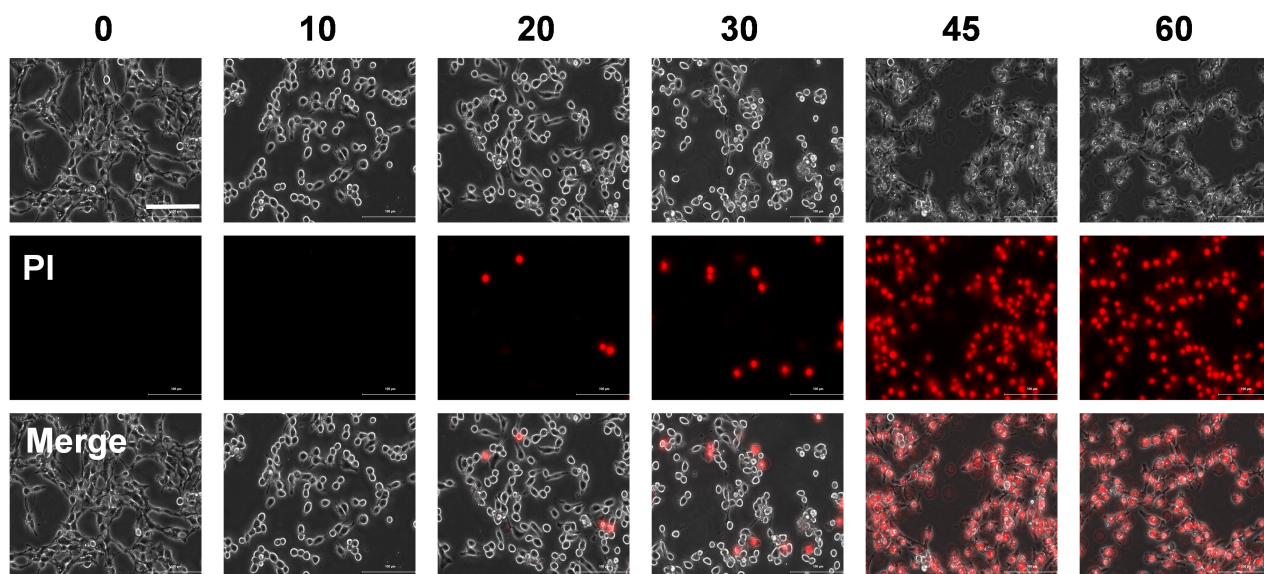
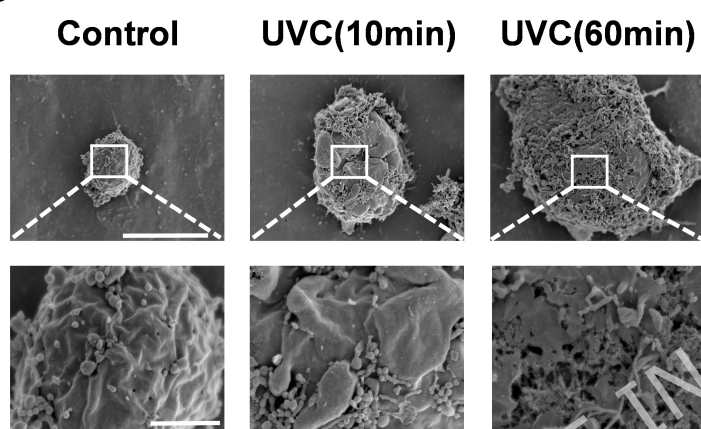
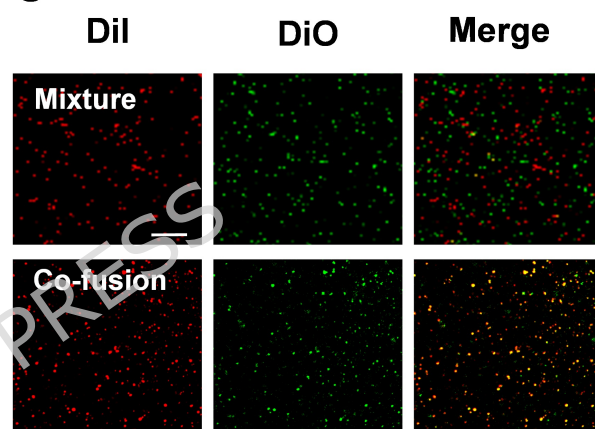
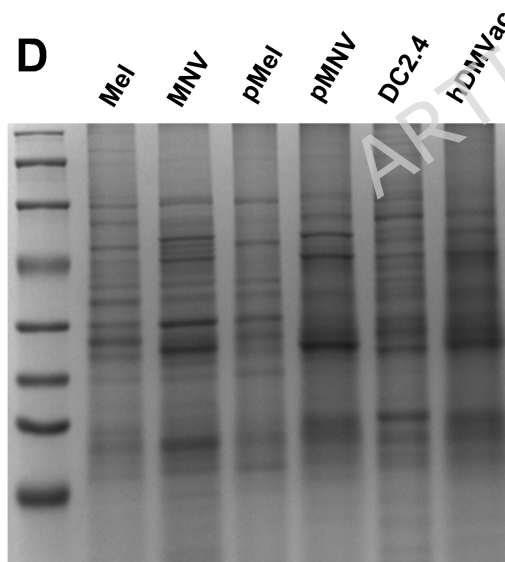
