Acta Pharmaceutica Sinica B 2026;16(4):1914−1942

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

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REVIEW

# Outer membrane vesicles: Versatile nanocarriers for therapeutic delivery and immune modulation

**Jinlong Yang[a,b,†], Tang Wang[b,†], Yuekai Zheng[b,†], Yuning Wei[b], Juan Tao[b], Zhongjian Chen[a,*], Jianping Qi[b,*]**

[a]*Shanghai Skin Disease Hospital, School of Medicine, Tongji University, Shanghai 200443, China*
[b]*School of Pharmaceutical Sciences, Fudan University, Key Laboratory of Smart Drug Delivery, Ministry of Education, State Key Laboratory of Advanced Drug Formulations for Overcoming Delivery Barriers, Shanghai 201203, China*

Received 24 May 2025; received in revised form 25 July 2025; accepted 22 September 2025

**KEY WORDS**

Outer membrane vesicles;
OMVs;
Nanocarriers;
Tumor immunotherapy;
Vaccine delivery;
Bioengineering;
Drug delivery;
Cancer therapy

**Abstract** Outer membrane vesicles (OMVs) are nanoscale lipid-bilayer vesicles naturally released by Gram-negative bacteria. By packaging membrane proteins, lipopolysaccharide-derived pathogen-associated molecular patterns, nucleic acids, and metabolites, OMVs integrate intrinsic bioactivity with cargo capacity and have emerged as a versatile platform for therapeutic delivery and immune modulation. In this review, we summarize current understanding of OMV biogenesis, composition, and physicochemical features that shape biodistribution and immunogenicity. We then discuss engineering strategies ranging from genetic rewiring of parental strains and detoxification of lipid A to surface functionalization, hybrid membrane assembly, and stimuli-responsive formulations that enable controllable targeting, loading, and release. Recent progress is highlighted in anti-tumor therapy, nanovaccines for infectious diseases and cancer, and nucleic acid delivery for gene regulation. Finally, we analyze key barriers to clinical translation, including endotoxin-associated safety, batch-to-batch heterogeneity, scalable manufacturing, and standardization of quality attributes. Addressing these challenges through rational design and robust production pipelines will be essential to advance OMV-based therapeutics toward safe, effective, and manufacturable clinical products.



This article is part of special issue entitled: Hot Topic Revs in Drug Delivery (II) published in Acta Pharmaceutica Sinica B.

*Corresponding authors.
E-mail addresses: qijianping@fudan.edu.cn (Jianping Qi), aajian818@163.com (Zhongjian Chen).
[†]These authors made equal contributions to this work.

Peer review under the responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2026.01.033

## 1. Introduction

Outer membrane vesicles (OMVs) are naturally released from Gram-negative bacteria and have emerged as a promising platform for biomedical research and therapeutic development. OMVs usually have spherical structures, typically ranging from 20 to 250 nm in diameter, and encapsulate a wide range of bacterial constituents, including lipopolysaccharides (LPS), outer membrane and periplasmic proteins, nucleic acids, and various metabolites[1,2]. As such, OMVs largely preserve the molecular signatures and biological functions of their parent bacteria while existing as non-replicative entities. Early studies regarded OMVs as inert debris during bacterial growth. However, accumulating evidence has redefined them as dynamic mediators of bacterial physiology, host−microbe communication, and environmental adaptation. In recent years, the inherent immunogenicity, biocompatibility, and engineering capacity of OMVs have made them increasingly valuable in therapeutic delivery and immune modulation[3-5].

In pathogenic contexts, OMVs play a central role in bacterial virulence and immune modulation. Several Gram-negative pathogens, such as *Neisseria meningitidis* and *Helicobacter pylori*, exploit OMVs as delivery vehicles for toxins and virulence factors, enabling their direct transfer into host cells while partially evading immune surveillance[6]. For example, *H. pylori* derived OMVs can transport the cytotoxin-associated gene A (CagA) into gastric epithelial cells, triggering inflammatory signaling cascades and contributing to gastric carcinogenesis[7]. These findings underscored OMVs' natural ability to cross biological barriers and deliver payloads precisely. Beyond cargo delivery, OMVs are enriched in pathogen-associated molecular patterns (PAMPs), including LPS and peptidoglycan fragments, which can easily activate innate immune receptors such as Toll-like receptors (TLRs)[8]. This intrinsic immunostimulatory capacity confers OMVs with self-adjuvanting properties, allowing them to simultaneously function as antigen carriers and immune activators.

The convergence of synthetic biology, genetic engineering, and nanotechnology has further expanded the biomedical potential of OMVs. Unlike synthetic nanoparticles, OMVs interact with host cells through several pathways, including receptor-mediated endocytosis and membrane fusion, resulting in efficient cellular uptake. Their lipid bilayer structure provides protection for encapsulated cargos against enzymatic degradation, while surface-exposed proteins can be genetically or chemically modified to achieve targeted delivery. OMVs derived from probiotic strains, such as *Escherichia coli* Nissle 1917, have been engineered to display tumor-targeting ligands and demonstrate selective accumulation in tumor tissues in colorectal cancer xenografts[9,10]. In addition, OMVs have been employed to deliver a broad spectrum of therapeutic payloads, including small-molecule drugs (Doxorubicin), nucleic acids (siRNA and mRNA), and immune checkpoint inhibitors (anti-PD-1 antibodies), thereby integrating drug delivery with immune regulation[11-14]. Moreover, the ability to engineer the composition and functionality of OMVs is critical to advancing their biomedical applications. Genetic knockout of endotoxin biosynthesis genes (such as *msbB*) can significantly reduce LPS toxicity while maintaining vesicle integrity. It has been successfully applied in *Salmonella* OMV vaccines[15]. Conversely, overexpression of autotransporter proteins (such as ClyA) has been used to display heterologous antigens on the surface of OMVs, as demonstrated in the development of vaccines against malaria and cancer. Emerging synthetic biology tools, including CRISPR-Cas9 and the SpyTag/SpyCatcher system, enable precise antigen incorporation and facilitate modular vaccine design. Innovations in bioprocessing, including magnetic nanoparticle-enhanced harvesting and tangential flow filtration, have increased OMV yields to 60 mg/L, which is 60 times higher than those achieved with conventional ultracentrifugation. These advances are paving the way toward scalable and clinically viable OMV-based therapeutics.

The applications of OMVs span a wide range of therapeutic domains. In oncology, they function as dual-purpose platforms capable of delivering chemotherapeutics while simultaneously stimulating antitumor immunity. For instance, OMVs loaded with doxorubicin have demonstrated enhanced bioavailability and tumor targeting in non-small cell lung cancer models, outperforming the free drug in terms of therapeutic efficacy[16]. OMVs engineered to display tumor antigens or immune checkpoint inhibitors can remodel the tumor microenvironment, converting immunologically "cold" tumors into "hot" ones that are more susceptible to T-cell infiltration. In the context of infectious diseases, OMVs serve as self-adjuvanting vaccines, eliciting robust humoral and cellular immune responses against pathogens such as *Shigella* and *Vibrio cholerae*[17]. Their ability to cross-present antigens *via* MHC-I pathways enables the activation of cytotoxic T lymphocytes (CTLs), a property that has been exploited in the development of personalized cancer vaccines[3,5,8]. Hybrid OMVs, produced by fusing bacterial vesicles with tumor cell membranes or synthetic nanoparticles, further enhance antigenicity and stability, showing promising results in preclinical models of melanoma and breast cancer[18,19]. Additionally, OMVs hold potential in gene therapy, particularly for delivering CRISPR-Cas9 components for targeted genome editing. Despite these advances, clinical translation of OMV-based therapies faces several challenges. Safety remains a key concern, especially regarding systemic inflammation induced by endotoxins. Although genetic modifications, such as lipid A truncation, can reduce toxicity, they may also compromise immunogenicity or structural stability[20]. The inherent heterogeneity of OMVs in size, cargo, and surface composition has posed significant hurdles for standardization. Therefore, it is necessary to produce stringent quality control measures. Rapid *in vivo* clearance also limits their systemic bioavailability. Surface modifications, such as PEGylation or albumin coating, could prolong their circulation time[21,22]. Moreover, the interaction between OMVs and the host microbiome remains poorly understood. While OMVs derived from commensal bacteria show potential in restoring gut homeostasis, their long-term immunological effects require further investigation.

The evolution of OMVs from microbial metabolites to therapeutic agents and drug delivery carriers underscores their remarkable versatility. However, this is still an emerging field. There is an urgent need to better understand the multifunctional effects of OMVs and be acquainted with new studies in this field. In this review, we summarize the fundamental biological characteristics of OMVs, highlight recent progress in their engineering and therapeutic applications, and discuss the key obstacles that must be addressed to enable their clinical translation. As interdisciplinary research continues to deepen our understanding of OMV biology and engineering, OMVs are poised to become a versatile and powerful platform in precision medicine.

## 2. Biogenesis and biological characteristics of OMVs

### *2.1. Biogenesis mechanisms and composition of OMVs*

Outer membrane vesicles (OMVs) are spherical and bilayer vesicles (100−250 nm) secreted from the outer membrane of Gram-negative bacteria into the bacterial environment. Unlike cell lysis byproducts, OMV biogenesis is a regulated process involving the dissociation of the outer membrane (OM) from the underlying peptidoglycan layer (Fig. 1). The bacterial envelope comprises an inner membrane (IM) and an asymmetric OM, separated by the periplasmic space containing a dense proteoglycan layer. The inner membrane (IM) is a phospholipid bilayer composed of repeated *N*-acetylglucosamine-*N*-acetylmuramic acid disaccharides with an outer membrane (OM) containing phospholipids and lipopolysaccharide (LPS) in the outer layer. The periphery is an aqueous space separated by outer and endoplasmic membranes that are densely packed with proteins[23,24]. OMVs are generated when a small portion of the outer membrane expands from the bacterial cell wall, squeezes out, and is released. Proteomic and biochemical analyses have revealed that OMVs contain many bacterial components, including DNA, RNA, LPS, proteins, enzymes, and phospholipids. Thus, OMVs contain many biological substances found in parental bacteria, but in a nonreplicating form. The stability of this architecture relies on cross-linking proteins, primarily Braun's lipoprotein (Lpp), outer membrane protein A (OmpA), and the Tol−Pal complex. Current models suggest that OMV formation is driven by the localized disruption of these linkages. Specific genetic factors are essential for the biogenesis and function of OMVs. For instance, the down-regulation or deletion of genes within the *tol−pal* cluster (encoding TolQ, TolR, TolA, TolB, and Pal) compromises the OM-peptidoglycan tethering, leading to membrane bulging and vesiculation. Consequently, *Salmonella typhimurium* mutants lacking *tolA* or *tolB* exhibit hyper-vesiculation phenotypes. Similarly, in *Salmonella choleraesuis*, the Tol system (*tolA*, *tolB, tolR*) is integral to envelope stability[25]. Beyond genetic factors, environmental stressors (such as antibiotic exposure) can also induce "explosive lysis", generating a distinct subtype of vesicles known as outer−inner membrane vesicles (O-IMVs). These O-IMVs encapsulate both periplasmic and cytoplasmic contents, including plasmid DNA, thereby facilitating horizontal gene transfer[26-28]. Quorum sensing molecules, such as the *Pseudomonas* quinolone signal (PQS) in *Pseudomonas aeruginosa*, also intercalate into the OM bilayer to physically induce curvature, linking population density to vesicle production[29].

OMVs are mainly composed of OM proteins, LPS, and periplasmic components and retain many characteristics of the OM[1,2,30]. The main phospholipids of OMVs are phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), which play key roles in maintaining stability[31,32]. The constituents of various phospholipids could also promote interaction with the host cells, regulate immune responses, and transport bioactive molecules[32-34]. Proteomic analyses reveals that virulence factors are often concentrated within OMVs. For example, *Neisseria gonorrhoeae* OMVs carry porins and opacity proteins that target host cells to induce apoptosis[35]. Compared with the IM and cytoplasmic proteins, the OM proteins of OMVs are abundant[36]. Interestingly, the loss of surface structures, such as the O-antigen in *Klebsiella pneumoniae*, can dramatically alter the protein sorting profile, suggesting a structural interplay between LPS length and protein packaging[23,37]. OMVs protect diverse genetic materials, ranging from small RNAs (sRNA), messenger RNA (mRNA), and ribosomal RNA (rRNA) to chromosomal DNA

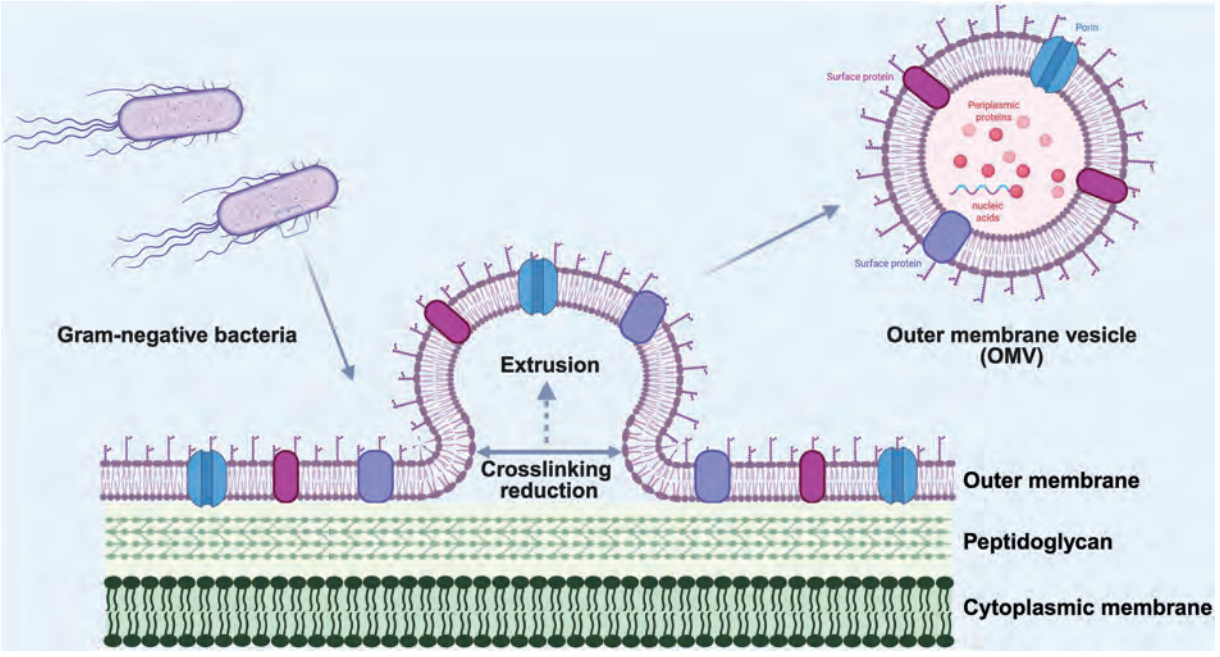



**Figure 1** Secretion mechanism of OMVs. The cell wall of Gram-negative bacteria is composed of two membranes that are chemically and structurally distinct. The inner membrane (cytoplasmic membrane) is a phospholipid bilayer composed of repetitive *N*-acetylamino-*N*-acetylaminoacetic acid disaccharides. The OM contains phospholipids in the inner layer and LPS in the outer layer. The periplasmic space is the water zone between the endoplasm and the endoplasm and densely wrapped by proteins. A small part of the OM of bacteria have probability to squeezed outwards, after extrusion, the OMVs are formed. Created with BioRender.com.

(cDNA) and plasmids, from extracellular nucleases[38]. Functional transfer of these cargoes modulates host gene expression potentially and has profound implications[39,40]. For instance, *Porphyromonas gingivalis* OMVs deliver specific sRNAs (*e.g.*, sRNA23392) to oral squamous cell carcinoma cells, modulating tumor metastasis pathways[40]. OMVs can also carry plasmids and cDNA, which can be significant for horizontal gene transfer among bacteria[41,42]. DNA fragments can be derived from cellular lysis or directly incorporated during OMV budding[43]. Furthermore, the encapsulation of DNA is associated with biofilm structural integrity and antibiotic resistance propagation. Recent studies emphasize that specific sorting mechanisms, potentially involving RNA-binding proteins, direct these nucleic acids into the vesicle lumen, distinguishing OMVs as sophisticated intercellular communicators rather than passive carriers. Nucleic acids may actively participate in modulating host responses or facilitating bacterial adaptation to diverse environments, such as antibiotic resistance among microbial populations[44,45]. Additionally, OMVs could deliver sRNAs to host cells and affect host signaling pathways, immune responses, and cellular behavior[39].

Elucidating the diverse bacterial sources of OMVs is essential for understanding their specific functions and potential biotechnological applications. Key bacterial sources of OMVs primarily include: *K. pneumoniae*[23], *Bacteroides fragilis*, *H. pylori*[46], *E. coli*, *Salmonella enterica*[47], *P. aeruginosa*[42], *N. gonorrhoeae*[36], *Salmonella* species[48], *Vibrio vulnificus*[49]. OMVs secreted by different Gram-negative bacteria (commensal/pathogenic) show significant heterogeneity in composition, function, and application (Table 1). Pathogenic OMVs mainly carry toxins (such as LPS, OmpA/C, and CirA) and virulence factors that directly trigger the activation of host inflammasomes (such as caspase-11) and promote the production of pro-inflammatory factors (TNF-$\alpha$, IL-8, and IL-1$\beta$). For instance, studies demonstrate that OMVs can prime macrophage inflammasomes and stimulate cytokine secretion both *in vitro* and *in vivo*, suggesting their role in exacerbating inflammatory responses in conditions like periodontal disease[50]. Similarly, *Acinetobacter baumannii* OMVs provoke pro-inflammatory signals *via* multiple signaling pathways, promoting the infiltration of macrophages and neutrophils[51]. These vesicles can traverse epithelial barriers to interact with underlying immune cells, delivering pro-inflammatory factors directly to the site of infection and enhancing immune cell recruitment[52]. Moreover, OMVs can inhibit autophagic flux in host cells, which exacerbates inflammation by preventing the clearance of damaged organelles and sustaining inflammasome activation. This mechanism can lead to a persistent inflammatory environment and increased susceptibility to secondary infections, potentially culminating in systemic inflammatory responses such as sepsis.

In contrast, OMVs derived from commensal bacteria play important roles in maintaining immune homeostasis and barrier integrity. OMVs from probiotic strains such as *E. coli* Nissle 1917 are internalized by intestinal epithelial cells *via* endocytic pathways, enhancing tight junction integrity and protecting against pathogen-induced barrier dysfunction[10]. Commensal OMVs can deliver peptidoglycan fragments to NOD1 receptors, modulating local immune responses without triggering excessive inflammation[53]. OMVs from *B. fragilis* preferentially activate regulatory immune pathways and contribute to immune tolerance in the gut. Through interactions with epithelial cells and regulatory T cells, commensal OMVs support mucosal defense and microbiota homeostasis[54,55].

OMVs derived from pathogenic bacteria are frequently utilized as vaccine vectors due to their high immunogenicity, as they naturally encapsulate LPS and virulence proteins that mimic natural infection. Conversely, OMVs from commensal bacteria engage the host immune system in a more nuanced manner, stimulating both innate and adaptive immunity without triggering overwhelming inflammatory cascades. Consequently, commensal OMVs represent a promising low-toxicity delivery system for mucosal vaccines. However, their antigen-loading capacity requires optimization to effectively regulate intestinal microbiota-related conditions, such as autoimmune diseases. Collectively, these findings highlight the structural complexity and functional diversity of OMVs. Their composition and biological activity are shaped by bacterial genetics, environmental conditions, and biogenesis pathways. Understanding the mechanisms governing OMV formation and cargo selection is essential for exploiting their potential as therapeutic delivery systems and immunomodulatory platforms.

### 2.2. *Isolation, purification and quality control of OMVs*

Optimization strategies to produce OMVs are critically important for enhancing their effectiveness in therapeutic applications, especially in vaccines and drug delivery systems. The yield, composition, and biological activity of OMVs are strongly

**Table 1** OMVs secreted by different Gram-negative bacteria (commensal/pathogenic) show significant heterogeneity in composition, function, and application.

| Feature | Pathogenic OMVs | Commensal OMVs |
|---|---|---|
| Primary function | Induce inflammation and aid in virulence | Promote immune homeostasis, barrier integrity, and tolerance |
| Composition | Enriched with toxins (LPS, OmpA/C, CirA), virulence factors, and enzymes | Contain commensal antigens, peptidoglycan, enzymes, and immunoregulatory molecules |
| Host immune response | Activate inflammasomes (caspase-11) → TNF-$\alpha$, IL-8, IL-1$\beta\uparrow$ → recruit macrophages & neutrophils | Activate NOD1 receptors → regulated cytokine release, promote Treg cells, and support immune tolerance |
| Barrier effect | Disrupts epithelial barriers and blocks autophagy → promotes systemic inflammation (sepsis) | Enhances tight junction proteins → maintains epithelial integrity and prevents pathogen invasion |
| Disease association | Linked to conditions like sepsis, periodontitis, and other inflammatory disorders | Associated with gut homeostasis, mucosal defense, and protection from pathogens |
| Key examples | *Helicobacter pylori*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Acinetobacter baumannii* | *Escherichia coli* Nissle 1917, *Bacteroides fragilis* |

influenced by bacterial culture conditions, induction strategies, and downstream isolation procedures. Therefore, optimization of OMV production and establishment of standardized purification and quality control workflows are critical for both experimental reproducibility and clinical translation.

Bacterial growth conditions play a central role in regulating OMV secretion. Parameters such as culture medium composition, pH, oxygen availability, and growth phase significantly affect vesicle yield. Studies using *S. enterica* serovar Typhimurium have demonstrated that fine-tuning culture pH and nutrient composition can substantially enhance OMV production. Similarly, *N. meningitidis* exhibits increased vesiculation under high dissolved oxygen conditions, suggesting that aerobic culture environments may favor OMV release in certain species[56]. In addition to physicochemical parameters, chemical stressors are frequently employed to induce vesiculation. Exposure to sublethal concentrations of antibiotics can stimulate OMV secretion as part of a bacterial stress response, whereas sulfate depletion has been shown to promote membrane remodeling and increase OMV output[57].

Genetic engineering provides a powerful strategy to enhance OMV yield and tailor vesicle composition. Mutations in genes involved in envelope stability often result in hypervesiculating phenotypes, leading to significantly higher OMV production compared with wild-type strains[58]. For example, deletion or modification of genes encoding membrane-anchoring proteins can destabilize the outer membrane-peptidoglycan linkage and facilitate vesicle budding. Beyond yield enhancement, genetic manipulation enables functional customization of OMVs, such as enrichment of specific proteins or antigens, which is particularly advantageous for vaccine and immunotherapy development[59].

Following bacterial cultivation, efficient recovery and purification of OMVs are required to ensure high yield and purity while preserving vesicle integrity. Ultracentrifugation remains the most widely used method for OMV isolation[59]. Typically, bacterial cultures are first subjected to low-speed centrifugation to remove intact cells and large debris, followed by high-speed ultracentrifugation to pellet OMVs from the clarified supernatant. Using this approach, OMV yields of approximately 2−3 mg/mL have been reported for *E. coli*[60]. However, ultracentrifugation may co-isolate protein aggregates and other contaminants, necessitating additional purification steps. Size-based separation techniques are commonly employed to improve OMV purity. Gel filtration chromatography allows separation of vesicles from smaller soluble proteins and metabolites based on hydrodynamic size, thereby enhancing sample homogeneity[26]. Ion-exchange chromatography has also been explored to purify OMVs according to surface charge characteristics, although this method may result in substantial vesicle loss and reduced yield. Size exclusion chromatography (SEC) has emerged as an attractive alternative, offering a balance between simplicity, scalability, and purification efficiency. SEC effectively separates OMVs from low-molecular-weight impurities while preserving vesicle structure and biological activity (Fig. 2)[61,62].

To address the limitations of ultracentrifugation-based workflows, ultracentrifugation-free strategies have been developed for large-scale OMV production. Hydrostatic filtration systems enable efficient vesicle concentration and purification with reduced mechanical stress, making them suitable for industrial-scale applications[63]. In addition, magnetic nanoparticle-assisted harvesting represents an emerging approach for OMV isolation. In this strategy, magnetic nanoparticles associate with bacterial membranes or OMVs, allowing rapid magnetic separation without extensive centrifugation[64]. Although promising, these methods require further validation across different bacterial species and production scales.

Comprehensive characterization and quality control are indispensable for evaluating OMVs intended for biomedical use. Morphological analysis using transmission electron microscopy (TEM) or scanning electron microscopy (SEM) is routinely performed to confirm vesicle integrity, spherical morphology, and size distribution[65,66]. Dynamic light scattering (DLS) provides complementary quantitative information on particle size and polydispersity[67]. Together, these techniques ensure consistency in vesicle dimensions and structural integrity across batches. Biochemical characterization focuses on OMV composition and functional components. Proteomic profiling using mass spectrometry enables identification and quantification of outer membrane proteins, enzymes, and virulence factors, providing insights into cargo selection and functional potential[68]. Lipid analysis is equally important, as LPS and phospholipid composition directly influence OMV immunogenicity and membrane stability. High-resolution flow cytometry has been applied to assess light-scattering properties and confirm the presence of intact lipid bilayers[69]. Evaluation of OMV immunogenicity and biological activity is particularly critical for vaccine and immunotherapy applications[70]. Western blotting and enzyme-linked immunosorbent assays are commonly used to verify the presence and accessibility of specific antigens[70,71]. *In vitro* assays using macrophages or dendritic cells assess cytokine production and immune activation potential, while cellular uptake studies confirm the ability of OMVs to deliver cargo to target cells[72,73]. *In vivo* validation in animal models further establishes therapeutic efficacy, biodistribution, and safety[73,74].

Stability testing represents another key aspect of OMV quality control. Vesicle integrity, size distribution, and biological activity should be monitored under different storage conditions, including temperature and formulation variables, to ensure long-term stability[66]. In addition, combining OMVs with adjuvants or other therapeutic agents may alter their physicochemical properties and immunological behavior, necessitating systematic evaluation during formulation development[74]. Overall, the isolation, purification, and quality control of OMVs require a multifaceted and standardized approach. Integration of optimized culture conditions, scalable purification strategies, and rigorous characterization protocols is essential to produce high-quality OMVs suitable for translational and clinical applications. Establishing reproducible workflows will be a decisive step toward the industrialization and regulatory approval of OMV-based therapeutics.