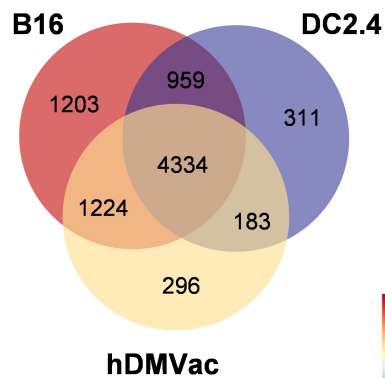
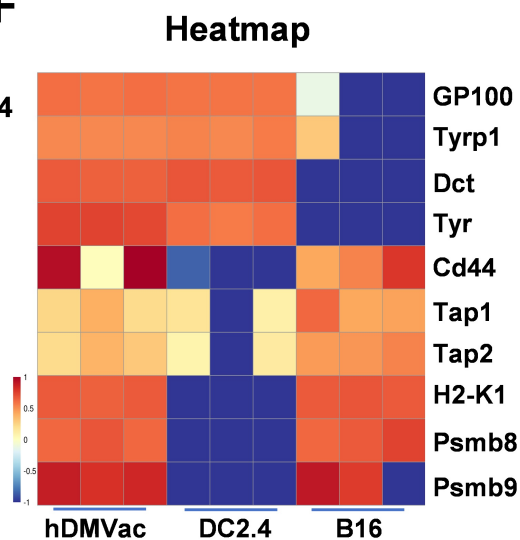
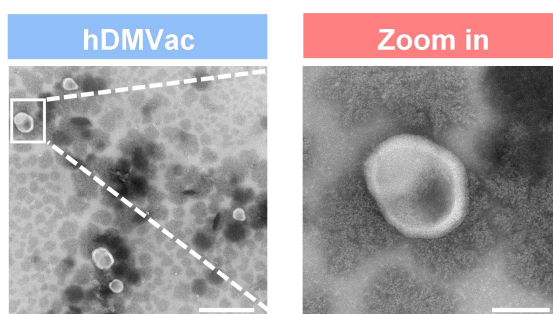
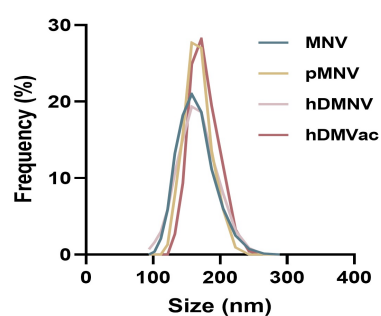