## 3. Engineering strategies: From gene editing to intelligent delivery systems

### *3.1. Genetic engineering to optimize OMVs components*

Genetic engineering has become a central strategy for optimizing the composition, safety, and functionality of outer membrane vesicles, enabling their rational design for vaccine development and therapeutic delivery. Because OMVs inherit many molecular features of their parental bacteria, targeted genetic modification of the producing strains offers a direct and efficient means to regulate vesicle yield, antigen presentation, and immunogenicity. OMVs are intrinsically immunogenic and capable of eliciting both humoral and cellular immune responses, making them attractive

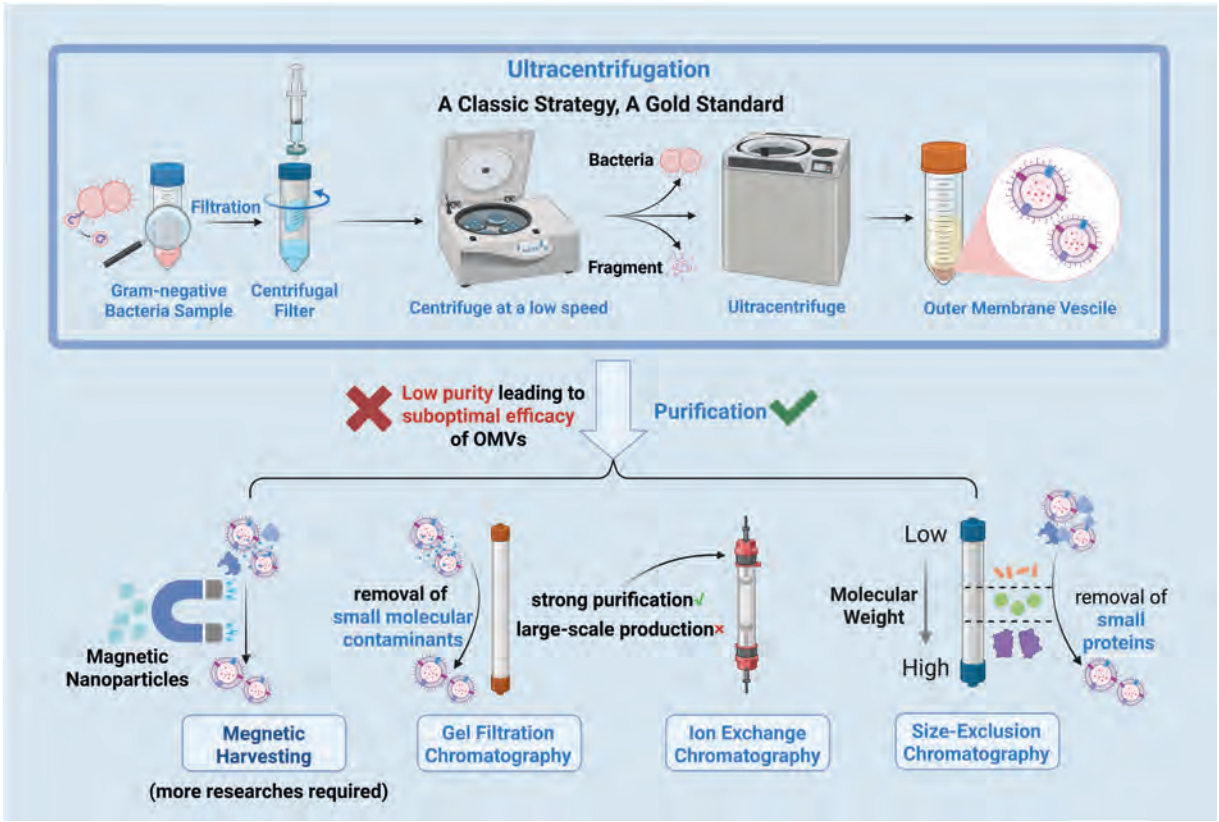



**Figure 2** OMVs purification is crucial for vaccine and therapeutic applications. Ultracentrifugation remains the gold standard: low-speed centrifugation removes cellular debris, followed by high-speed pelleting of OMVs, though co-purification of contaminants persists. Gel filtration chromatography (Sephadex G-150) refines purity by size exclusion, confirmed *via* TEM. Ion-exchange chromatography separates OMVs by charge, but risks yield reduction. Size exclusion chromatography (SEC) balances simplicity and efficiency. Sequential methods, like ultracentrifugation paired with gel filtration, enhance purity and yield. Emerging techniques like magnetic nanoparticle isolation offer rapid, scalable alternatives but require validation. Created with BioRender.com.

candidates for vaccine platforms[75]. However, unmodified OMVs derived from pathogenic bacteria often carry excessive virulence factors or endotoxins, which may induce undesired inflammatory responses. To address this limitation, attenuated OMVs are commonly generated through genetic modification of bacterial strains[76]. For example, OMVs derived from attenuated strains of *S. enterica* serovar Typhimurium or *A. baumannii* have been shown to induce robust immune responses while exhibiting reduced pathogenicity[75]. These attenuated OMVs retain their antigenic complexity but demonstrate improved safety profiles, supporting their use in prophylactic vaccination strategies[77].

One of the most widely adopted genetic approaches involves modifying lipid A biosynthesis to reduce endotoxin activity. Deletion of the *msbB* gene, which encodes a lipid A acyltransferase, results in underacylated lipid A with markedly reduced inflammatory potency[78,79]. OMVs produced by *msbB*-deficient strains maintain structural integrity and antigen presentation capacity while exhibiting substantially attenuated Toll-like receptor 4 activation. This strategy has been successfully applied in the development of low-toxicity OMV vaccines and therapeutic delivery systems. Beyond endotoxin attenuation, genetic engineering enables the controlled display of heterologous antigens on the OMV surface[80]. Autotransporter-based systems have been extensively used to anchor recombinant antigens to the outer membrane, ensuring their stable incorporation into OMVs. Among these, fusion of antigens to cytolysin A (ClyA), a highly abundant outer membrane protein, has proven particularly effective[80,81]. This approach facilitates high-density antigen presentation and promotes strong T helper 1-biased immune responses, which are critical for protection against intracellular pathogens and tumors[82].

The immunogenic properties of OMVs are further influenced by their lipid and protein composition[83]. Studies have shown that selective deletion or modification of specific virulence-associated proteins can reduce inflammatory toxicity without compromising immunogenicity. Conversely, overexpression of immunostimulatory outer membrane proteins can enhance antigen-specific immune responses[84]. For instance, OMVs enriched with outer membrane proteins from *Salmonella* have been reported to significantly boost antibody production, underscoring the importance of rational protein selection in OMV engineering. Genetic regulation of OMV biogenesis pathways also provides a means to control vesicle yield and composition[85]. Alterations in genes associated with envelope integrity or stress response signaling can induce hypervesiculation phenotypes. For example, manipulation of regulatory systems such as PmrAB and PmrC in *Citrobacter rodentium* has been shown to modulate OMV production, highlighting the role of membrane remodeling pathways in vesicle biogenesis[86]. Similarly, overexpression of the sensor kinase RscS in *Vibrio fischeri* leads to the secretion of antigenically distinct

OMVs, demonstrating that vesicle composition can be dynamically regulated through signal transduction pathways[87].

Recent advances in synthetic biology have further expanded the genetic toolbox available for OMV engineering. CRISPR-Cas-based genome editing allows precise and multiplexed modification of bacterial genomes, facilitating fine-tuning of vesicle composition and function. Combined with modular antigen-display systems, such as covalent tag-catcher platforms, these tools enable the rapid generation of customizable OMV-based vaccines capable of presenting multiple antigens simultaneously. Collectively, genetic engineering strategies provide a versatile framework for tailoring OMVs to specific biomedical applications. By regulating endotoxin content, antigen density, vesicle yield, and immunostimulatory properties, engineered OMVs can be optimized as safe and effective platforms for vaccination and immunotherapy[17,47,84,88,89]. These advances lay the foundation for the development of next-generation OMV-based delivery systems with enhanced precision and translational potential.

### *3.2. Surface functionalization and targeted delivery*

Surface functionalization represents a critical engineering strategy for transforming outer membrane vesicles from passive carriers into intelligent and programmable delivery systems. By modifying the surface properties of OMVs, their targeting specificity, circulation behavior, cellular uptake, and immunological effects can be precisely regulated, thereby expanding their applicability in cancer therapy, immunomodulation, and vaccine development[90].

One of the most direct approaches to surface functionalization is genetic engineering-mediated display of targeting ligands or functional proteins on the OMV membrane. By fusing specific ligands to abundant outer membrane proteins, OMVs can be endowed with selective affinity toward defined cell populations[91]. For example, incorporation of tumor-targeting peptides enables OMVs to preferentially accumulate in tumor tissues, enhancing local drug concentration while minimizing off-target effects. Similarly, surface expression of immune regulatory molecules, such as programmed death ligand 1 (PD-L1) or its binding partners, allows OMVs to actively engage immune checkpoint pathways and modulate antitumor immune responses. Beyond genetic display, chemical modification offers a versatile and modular strategy for OMV surface engineering. Bioorthogonal click chemistry enables the covalent attachment of functional molecules to OMV surfaces with high specificity and efficiency, without disrupting vesicle integrity[92]. Using this approach, antigens, targeting moieties, or imaging agents can be precisely positioned on the OMV membrane, facilitating controlled immune activation and targeted delivery. Plug-and-Display technologies further extend this concept by allowing interchangeable and rapid conjugation of diverse antigens onto a common OMV scaffold, thereby supporting the development of modular and personalized vaccine platforms[92].

Surface functionalization can also be exploited to achieve environment-responsive delivery. Biomineralization strategies, such as coating OMVs with calcium phosphate shells, provide a means to shield immunogenic components during systemic circulation while enabling pH-responsive release within acidic tumor microenvironments. Upon exposure to acidic conditions, the mineral shell dissolves, releasing OMVs and their cargo selectively at the target site. Although such approaches offer improved safety and spatial control, challenges related to shell uniformity and *in vivo* release kinetics remain to be addressed.

In addition to enhancing targeting efficiency, surface modification can significantly influence OMV interactions with the immune system. Incorporation of ligands that promote uptake by antigen-presenting cells, such as macrophages and dendritic cells, enhances phagocytosis and antigen presentation, thereby improving vaccine efficacy[93,94]. Conversely, surface engineering can be designed to attenuate immune recognition and prolong circulation time. Masking immunogenic epitopes through polysaccharide or polymer coatings reduces opsonization and clearance by the mononuclear phagocyte system. Polyethylene glycol conjugation has been widely explored to improve OMV stability and pharmacokinetics, although potential immune sensitization associated with repeated administration warrants careful consideration[95]. Engineering OMVs for immune evasion represents a complementary strategy for therapeutic delivery, particularly in oncology. By altering surface glycan structures or displaying immunosuppressive molecules, OMVs can partially evade host immune surveillance while retaining delivery efficiency[96]. For instance, OMVs presenting PD-L1 or other inhibitory ligands can locally suppress T-cell activation within the tumor microenvironment, facilitating sustained drug delivery and enhancing therapeutic efficacy[97]. In parallel, targeting immunosuppressive cell populations, such as regulatory T cells or myeloid-derived suppressor cells, through surface-displayed antibodies or peptides provides an additional layer of immune modulation (Fig. 3).

Nanocomposite coatings further expand the functional repertoire of OMVs. Electrostatic or covalent association with inorganic nanoparticles, such as gold or iron oxide, can enhance vesicle stability, enable external guidance, or introduce photothermal and imaging capabilities[98,99]. These hybrid systems combine the biological advantages of OMVs with the physical properties of synthetic nanomaterials, allowing multifunctional therapeutic strategies that integrate targeted delivery, immune activation, and stimulus-responsive treatment.

Collectively, surface functionalization strategies enable precise control over OMV biodistribution, cellular interactions, and immunological behavior. By integrating genetic engineering, chemical modification, and nanomaterial hybridization, OMVs can be rationally programmed to function as intelligent delivery platforms. These advances underscore the potential of OMVs to bridge biological specificity with engineering versatility, laying the groundwork for next-generation nanotherapeutics.

## 4. Application of OMVs in disease treatment

### *4.1. Drug loading and antitumor therapy*

Outer membrane vesicles have emerged as a promising class of nanocarriers for antitumor therapy owing to their intrinsic biocompatibility, immunostimulatory properties, and capacity for efficient drug encapsulation and delivery[100]. Unlike synthetic nanoparticles, OMVs possess a natural lipid bilayer and surface proteins that facilitate cellular uptake and interaction with the tumor microenvironment, making them well suited for transporting therapeutic agents to malignant tissues[101].

OMVs can encapsulate a wide range of chemotherapeutic drugs, including doxorubicin and vincristine, either within their lumen or through association with the vesicle membrane[102,103]. Encapsulation within OMVs protects drugs from premature degradation and improves their pharmacokinetic stability[97]. In preclinical models of non-small cell lung cancer, doxorubicin-loaded OMVs exhibited

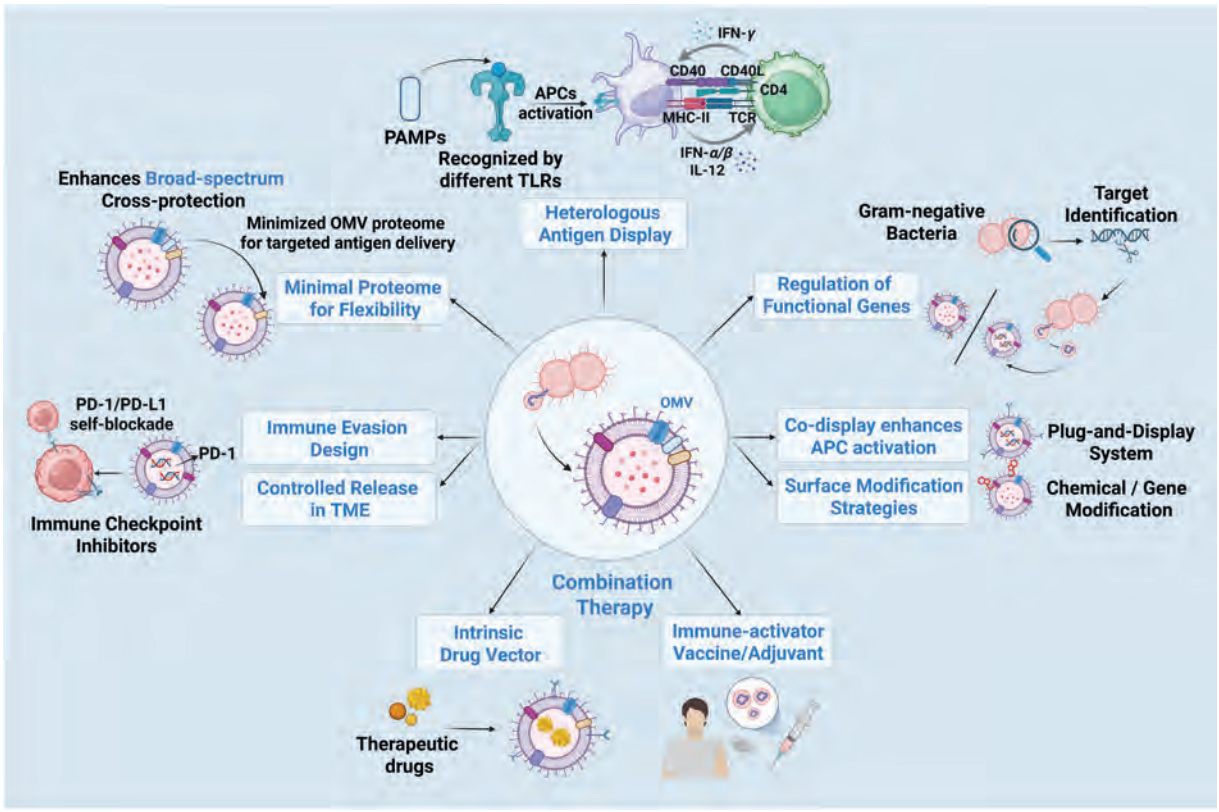



**Figure 3** OMVs play critical roles in drug delivery, immune modulators, microbial ecology and pathogenesis by mediating quorum-sensing communication, facilitating horizontal gene transfer, and strategically modulating host interactions through virulence factor delivery and TLR-dependent immune evasion mechanisms. Created with BioRender.com.

enhanced antitumor efficacy compared with free drug administration, resulting in greater tumor regression and reduced systemic toxicity[104]. The lipid bilayer of OMVs not only enables sustained drug release but also promotes efficient internalization by tumor cells due to membrane similarity and receptor-mediated uptake mechanisms[105]. Targeted delivery represents a major advantage of OMV-based drug carriers. Through surface functionalization with ligands or antibodies recognizing tumor-associated antigens, OMVs can selectively accumulate in tumor tissues and enhance intratumoral drug concentration[106]. For example, OMVs engineered to display antibodies against epidermal growth factor receptor have demonstrated improved binding to receptor-overexpressing cancer cells, leading to enhanced cytotoxic effects of the loaded chemotherapeutic agents[74]. Such targeting strategies reduce off-target exposure and mitigate adverse effects commonly associated with conventional chemotherapy.

In addition to serving as passive drug carriers, OMVs actively modulate the tumor immune microenvironment[95]. The presence of pathogen-associated molecular patterns within OMVs stimulates innate immune responses, which can synergize with chemotherapeutic-induced tumor cell death[104]. OMV-mediated immune activation has been shown to promote T lymphocyte proliferation and enhance immune-mediated tumor clearance, thereby augmenting the overall therapeutic outcome[106]. Genetic modification of OMVs to present tumor-associated antigens further amplifies antitumor immunity, especially when combined with immune checkpoint inhibitors[97]. OMVs also provide a versatile platform for the delivery of genetic therapeutics in cancer treatment. Their ability to encapsulate nucleic acids, including DNA, messenger RNA, and small interfering RNA, enables targeted modulation of oncogenic pathways. By displaying tumor-homing ligands on their surface, engineered OMVs preferentially deliver genetic cargos to tumor tissues, inducing apoptosis or suppressing tumor growth[107]. For instance, OMVs carrying tumor antigen-encoding mRNA have been shown to elicit robust cytotoxic T cell responses, highlighting their potential in personalized cancer immunotherapy[108].

Co-delivery of multiple therapeutic modalities using OMVs offers further advantages[109]. OMVs can simultaneously transport chemotherapeutic agents and genetic drugs, achieving synergistic antitumor effects[110]. In non-small cell lung cancer models, OMV-mediated co-delivery of doxorubicin and gene therapy constructs significantly enhanced therapeutic efficacy compared with monotherapy. This combinatorial approach integrates direct cytotoxicity with immune activation, addressing tumor heterogeneity and resistance mechanisms. Surface engineering strategies further optimize OMV-based antitumor therapy by improving circulation time and targeting accuracy[111]. Modification with polyethylene glycol or tumor-homing peptides reduces nonspecific clearance and enhances accumulation within tumor tissues. In addition, incorporation of photothermal or photodynamic agents into OMVs enables externally triggered tumor ablation while simultaneously delivering immunostimulatory signals. OMVs combined with photothermal materials can convert near-infrared light into localized heat, inducing tumor cell death and releasing tumor antigens that further activate antitumor immune responses[112].

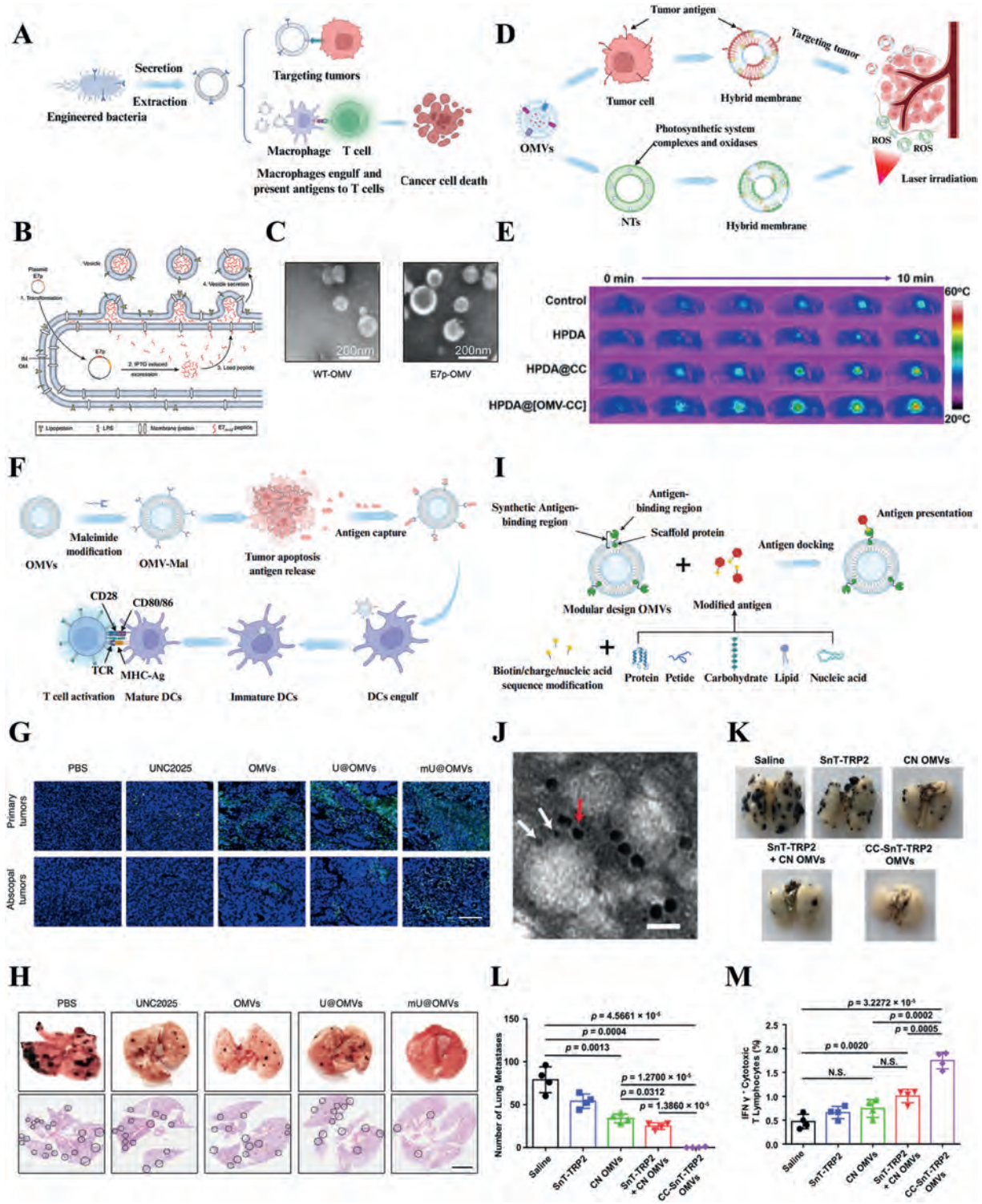



**Figure 4** Representative engineering strategies of OMV-based tumor vaccines for enhanced tumor targeting, antigen presentation, and anti-tumor immunotherapy. The strategy of genetic engineering modification of OMV (A–C). (A) Genetic engineering equips OMVs with the capability to express tumor-targeting peptides and tumor antigens. This enables OMVs to exhibit tumorigenic properties and present tumor antigens to immune cells, thereby exerting anti-tumor effects. (B) The peptide antigen of HPV tumors efficiently loaded into the bacterial OMV through the natural secretion system of the bacteria. (C) The HPV cancer peptide antigen E7p can be easily and efficiently loaded into bacterial OMVs[119]. The strategy of membrane fusion OMV tumor vaccine and the tumor targeting ability of membrane fusion OMV (D, E). (D) OMV hybridizes with tumor cell membrane to obtain the ability to target tumors; Hybrid with NTs membrane to achieve combined anti-tumor effects of immune activation and photodynamic therapy (PDT). (E) Infrared thermography of melanoma-bearing mice under various treatments (tail intravenously injected with PBS, HPDA, HPDA@CC, and HPDA@[OMV-CC] NPs) revealed that the membrane-fused HPDA@[OMV-CC] NPs

### 4.2. *Vaccine development*

As natural immunostimulants, OMVs exhibit unique self-adjuvanting properties and broad, multidimensional immunomodulatory capabilities. Their surfaces are enriched with various PAMPs, including LPS, flagellin, lipoproteins, and unmethylated CpG DNA[113,114]. In the immune system, these PAMPs play a pivotal role by activating the pattern recognition receptor (PRR) system, particularly Toll-like receptors (TLRs), thereby triggering a cascade of pro-inflammatory signaling pathways[113-115]. OMVs are internalized by dendritic cells (DCs) *via* endocytosis. Once inside DCs, they activate the cytoplasmic LPS-sensing pathway and simultaneously engage multiple TLR pathways (such as TLR2, TLR4, and TLR5) through diverse PAMPs, leading to comprehensive DC activation[114,116-118]. This intricate activation process produces dual effects: on the one hand, it drives the maturation of DCs from a naive state; on the other hand, it upregulates the expression of co-stimulatory molecules (CD80 and CD86) and induces the secretion of pro-inflammatory cytokines such as IL-6, TNF-$\alpha$, and IL-1$\beta$, thereby potentiating immune responses[115]. Compared to soluble antigens, OMVs demonstrate superior advantages by sustaining prolonged antigen presentation and eliciting stronger immune responses, which maintain T-cell activation[113]. Furthermore, the nano-vesicular nature of OMVs enables passive targeting to lymph nodes and significantly prolongs their retention therein[113,116]. Additionally, OMVs can actively home to lymph nodes through uptake by antigen-presenting cells (APCs), further enhancing their antigen delivery efficiency[116]. Consequently, the immune system more rapidly and precisely recognizes and responds to OMV-associated antigens.

OMVs have become a hotspot in tumor vaccine development because of their natural immunostimulatory properties and their advantages as nano-carriers. Interestingly, OMVs can efficiently utilize the Enhanced Permeability and Retention (EPR) effect for targeted delivery by leveraging their nanoparticle size and tumor microenvironment characteristics, demonstrating inherent advantages for tumor therapy. Therefore, researchers have developed diverse tumor vaccines using OMVs, which have shown promising therapeutic efficacy.