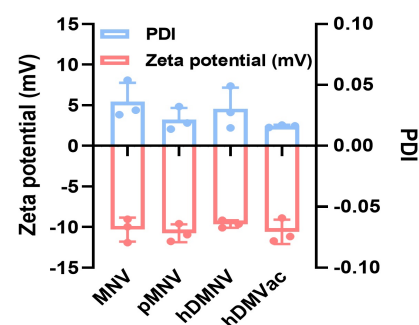
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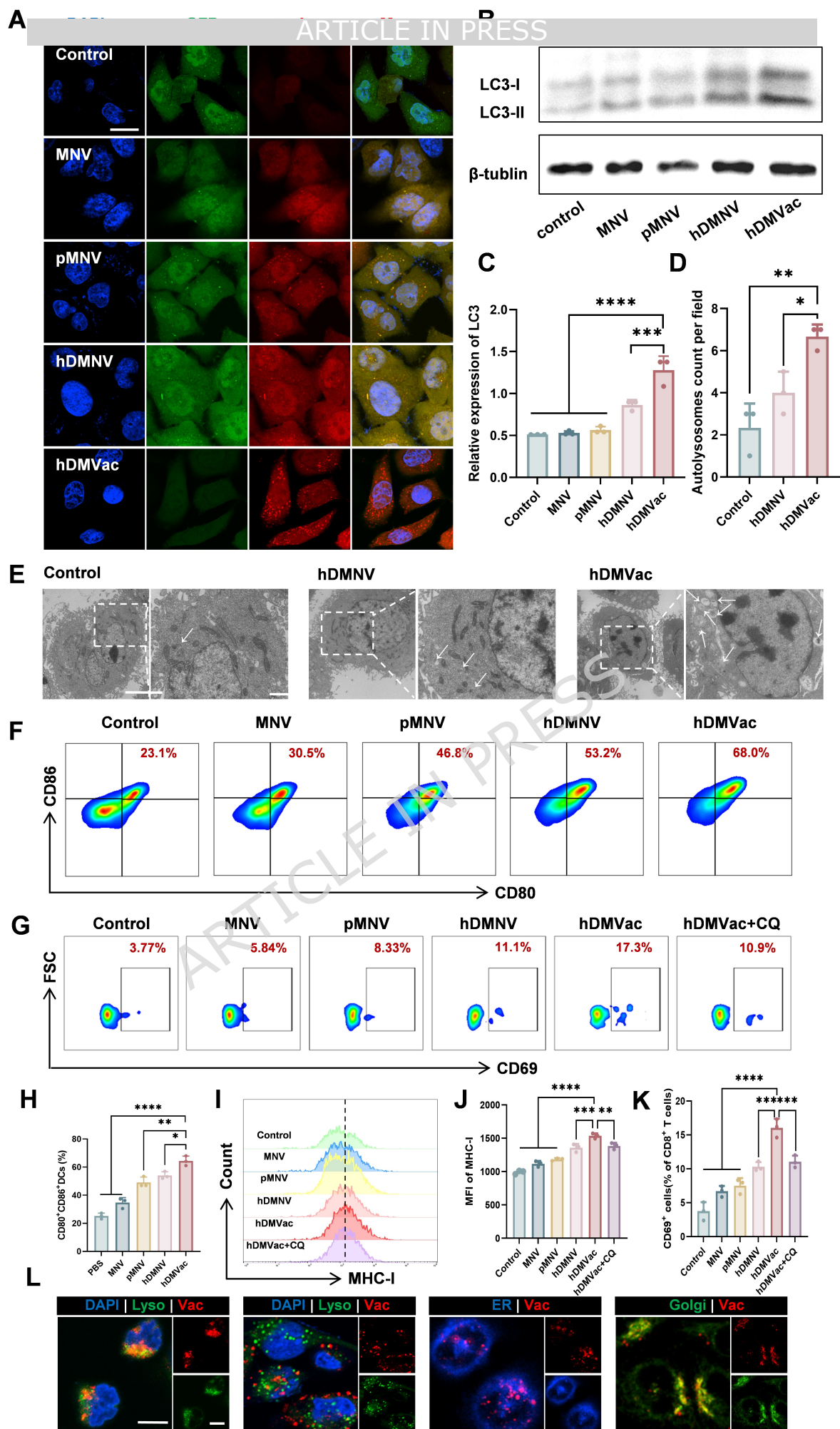
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