**Genetically engineered OMV tumor vaccines:** Through genetic engineering modifications of bacteria, such as *E. coli*, researchers have successfully prepared OMVs expressing tumor-targeting peptides that efficiently home to tumor tissues (Fig. 4A). Specifically, OMVs expressing the tumor-targeting peptide LyP1 (LOMVs) were constructed by introducing plasmids into *E. coli*. In these LOMVs, the LyP1 peptide is fused to the highly abundant membrane protein ClyA on the surface of OMVs, leveraging the natural membrane-localization properties of ClyA to ensure stable exposure of LyP1 on the OMV surface. Following intravenous injection, LOMVs effectively reach tumor tissues *via* the circulatory system owing to LyP1's targeting capability, achieving specific accumulation at tumor sites[13]. Additionally, as an excellent antigen-presenting platform, genetically engineered OMVs that encapsulate disease-specific antigens can significantly enhance antigen stability and prolong immune activation, thereby leading to improved therapeutic outcomes. The human papillomavirus (HPV) E7p peptide contains cytotoxic T lymphocyte (CTL) and T helper (Th) epitopes from the oncogenic E7 protein. Through plasmid introduction into *E. coli*, intracellular biosynthesis of E7p was achieved, and the peptide was successfully encapsulated into OMVs during bacterial secretion, yielding E7p-OMVs with high-efficiency antigen delivery capability. *In vivo* administration experiments demonstrated that OMV encapsulation markedly enhanced the stability of the E7p peptide. After uptake and processing by DCs, E7p-OMVs robustly activated HPV tumor-specific $CD4^+$ Th1 and $CD8^+$ CTL immune responses, exhibiting sustained immune activation and superior therapeutic effects compared with the free E7p peptide (Fig. 4B and C)[119]. In recent years, researchers have moved beyond traditional injectable OMV vaccines to innovatively develop an orally administered OMV vaccine for tumor treatment, achieving promising results. Using genetic engineering, *E. coli* strains expressing the tumor antigen Ag and mouse IgG Fc fragments (mFc) were constructed. Upon oral administration of these engineered bacteria, arabinose (Ara) was administered to induce secretion of OMVs carrying Ag and mFc (OMV-Ag-mFc) in the intestinal tract. OMV-Ag-mFc traverses the intestinal barrier *via* its natural mucosal penetrability and interacts with FcRn on intestinal lamina propria DCs through surface mFc, thus enabling DC recognition and uptake. Subsequently, Ag is presented *via* MHC-I to activate tumor-specific $CD8^+$ and memory T cells, demonstrating significant efficacy in multiple murine tumor models[3].

**Membrane hybridization OMV tumor vaccines:** In recent years, various OMV-tumor vaccines based on membrane hybridization technology have been developed to combine the advantages and characteristics of different cell membranes for synergistic therapeutic effects (Fig. 4D). Usually, owing to the high toxicity of OMVs and the severe off-target adverse reactions they induce, their practical application potential in tumor vaccine development has been significantly limited. Although genetic engineering techniques offer the possibility of targeted modification of OMVs, their widespread application is hindered by the

group exhibited strong infrared thermal imaging, indicating a robust thermogenic and tumor-homing accumulation capability of the fused OMVs[18]. Adapted with permission from Ref. 18. Copyright © 2020 American Chemical Society. Maleimide (Mal) confers OMV with the capability to capture and present tumor antigens, thereby inducing significant apoptosis and inhibiting lung metastasis in tumor tissues (F−H). (F) OMV-Mal captures tumor antigens and presents them to DCs to activate anti-tumor immune response. (G) mU@OMVs group exhibited more extensive apoptotic cells on the bilateral tumor tissues as determined by the TUNEL staining, further proving its strong curative effects. (H) mU@OMVs significantly reduced lung metastasis, and the number of metastasis nodules decreased by ∼60% compared with other groups, indicating its great potential in preventing metastatic tumors[11]. Modular OMV antigen presentation vaccine strategy and SpyTag (SpT)/SpyCatcher (SpC) and SnoopTag (SnT)/SnoopCatcher (SnC) pairs bestow OMVs with Plug-and-Display modular antigen presentation capability and tumor treatment efficacy (I−M). (I) SNAP-OMV antigen presentation platform. (J) The simultaneous display of two Catcher/Tag pairs by the same OMVs. SpT-Cys- and SnT-Cys labeled with 5 nm (white arrow) and 10 nm (red arrow) gold nanoparticles, respectively, were used to identify SpC and SnC on CC OMVs. Scale bar, 20 nm. (K, L) CC-SnT-TRP2 OMVs almost eliminated tumor lung metastasis. (M) CC-SnT-TRP2 OMVs elicited a strong increase in the numbers of IFN $\gamma^+$ cytotoxic T lymphocytes[92]. Adapted with permission from Ref. 92. Copyright © 2021 Springer Nature. Schematics in A, D, F, I were created with BioRender.com.

complexity and high cost of this technology. To explore new approaches for OMV-targeting modification, researchers have turned their attention to tumor cell membranes, which naturally carry tumor antigens, to achieve a simpler and more effective tumor-targeting modification method through membrane fusion or membrane coating techniques. For instance, one study successfully prepared a hybrid membrane composed of OMVs and cancer cell membranes (CC), termed OMV-CC, by ultrasonically fragmenting OMVs derived from *E. coli DH5α* and mixing them with B16-F10 cancer cell membranes for further sonication. *In vivo* experiments demonstrated that OMV-CC could target melanoma *via* the cancer cell membrane, stimulate the maturation of DCs in mice, effectively activate immune responses, and exert antitumor effects (Fig. 4E)[18]. Additionally, hybridizing different biological membranes can complement their advantages and achieve more effective anti-tumor outcomes. For example, spinach-derived thylakoid nanovesicles (NTs) are rich in photosynthetic system complexes and oxidases, capable of generating reactive oxygen species (ROS). Bacterial-plant hybrid nanovesicles (BPN) were prepared by fusing NTs with OMVs *via* extrusion. Following intravenous injection, BPN selectively accumulates in tumor tissues owing to the targeting and natural adjuvant properties of OMVs, activating the immune system. Under external laser irradiation, enzyme systems on the NTs membrane are activated, producing large amounts of ROS, thereby inducing immunogenic cell death in tumors and improving the tumor microenvironment. Ultimately, this approach achieved combined antitumor effects through immune activation and photodynamic therapy[112].

**Multifunctional OMV tumor vaccines:** Compared to other vaccines, OMVs have the advantage of being easy to modify and engineer. Researchers have employed various materials and technologies to optimize OMVs and successfully develop OMV-based vaccines with multifaceted antitumor effects, which have demonstrated remarkable efficacy in tumor therapy. Tumor immune suppression hinders the immune system from recognizing and eliminating tumors. To address this, a genetically engineered OMV featuring surface optimization through the incorporation of the extracellular domain of PD-1 was developed. Upon intravenous injection, OMV-PD1 not only activates the immune system but also competitively binds to PD-L1 on tumor cells *via* the PD-1 extracellular domain, thereby blocking the PD-1/PD-L1 signaling axis and alleviating the immunosuppressive tumor microenvironment caused by T-cell exhaustion, exerting dual antitumor effects[115]. Photothermal therapy (PTT) is frequently employed in the development of OMV-based tumor vaccines to mitigate systemic toxicity and achieve localized antitumor effects. PTT utilizes photothermal agents (PTAs) to absorb near-infrared (NIR) light and convert it into thermal energy, inducing localized hyperthermia in tumor tissues and directly triggering necrosis or apoptosis of tumor cells. Numerous studies have developed OMV-delivered tumor vaccines by integrating various PTAs with OMVs, combining the advantages of immunotherapy and PTT to transform "cold tumors" into "hot tumors", significantly activating antitumor immunity and achieving satisfactory therapeutic outcomes[18,120,121]. Additionally, exploiting the characteristic abundance of hydrogen peroxide ($H_2O_2$) in the tumor microenvironment, researchers have utilized OMVs as a delivery platform for $Fe^{3+}$, which reacts with $H_2O_2$ *via* the Fenton reaction to generate hydroxyl radicals and reactive oxygen species (ROS). This approach modulates tumor hypoxia, induces immunogenic cell death in tumor cells, and releases abundant tumor antigens to effectively activate immune cells, eliciting specific antitumor immune responses and inducing potent tumor ablation, thereby realizing chemodynamic therapy[121,122]. Furthermore, to overcome antibody-dependent clearance and systemic inflammatory toxicity induced by intravenous OMV administration, researchers have used calcium phosphate (CaP) shells to coat and mineralize OMVs. CaP modification not only masks OMV membrane antigens, reduces immunogenicity, and enhances safety but also facilitates dissolution triggered by acidic tumor tissue, neutralizes the acidic TME, prevents OMV leakage into healthy tissues, and promotes macrophage polarization from anti-inflammatory M2 to pro-inflammatory M1 phenotypes, thereby enhancing antitumor efficacy[20,22].

**Specific antigen-presenting vaccines:** OMVs have also been applied as outstanding vector platforms in the design of tumor vaccine antigen-presenting carriers, demonstrating promising therapeutic prospects. Damage-associated molecular patterns (DAMPs) and tumor-associated antigens (TAAs) released by dead tumor cells exhibit strong immunogenicity. However, macrophage-mediated efferocytosis clears these antigen molecules and weakens antitumor immune responses. Therefore, strategies to inhibit macrophage efferocytosis are gradually emerging as a new focus in antitumor immunotherapy. In this context, studies have utilized OMVs loaded with the efferocytosis inhibitor UNC2025 to selectively suppress MerTK phosphorylation in macrophages, thereby blocking the clearance of apoptotic cells. This leads to the accumulation of dead tumor cells and subsequent secondary necrosis, releasing large amounts of TAAs and DAMPs to activate robust antitumor immunity, achieving an "*in situ* tumor vaccine" effect (Fig. 4G and H)[11]. Additionally, by targeting antigens released by tumor cells after antitumor therapies, the research team designed an OMV-based antigen-presenting vaccine. This vaccine modifies maleimide (Mal) on the OMV surface to form OMV-Mal, leveraging the stable thioether bond formed between maleimide and antigens to capture tumor antigens and present them to DCs, thereby activating potent antitumor immunity (Fig. 4F)[11,123]. In addition to utilizing antigens from apoptotic tumor cells, another effective anticancer strategy involves delivering nucleic acids *via* OMVs to express tumor antigens and subsequently activate the immune system. The research team engineered OMVs expressing the RNA-binding protein L7Ae on their membrane surface along with listeriolysin O (LLO) to promote endosomal escape. This platform efficiently delivers tumor antigen-encoding mRNA into dendritic cells through L7Ae-mediated mRNA loading and LLO-driven endosomal escape mechanisms. The mRNA is then translated within the cells, generating tumor-specific antigens that are presented to T cells *via* the MHC-I pathway, successfully activating specific antitumor T cell responses to eliminate tumors[124].

**Modular design of OMV tumor vaccines:** To advance the field of antitumor vaccines, researchers have committed to developing a more versatile and broadly applicable universal vaccine platform. Against this backdrop, the modular-designed OMV vaccines have emerged to achieve broad-spectrum therapeutic effects. Currently, research approaches primarily focus on modifying target antigens or engineering OMVs to enable rapid vaccine preparation. For example, scientists introduce cationic groups through chemical modifications to the target antigen, endowing it with a positive charge, and co-incubating it with OMVs. Utilizing electrostatic interactions, these positively charged antigens can spontaneously assemble onto the negatively charged OMV membrane, thereby enabling the rapid preparation of OMV vaccines. Moreover, simply by replacing the positively

charged modified antigens, rapid loading of different antigens can be achieved[125]. Cheng et al.[92] fused the SpyCatcher domain onto the surface protein ClyA of OMVs. By mixing and incubating with tumor antigens pre-labeled with SpyTag short peptides (which can covalently bind to SpyCatcher), rapid and flexible covalent conjugation of antigens to OMVs was achieved. Furthermore, this platform possesses the capability to simultaneously display multiple tumor antigens, making it an ideal choice for presenting multivalent tumor antigens (Fig. 4J−M)[92]. For antigens that are challenging to transfect or express on biological membranes, the research team utilized plasmid transfection technology to produce OMVs expressing synthetic antigen-binding protein (SNAP) in an *E. coli* hypervesiculating strain. By biotinylating the target antigen and co-incubating it with SNAP-OMVs, rapid antigen-OMV conjugation was achieved (Fig. 4I)[8]. Additionally, based on the RNA-binding protein L7Ae, an OMV-modular RNA vaccine delivery system was designed specifically for delivering nucleic acids to express antigens. This platform can rapidly adapt to various mRNA tumor antigens by exchanging box C/D-mRNA sequences, demonstrating its outstanding modular platform characteristics[124].

Scientists have pioneered a novel approach to enhance disease treatment efficacy by combining OMVs with other drugs or therapeutic agents, offering new possibilities for therapeutic strategies. Researchers have constructed manganese dioxide ($MnO_2$)-encapsulated OMVs through extrusion loading. Administration in mice demonstrated that these $MnO_2$-loaded OMVs could tightly adhere to the inflamed colonic epithelium, catalytically eliminate excessive intestinal ROS, significantly reduce pro-inflammatory cytokine levels, and elevate anti-inflammatory cytokine levels. When combined with the anti-inflammatory drug metformin, they further remodeled the dysbiotic gastrointestinal microbiome in colitis mice, improved the overall richness and diversity of the gut microbiota, and exhibited superior therapeutic effects compared with commercial IBD chemotherapy drugs[126,127]. In the field of tumor therapy, although oncolytic adenovirus (Ad) infection has been shown to promote autophagy in tumor cells, intravenously delivered Ads often exhibit low accumulation in tumor tissues, making it difficult to effectively activate tumor autophagy. By encapsulating Ads into OMVs, not only was the protection of Ads achieved after intravenous injection, but their intratumoral accumulation was also significantly enhanced. Moreover, as potent immune stimulators, OMVs can reshape the immunosuppressive tumor microenvironment, further promote antitumor immune responses, and thereby exert combined anti-tumor therapeutic effects[128].

Currently, combination therapies involving OMV vaccines have demonstrated immense potential and broad application prospects for enhancing disease treatment efficacy. Through co-administration with other drugs or therapeutic agents, OMVs can exert extensive and profound immunomodulatory effects, providing new insights and strategies for disease treatment. With continuous research advancements and technological progress, OMV-based combination therapy is expected to play a significant role in the treatment of a wide range of diseases.

### *4.3. Applications of OMVs in gene-editing therapies*

Through targeted modification of DNA sequences, gene editing technologies have provided revolutionary tools for treating various diseases, including cancer, hereditary diseases, and viral infections. However, their clinical applications are often limited by the efficiency and safety of delivery systems[129]. While traditional vector systems, such as viruses or lipid nanoparticles, have various disadvantages such as high immunogenicity and low targeting specificity, OMVs are intrinsically superior owing to their natural immune-activating ability, efficient cellular penetration, and programmable properties. Owing to these advantages, OMVs have become a cutting-edge research focus for gene editing delivery.

The CRISPR-Cas9 system, a globally renowned and revolutionary gene-editing tool, has demonstrated tremendous potential for treating hereditary diseases, tumors, and even infectious diseases[129]. Typically, the target gene resides on a specific plasmid, and the CRISPR-Cas9 system acts like molecular "scissors" to edit, modify, or silence relevant gene fragments, ultimately yielding the desired target DNA. However, establishing an efficient and safe *in vivo* delivery system remains a major bottleneck in clinical translation[130]. In contrast, CRISPR-dCas9 acts as a precise molecular switch that can bind specifically to DNA to either silence or activate the target genes[131]. As spherical vesicles that can encapsulate RNA and DNA fragments during their secretion[41], OMVs have been shown to possess a stable lipid bilayer membrane, allowing robust encapsulation and protection of nucleic acid fragments from degradation or denaturation. Furthermore, to achieve surface modification of OMVs, Gram-negative bacteria can be targeted before secretion by employing CRISPR-Cas9 and CRISPR-dCas9 systems to edit, modify, or silence specific gene fragments. This process endows secreted OMVs with the same genetic modifications and characteristics as their parental bacteria[132]. Based on this logic and theory, researchers have proposed various delivery strategies for achieving precise targeting and stable expression. These strategies can even attenuate the immunogenicity induced by OMVs[20], rendering genetically engineered OMVs a stable and reliable new immune activator and drug delivery vector with promising prospects.

However, multiple simultaneous therapies may impose a heavy burden on organisms. Therefore, it is crucial to minimize the systemic toxicity caused by OMV carriers. The CRISPR-Cas9 system plays a pivotal role in this process. CRISPR-Cas9 was used to knock out *msbB* in *E. coli* to reduce the expression of lipid A acyltransferase, thus lowering the inflammatory activity of LPS[93]. This enabled the construction of low-inflammation *m*-OMVs, which facilitated low-toxicity targeted delivery in a meningitis model using antibiotics combined with sonodynamic therapy[132]. The CRISPR-mediated engineering strategy significantly reduced the toxicity of OMVs. Through magnetic guidance and ultrasound activation, it enhanced the efficiency of drug penetration across the blood−brain barrier and bactericidal efficacy, demonstrating substantial advantages in the clinical treatment of refractory brain infections (Fig. 5A)[132]. In addition to controlling cytotoxicity *via* CRISPR-Cas9, researchers have discovered that CRISPR-dCas9 technology can be employed to suppress the transcription of genes involved in peptidoglycan layer synthesis in Gram-negative bacteria, such as *mcrA*, *mrcB*, *pbpC*, *murE*, *mrdB*, and *mreB*, which in turn accelerates the production rate of OMVs[131].

In recent years, the combination of gene editing strategies with delivery platforms, along with the rapid development and maturation of technologies such as CRISPR, has demonstrated significant application potential in tumor immune modulation with low-toxicity engineering strategies, antiviral therapy, and intracorporeal imaging tracking.

In the CRISPR-Cas9-based tumor therapy mentioned above, engineered bacterial strains with the *msbB* gene for lipid A acyltransferase knockout showed a significant reduction in toxicity (Fig. 5B), substantially ensuring the survival of the test

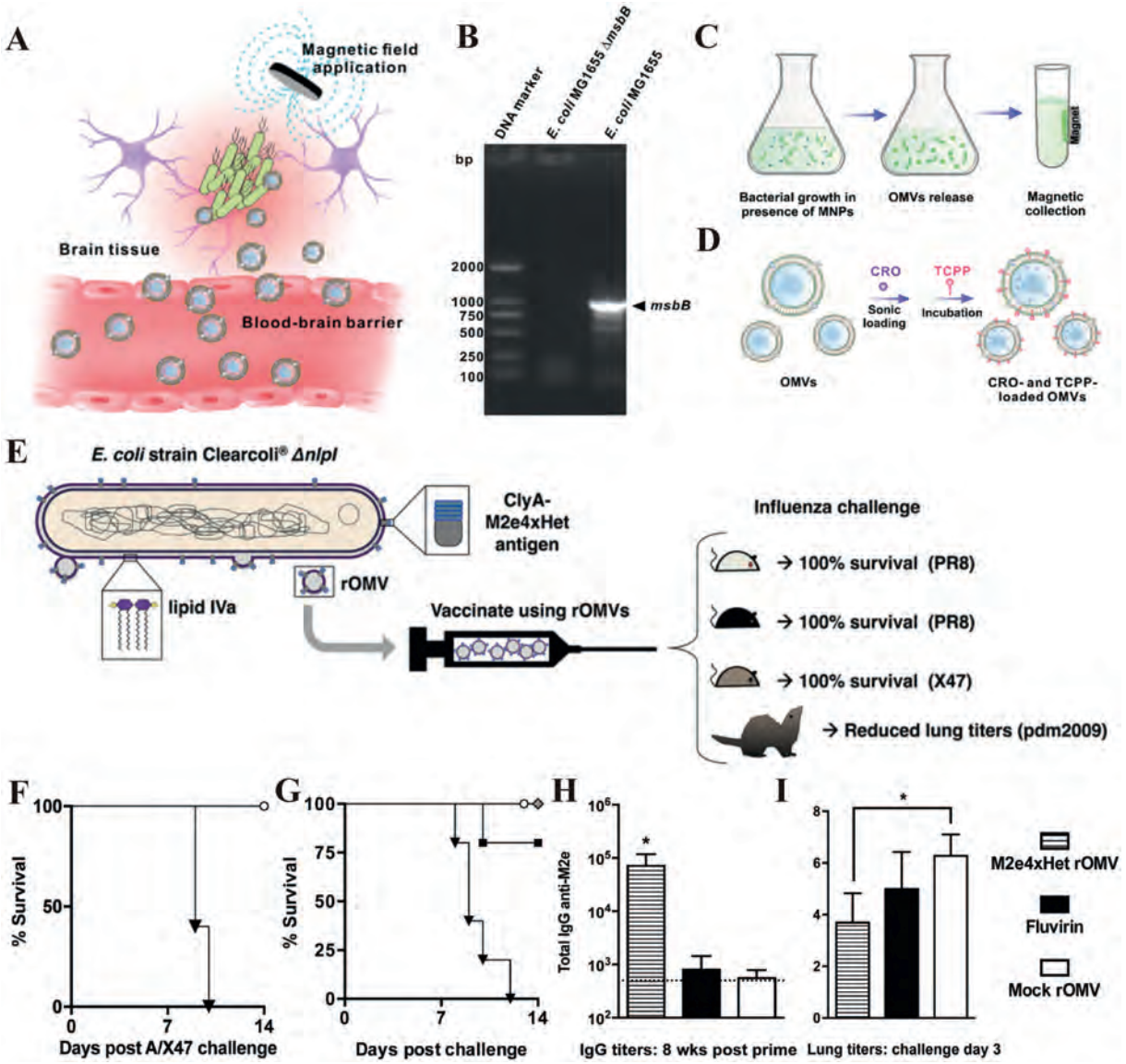



**Figure 5** Engineered OMV platforms for low-toxicity delivery and immune protection in infectious disease therapy and vaccination. (A) Through magnetic guidance and ultrasound activation, it enhanced the efficiency of drug penetration across the blood−brain barrier and bactericidal efficacy. (B) CRISPR-Cas9-mediated *msbB* gene knockout could produce low-toxicity *m*-OMVs with good therapeutic outcomes. (C, D) Magnetic harvesting of OMVs secreted by *E. coli* grown in presence of iron-oxide magnetic nanoparticles and loading of CRO and TCPP[132]. Adapted with permission from Ref. 132. Copyright © 2023 Elsevier. rOMV injections significantly attenuated febrile reactions and helped conquer influenza challenge in mouse and ferret models. (E) Graphical abstract, theoretical basis, and consequences. (F) Mortality of mice challenged with a lethal dose (50 FFU) of influenza A/PR8 ($n = 5$ CC rOMV-vaccinated, $n = 5$ Nsl rOMV-vaccinated, $n = 5$ PBS-vaccinated, and $n = 5$ pre-exposed). Kaplan−Meier survival curves were analyzed with a log-rank test using the Bonferroni method to account for multiple comparisons. (G) Mortality of DBA/2J mice challenged with a lethal dose (5000 PFU, ~2.5 × LD50) of influenza A/X-47 ($n = 5$ CC rOMV vaccinated, $n = 5$ PBS vaccinated). Kaplan−Meier survival curves were analyzed using a log-rank test. (H) Total IgG anti-M2e titers of ferrets at eight weeks post-prime vaccination. (I) Lung viral titers of ferrets three days post-challenge with influenza strain pdmH1N1 ($n = 3$ ferrets per group). Lung titers were compared using ANOVA, followed by comparison with mock CC rOMVs using the Bonferroni correction method ($n = 3$)[135]. Adapted with permission from Ref. 135. Copyright © 2017 Elsevier.

mice without altering the loading amount. According to the research, wild-type OMVs were lethal at 6 mg/kg, whereas *ΔmsbB* OMVs were non-lethal even at 60 mg/kg, which was apparently a 10-times dose. Additionally, inflammatory cytokine TNF-$\alpha$ and IL-1$\beta$ levels in the mice serum decreased by more than 10 and 3 times, respectively[132]. This result demonstrates that CRISPR-mediated low-toxicity engineering strategy can be utilized to treat drug-resistant bacterial infections. Moreover, the engineering modification properties of OMVs are highly favorable for gene editing. It is conceivable that by directly modifying tumor-specific ligand chimeric receptors on the surface of OMVs, OMVs can actively target and recognize tumor cells to successfully accomplish targeted delivery of the gene-regulatory molecules. As a non-coding miRNA, miR-34a could inhibit the

expression of silent information regulator 1 (*Sirt1*), leading to reduced SIRT1 protein levels and marked efficacy in breast cancer treatments Based on this theory, researchers have proposed using OMVs as miRNA delivery platforms to enhance therapeutic efficacy by displaying anti-PD-1 functional proteins on the OMV surface while encapsulating acid-sensitive ZIF-8 nanostructures to deliver miR-34a to tumor tissues. These engineering strategies have helped achieve immune co-regulation and tumor-specific therapy[14]. In the mouse breast cancer model, the tumor volume was reduced by approximately 70% through the construction of such OMVs, and $CD8^+$ T-cell infiltration density in the tumor tissue was significantly increased by about tenfold, demonstrating that OMVs carrying miRNA after gene editing and engineering modification can largely enhance efficacy by combining immune checkpoint blockade with gene silencing.

OMVs have also made significant contributions to the field of antiviral therapy. In a previous study, the regulatory ability of bacterial outer membrane vesicles in viral infections was evaluated. OMVs from *Klebsiella*, *E. coli*, and *Salmonella* can activate the TLR4−TRIF signaling pathway in human alveolar macrophages *in vitro*, prolong STAT1 phosphorylation, significantly upregulate *Mx1* expression, and finally induce a strong IFN-I response. This established an antiviral state in macrophages, significantly inhibiting influenza virus replication and spread in macrophages, alveolar epithelial cells, and overall lung tissues, thus rapidly reducing influenza virus replication. Given that this antiviral state disappeared in TRIF-knockout macrophages, it can be concluded that OMVs successfully activated the TLR4−TRIF pathway in human alveolar macrophages to enhance antiviral effects[133]. Another study using a mouse norovirus model showed that OMVs could be co-delivered with the virus into target cells to induce stronger pro-inflammatory cytokine release (such as IL-6 and TNF-$\alpha$) and inhibit norovirus replication[134]. In the co-delivery group, norovirus RNA levels in mice decreased by more than tenfold, and the duration of infection was shortened by approximately 36 h, suggesting that OMVs may assist in the rapid immune clearance of viruses, although attention must be paid to whether strong systemic toxicity is caused by co-stimulation of the immune system.