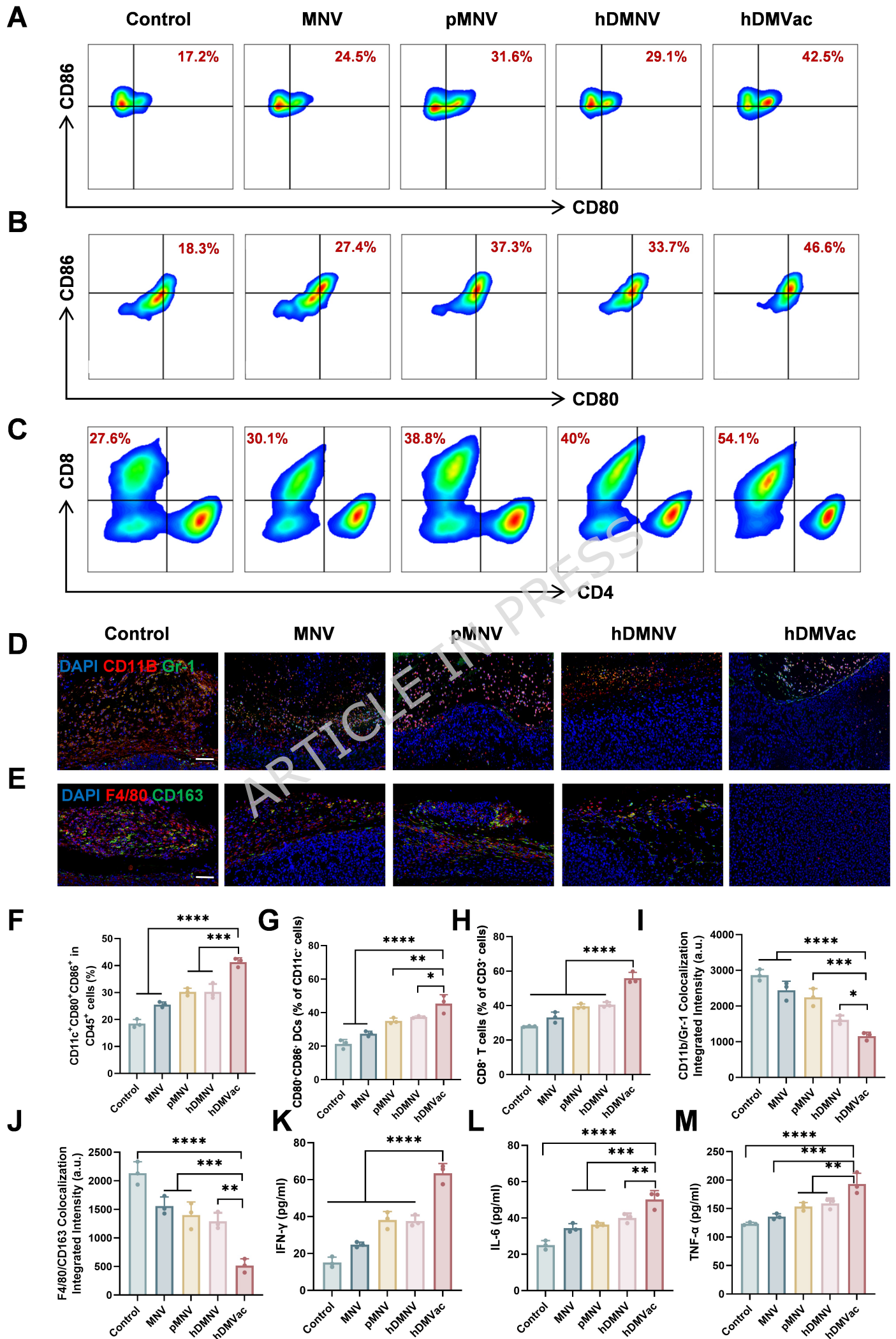
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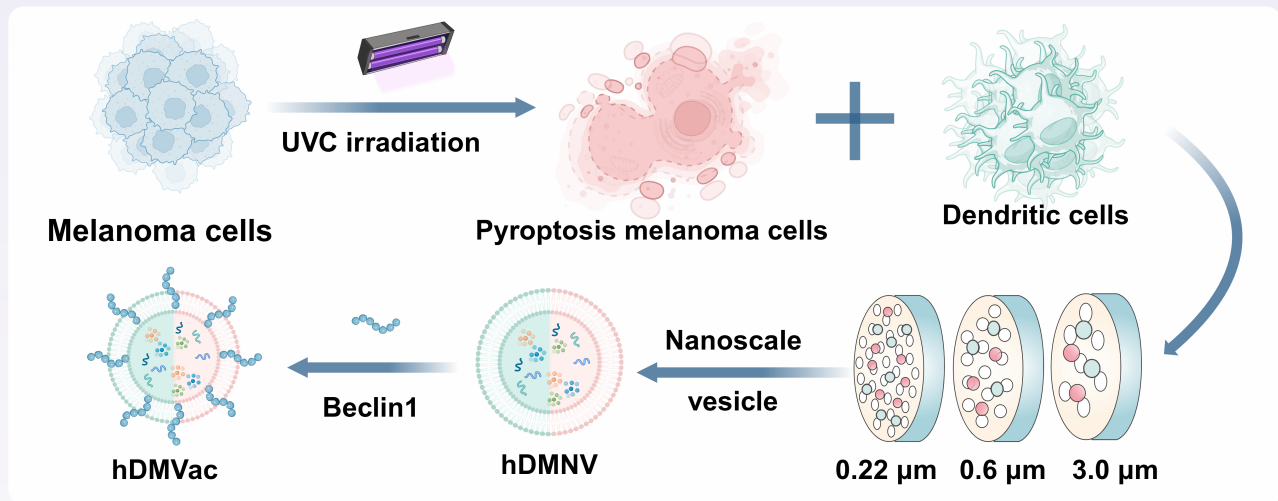
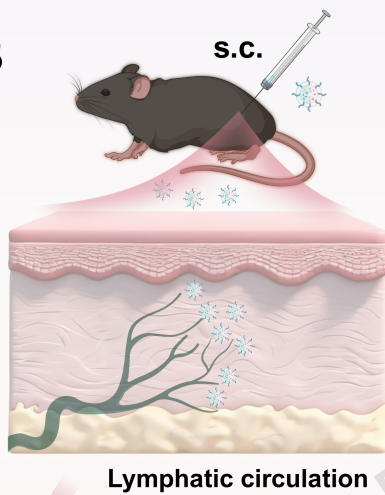