OMVs based on gene knockouts can also be used for *in vivo* imaging and tracking. Before Gram-negative bacteria secrete OMVs, gene-editing techniques can be employed to silence or modify specific genes in the bacterial population, resulting in OMVs that carry the same genetic modifications. This theory lays the groundwork for constructing diversified genetically engineered OMVs, and *in vivo* tracking of OMVs can be achieved using such approaches. Researchers have combined genetic engineering and antibiotic-induced release techniques to prepare nanoprobes with fluorescence activation and absorption-shifting labels, which can be conjugated to OMVs and serve as novel fluorescent probes[4]. These engineered OMVs can be non-invasively tracked in real time under anaerobic conditions, enabling controllable fluorescence switching and reversible dual-color imaging, which offers higher application value in drug delivery and therapeutic monitoring. Based on this technology, clinical medicine, such as real-time imaging tracking and combined immune regulation, may further expand the applications of OMVs in disease prevention and treatment.

As a nano-delivery platform with strong immune-activating capabilities, OMVs still face numerous biosafety challenges. Principal obstacles include excessive immune responses induced by endotoxins, toxicity risks caused by non-specific organ accumulation, and batch-to-batch heterogeneity, which limit clinical translation due to inconsistencies in production.

In terms of immunogenicity regulation, engineering strategies have mainly focused on the structural optimization of lipopolysaccharides. Beyond CRISPR-Cas9-mediated *msbB* gene knockout (Fig. 5C and D), which produces low-toxicity *m*-OMVs with good therapeutic outcomes. Researchers have also directly modified LPS structures to reduce excessive activation of immune cells[132]. Recombinant OMV (rOMV) produced by *ClearColi* strains, in which LPS was modified to contain only the Lipid IVa structure, has been demonstrated to be incapable of activating the human TLR4 pathway. Moreover, in mouse and ferret models, the rOMV system can stimulate immune responses while significantly attenuating febrile reactions in animals (Fig. 5E)[135]. Even with a reduction in immunogenicity, this type of rOMV could still induce a relatively balanced Th1/Th2 immune response, achieving complete protection against H1N1 and H3N2 influenza viruses in mice with various genetic backgrounds and a 100-fold reduction in viral titers in the ferret model (Fig. 5F−I).

Biodistribution studies have shown that OMVs exhibit a rapid systemic distribution *in vivo*. After intraperitoneal injection in mice, OMVs were distributed to the liver, lungs, spleen, kidneys, and other organs within 3 h. Systemic inflammatory responses began to subside within 6 h and reverted by 12 h. However, early elevations of serum TNF-$\alpha$ and IL-6 were detectable, accompanied by leukopenia and increased expression of ICAM-1 in the lungs, indicating that further optimization of the immune activation intensity is necessary to improve the dosing safety of OMVs[136]. To further control immune toxicity, researchers utilized metal-polyphenol networks to encapsulate OMVs, constructing a "nano-prison" that enables alleviated systemic toxicity, mitigates cytokine upregulation, and enhances safety. This carrier targets tumor immune microenvironments characterized by low pH and high ATP levels, locally releasing iron ions to induce immunogenic cell death, thereby promoting tumor antigen release and dendritic cell activation. In bilateral tumor models, it achieves pronounced tumor suppression while significantly reducing systemic toxicity[122].

In terms of standardized production, the batch-to-batch controllability and yield of OMVs have long limited their industrialization, especially because batch variations often result in inconsistent actual injection doses and individual differences in delivery efficacy. Furthermore, protein expression control and membrane localization in genetically edited OMVs still remain key bottlenecks affecting antigen-loading efficiency and easily lead to OMV heterogeneity[8], which poses potential safety risks. In summary, endotoxin modification, targeted release design, and engineering expression systems urgently require long-term improvements, and the biosafety of OMVs still requires significant enhancement.

## 5. Immunomodulatory mechanisms and precision regulation

### 5.1. *Activation pathways of innate immunity*

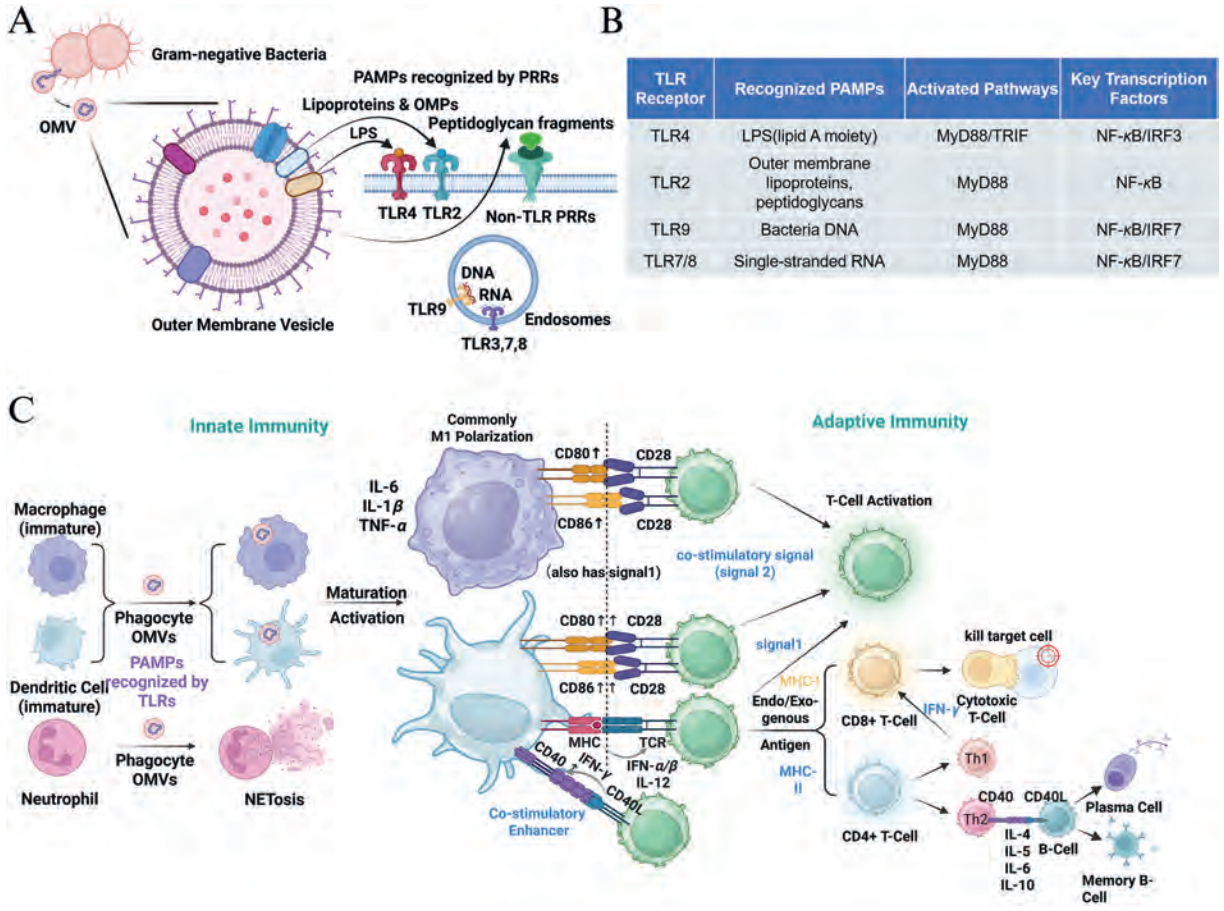
As a vital communication mode among Gram-negative bacteria, OMVs inherently retain many of their parent bacteria's intrinsic properties. Most importantly, like typical foreign pathogens, OMVs can efficiently utilize PAMPs carried during secretion, including those from the bacterial outer membrane and periplasmic space, so that they can be rapidly recognized by PRRs on host cell surfaces and within the host cell, such as TLRs and NLRs.

This recognition activates the innate immune pathways and builds a bridge to adaptive immunity (Fig. 6).

PAMPs on the OMV surface mainly include lipopolysaccharides and lipoproteins from the bacterial outer membrane, as well as DNA, RNA fragments, and peptidoglycans from the membrane interior. However, not all PAMPs are present on the surface of OMVs; some are distributed internally, leading to different modes of PRR activation. In a study on *B. fragilis*, a typical Gram-negative bacterium[137], researchers found that LPS, lipoproteins, and peptidoglycans are primarily displayed on the OMV surface, whereas DNA and RNA fragments are usually enclosed inside OMVs and protected by the bilayer membrane structure. Their recognition by PRRs also differs: non-DNA/RNA PAMPs such as LPS are generally recognized by TLRs on the host cell surface, such as LPS by TLR-4 and lipoproteins/peptidoglycans by TLR-1, 2, 5, and 6, whereas DNA and RNA fragments, after entering the host cells as intravesicular contents, activate intracellular TLRs, with RNA recognized by TLR7 and TLR8, and DNA binding to TLR3 and TLR9 (Fig. 6A and B)[138].

Host cells capable of recognizing PAMPs include neutrophils, macrophages, and dendritic cells. In addition to PAMP recognition by surface TLRs, these host cells internalize OMVs, allowing their contents to act as endosomal cargo. This step is critical for the maturation and activation of macrophages and dendritic cells[139,140] and is crucial for the activation of neutrophils. As for neutrophils, beyond their role as targeted delivery agents, OMVs can also activate the induction of NETosis[141], prompting neutrophils to release DNA and proteins that form a net-like structure, which traps and directly kills invading pathogens and tumor cells[142]. In macrophages and dendritic cells, internalized OMVs allow the recognition of PAMPs by TLRs and NLRs, leading to the activation of downstream immune signaling pathways, notably NF-$\kappa$B and IRF3/7[143]. Activation of these pathways results in the production of pro-inflammatory cytokines such as IL-6, TNF-$\alpha$ and IL-1$\beta$. Concurrently, important costimulatory molecules, including MHC, CD80 and CD86 are upregulated[144], priming the activation of T cells. Subsequently, these signaling pathways, cytokines, and co-stimulatory molecules maintain a relatively coordinated state over an extended period, mutually regulating and activating or inhibiting one another, thereby constructing a crucial bridge between innate and adaptive immunity and maintaining host immune homeostasis (Fig. 6C)[145]. This reactivation of immune balance not only enhances the body's ability to clear pathogens but also provides a theoretical basis for the use of OMVs as vaccine platforms and immunotherapeutic carriers. Furthermore, this balance can synergize with immune checkpoint inhibitors as well.

The lipid cavity structure of OMVs endows them with intrinsic drug loading capabilities. By performing lipid-OMV hybridization, their drug-loading capacity can be further enhanced while retaining their inherent immune-activating properties[146],




| TLR Receptor | Recognized PAMPs | Activated Pathways | Key Transcription Factors |
|---|---|---|---|
| TLR4 | LPS(lipid A moiety) | MyD88/TRIF | NF-κB/IRF3 |
| TLR2 | Outer membrane lipoproteins, peptidoglycans | MyD88 | NF-κB |
| TLR9 | Bacteria DNA | MyD88 | NF-κB/IRF7 |
| TLR7/8 | Single-stranded RNA | MyD88 | NF-κB/IRF7 |

**Figure 6** The immunomodulatory mechanisms of OMVs. (A) PAMPs on the OMV surface are recognized by TLRs and NLRs. (B) TLRs and their corresponding PAMPs activated pathways, key transcription factors, and induced immune factors. (C) The bridge that OMVs have constructed between innate and adaptive immunities. It mainly involves APCs maturation, pathway activation, and immune cell activation. Schematics in A, C were created with BioRender.com.

positioning them as a new generation of immune adjuvants or vaccines capable of killing pathogens or tumor cells while simultaneously enhancing immune responses. Recent studies have demonstrated that OMVs derived from *Akkermansia muciniphila* (Akk-OMV) can induce anti-tumor immune responses and, owing to their excellent safety profile, hold great promise as self-adjuvanted, biocompatible vesicles[147]. Moreover, by hybridizing Akk-OMVs with liposomes, researchers have developed extracellular vesicle-lipid hybrid nanovesicle cancer vaccine formulations capable of promoting dendritic cell maturation in lymph nodes, activating cytotoxic T cells, and achieving enhanced prophylactic and therapeutic effects. In addition, by utilizing the enhanced drug-loading capacity, plasmids can be loaded and injected peritumorally to mediate PD-L1 blockade gene therapy. This strategy highlights a novel multifunctional approach for synergistic cancer immunotherapy vaccines based on immune checkpoint blockade and vesicle-based drug delivery.