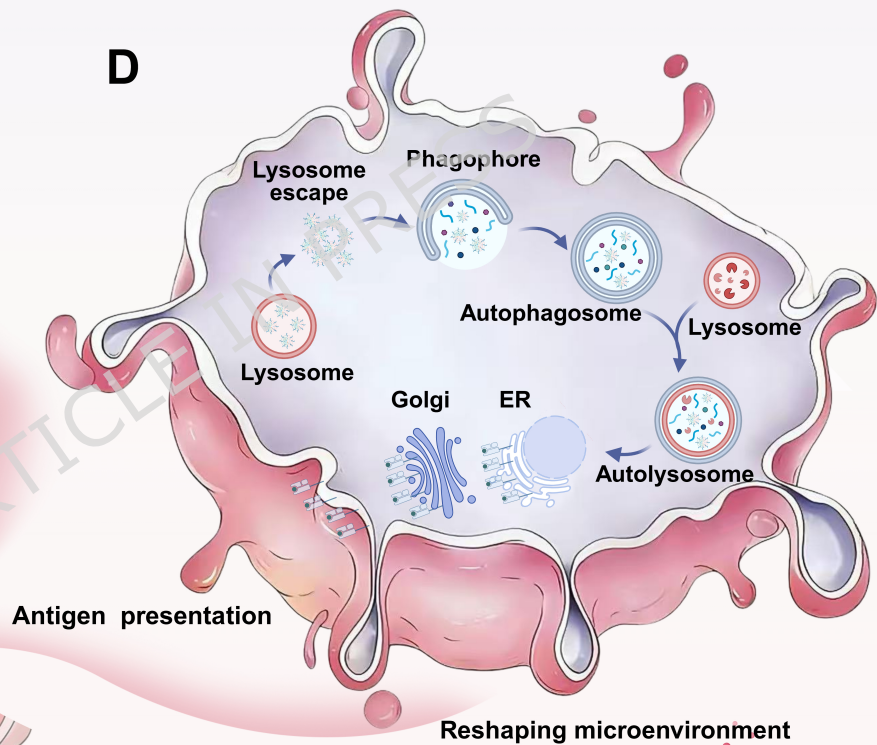
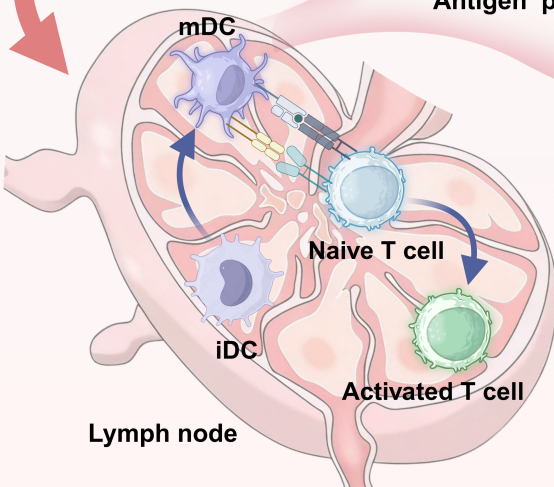
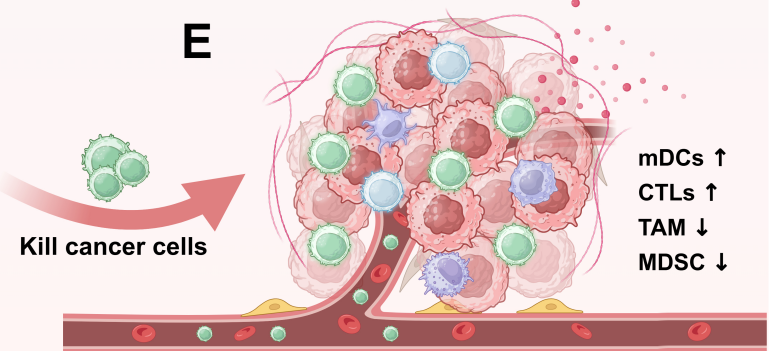
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