While constructing immune balance, OMVs can also act on innate immune cells *in vivo* to induce trained immunity, thus becoming a new type of trained immunity vaccine. It is traditionally believed that one of the major distinctions between innate and adaptive immunity lies in the absence of immune memory within the innate immune system. However, it is increasingly evident that organisms lacking T and B lymphocytes are not devoid of immunological memory, a fact underscored by the observation that approximately 95% of all animal species on Earth rely solely on innate immunity[148]. For instance, a study published in 2003 demonstrated that copepods, which possess only innate immunity, exhibit memory-like immune responses that protect them against reinfection by parasitic tapeworms[149]. This phenomenon, termed "trained innate immunity", has since been observed not only in other invertebrates but also in vertebrates, including humans[150]. Notably, Bacillus Calmette-Guérin (BCG) vaccination was found to confer cross-protective effects and reduce mortality in severe combined immunodeficiency (SCID) mice, which lack functional B and T-cells[151].

Classically, PAMPs and DAMPs are regarded as key inducers of trained immunity. They stimulate innate immune cells in both peripheral tissues and within central reservoirs such as the bone marrow. Even after these cells return to a resting state post-initial stimulation, they exhibit enhanced and accelerated responses upon re-exposure, which could be mediated through long-lasting epigenetic and transcriptional reprogramming[152,153]. As discussed previously, OMVs inherit a variety of PAMPs from their parent Gram-negative bacteria, which are readily recognized by PRRs on innate immune cells. This unique composition endows OMVs with the capacity to act as potent inducers and carriers for trained innate immunity.

Such OMVs are used to "pre-train" innate immature immune cells and strengthen them during the early developmental stages. For example, GM-CSF stimulates hematopoietic stem cells to differentiate into monocytes and macrophages. In some studies, researchers co-administered GM-CSF and OMVs during the bone marrow phase to pre-train immature macrophages, endowing them with enhanced antitumor and proinflammatory capabilities upon maturation, thereby creating a lineage of memory-like tumor-fighting macrophages[145]. This form of immune activation provides the immune system with a form of prestimulation, resulting in immune cells with stronger cytotoxic capabilities after maturation, improving the body's early adaptation to external stimuli, and ultimately reducing the severity of systemic toxicity.

### *5.2. Programming of adaptive immunity*

As shown in Fig. 6, after the activation of signaling pathways, cytokines, and the upregulation of various co-stimulatory molecules, antigen-presenting cells activate immune cells, which is the most critical step mediated by OMV-based immunotherapies. Various current combination therapies are based on this vital adaptive immune activation pathway.

It is well known that both exogenous and endogenous antigens can be presented by MHC molecules to TCRs, serving as one of the most important co-stimulatory signals to induce T-cell activation. Another remarkable advantage of OMVs is as follows. According to conventional immunological thinking, OMVs, which are exogenous antigens secreted by Gram-negative bacteria, primarily activate MHC-II molecules and thus stimulate $CD4^+$ T cells after binding to TCRs. However, researchers have surprisingly discovered that exogenous OMV antigens can also undergo cross-presentation *via* the MHC-I pathway, therefore co-activating $CD8^+$ T cells[154]. After OMV-associated PAMPs enter dendritic cells or other APCs, a portion of the antigens escapes into the cytoplasm. PAMPs binding to TLR-4 receptors on dendritic cells activate the TLR-4−MyD88 signaling pathway, indirectly enhancing proteasomal antigen processing and cross-presentation. Consequently, dendritic cells present OMV-carrying antigens through the activated MHC-I pathway to $CD8^+$ T cells, promoting their differentiation into CTLs[155] and becoming one of the key killing tools in OMV-mediated immunotherapies. In addition to the activation of cytotoxic T cells induced by MHC-I pathway-mediated cross-presentation, the MHC-II pathway, through OMVs serving as exogenous antigens, facilitates the differentiation of T cells into helper T cells (Th1 and Th2), thus providing critical support for the activation of cytotoxic T cells and B cells. On this basis, B cells, with the assistance of Th2 cells, further differentiate into plasma cells and memory B cells. Plasma cells produce specific antibodies to establish humoral immunity, while memory B cells form immune memory after multiple immunizations, thereby providing a durable secondary layer of protection for specific immunity[135,156]. Consequently, OMVs are competent to serve as adjuvants that enhance the magnitude and breadth of humoral immune responses, especially for recombinant antigen vaccine strategies with inherently weak immunogenicity.

As previously mentioned, OMVs exhibit extremely close and complex interactions with immune cells such as macrophages, dendritic cells, and neutrophils. They not merely promote antigen presentation by being endocytosed into these cells, thereby activating T cells, but rather directly regulate the activity and secretory functions of these immune cells through signaling pathway modulation. Combined with additional features of OMVs, such as engineering modifications and lipid bilayer structures, there is great potential to construct more refined adaptive immune programming pathways. At present, gene editing techniques and chemical modifications are the primary methods for engineering OMVs, and research has mainly focused on the further activating antigen cross-presentation pathways to enhance cytotoxic T cell responses, precision targeting of tumor-associated cells through modification of specific immune regulatory molecules, and surface engineering of OMVs to construct multifunctional platforms incorporating therapy systems, including photothermal therapy and chemo-immuno-combination therapy.

Taking advantage of the surface-modification capabilities of OMVs, a variety of composite OMV-based nanoplatforms have been developed. Through engineering techniques, these

nanoplatforms not only retain the intrinsic stability of OMVs but also integrate various additional functionalities, such as photothermal conversion capacity and localized immune modulation, thereby exhibiting enhanced efficacy and precision in antitumor immunotherapy[20]. For example, in a representative case of synergistic photothermal therapy mediated by functional surface modification, OMVs were engineered to form $Fe_3O_4$-$MnO_2$ (FMO) nanoparticle platforms. Upon neutrophil-mediated targeted delivery to the tumor immune microenvironment, the particles underwent external near-infrared photothermal stimulation and could enhance T-cell activation, largely promoting their differentiation into highly cytotoxic T cells, augmenting the killing of tumor cells, and suppressing metastatic lesion formation caused by the proliferation of distal cancer cells (Fig. 7A)[121]. Experimental observations demonstrated significant reductions in both tumor volume and weight in B16-F10 melanoma mice, suggesting excellent tumor suppression (Fig. 7B−E).

Considering the strong immunostimulatory properties of OMVs and their engineering plasticity, the construction of vaccines and adjuvant platforms based on OMVs has emerged as a highly active research direction. As discussed previously, in the context of MHC-mediated antigen presentation, the presence of specific tumor-associated antigens on the surface of cancer cells, antigens absent from normal tissue, provides a viable strategy for tumor immunotherapy by enhancing the immune system's recognition of tumor-specific antigens[146]. Conceptually, if OMVs can be designed to present tumor-specific antigens on their surface, thereby enabling MHC molecules to actively present these antigens to immune cells for "specific activation", it would empower the immune system to recognize and target tumor cell surface antigens actively. As a novel adjuvant strategy, OMVs promote active immune surveillance and target cancer cells, achieving broad tumor coverage and potent cytotoxicity. Researchers have validated this theory by demonstrating that tumor antigens can be displayed on the surface of OMVs through fusion with the ClyA protein and by further utilizing Plug-and-Display technology[157], which allows protein tags to spontaneously form isopeptide bonds with protein catchers (Fig. 7F)[158,159]. By fusing these protein catchers with ClyA, various tumor antigens conjugated with specific tags can be rapidly and simultaneously displayed on the OMV surface. For instance, dual tumor antigens, such as $OVA_{257-264}$ and $TRP2_{180-188}$, successfully stimulated $CD8^+$ T cell-mediated synergistic immune responses, thereby robustly inhibiting lung metastatic melanoma (Fig. 7G−I). This construction provides a valuable foundation for the development of multifunctional OMV-based platforms tailored for personalized tumor vaccine applications.

In the context of tumor treatment, given the substantial heterogeneity typically exhibited by tumor cells, the customization of personalized therapeutic regimens has become a prominent and innovative developmental direction[160]. However, technological and cost barriers associated with the identification of individual tumor antigens have limited the advancement of personalized therapies[161]. Nonetheless, in most current tumor surgeries, cancer cells from patients are directly accessible during the resection procedures. Although the direct application of excised tumor cells might risk unnoticed dissemination during surgery[162], owing to their inherent homologous targeting capabilities and abundant tumor antigen content, tumor cell membranes appear to be highly suitable candidate components for tumor vaccines. Based on this concept, researchers harvested tumor cell membranes from primary solid tumors and hybridized them with OMVs extracted from *E. coli*, thus constructing hybrid vesicles termed mTOMV (Fig. 7J)[163]. Due to the strong immunostimulatory properties of OMVs, mTOMVs exhibited enhanced accumulation within the inguinal lymph nodes compared to mT alone and more potently activated both innate and adaptive immune responses (Fig. 7K−M), thereby achieving significant suppression of tumor metastasis and indicating tremendous promise for personalized immunotherapy.

### 5.3. *Strategies for immune checkpoint blockade*

Given the features of OMVs, such as engineering modification capability, nanoscale size for high permeability, and the presence of a lipid bilayer structure for drug carriage, researchers have designed various OMV-based strategies for immune checkpoint blockade beyond simply constructing immune activation and combination therapies. However, it is necessary to first briefly outline certain theoretical aspects regarding immune checkpoint blockade therapy.

Under the current medical standards, tumor therapies mainly focus on direct surgical resection, radiotherapy, chemotherapy, and immunotherapy. Immune checkpoint blockade is a major branch of immunotherapy. In laboratory settings, melanoma is often used to establish cancer models, while in clinical practice, immune checkpoint therapies have already been approved by the U.S. Food and Drug Administration (FDA) for melanoma treatment Owing to their powerful therapeutic potential, numerous checkpoint inhibitors targeting lung cancer, renal cell carcinoma, and other tumors are either undergoing extensive preclinical research or in various stages of clinical trials. Many of the checkpoint inhibitors have already been approved for market use or anticipated to receive approval in the coming years[164]. Currently, the most intensively studied immune checkpoint blockade therapies focus on anti-CTLA-4 and anti-PD-1 antibodies. In addition, emerging targets such as LAG-3, TIM-3, and TIGIT have garnered increasing attention, along with therapeutics targeting the CD47−SIRP$\alpha$ axis, which also play a critical role in modulating and training the innate immune system.

Serving as nanoscale carriers that are highly amenable to surface engineering, OMVs can easily target specific immune regulatory molecules after engineering modifications and further improve the precise targeting of tumor cells based on their intrinsic permeability and enhanced permeability and retention (EPR) effect[13]. OMVs also possess the potential to target immune cells within the tumor microenvironment, such as tumor-associated macrophages and other immune cells. It can bind to their surface and internal PRRs, which further stimulate and modulate intercellular signaling pathways, optimize antigen presentation, and enhance immune activation as well. Through these mechanisms, OMVs can achieve synergistic effects between immune activation and checkpoint blockade in cancer immunotherapy.

As previously mentioned, researchers used *E. coli*, a typical Gram-negative bacterium, as the secretion host and introduced the LyP-1 peptide onto the OMV surface *via* engineering modification prior to OMV secretion, thereby endowing OMVs with the ability to actively recognize and adhere to tumor cells. Subsequently, plasmids carrying the PD-1 gene were introduced and DNA fragments encoding PD-1 were encapsulated within LyP1-OMVs to form the final delivery vehicle, termed LyP1-OMV@PD-1 (Fig. 7N)[13]. Upon recognition and adhesion to tumor cells *via* LyP-1, LyP1-OMV@PD-1 is endocytosed into tumor cells, where the PD-1 gene fragment enters the nucleus, undergoes

transcription and translation into PD-1 mRNA and protein, and is ultimately expressed on the tumor cell surface as a binding site. Since tumor cells inherently express PD-L1 ligands, the newly synthesized PD-1 molecules on the tumor cell surface can bind to the endogenous PD-L1, resulting in a phenomenon termed "self-blockade". Interestingly, this self-blockade process can also occur among different tumor cells, wherein PD-1 molecules expressed on the surface of cell A bind specifically to PD-L1 molecules on the surface of cell B, leading to widespread mutual blockade across the tumor region. As a result, the probability of each tumor cell being recognized by CTLs is significantly reduced, thereby alleviating immune suppression, enhancing CTL-mediated cytotoxicity, and markedly improving overall tumor immunotherapy efficacy.

A more straightforward approach than inducing PD-1/PD-L1 self-blockade is to directly remove PD-L1 from the tumor cell surface, thus blocking the PD-1/PD-L1 signaling axis between T cells and cancer cells. Lysosomes, as endogenous degradation machinery for secreted and membrane proteins, appear to be ideal tools to eliminate surface PD-L1 expression. Existing strategies employing lysosome-targeting chimeras (LYTACs) have shown promising applications in the targeted degradation of extracellular proteins. Researchers have designed a genetically engineered transferrin receptor (TfR)-mediated lysosome-targeting chimera, TfR−LYTAC, which utilizes TfR-mediated endocytosis to internalize into cells and direct PD-L1 for lysosomal degradation[165]. However, the current TfR−LYTAC constructs have short half-lives and poor tumor-targeting capabilities. To address this, a novel delivery platform was developed by fusing TfR−LYTACs onto the surface of bacterial OMVs to form OMV-LYTACs. These engineered OMVs not only achieve targeted degradation of PD-L1 through the LYTAC strategy but also leverage the intrinsic immune activation properties of OMVs to further enhance tumor growth suppression, offering a new pathway for realizing extracellular protein degradation *via* the TfR−LYTAC system[165].

In addition to PD-1/PD-L1 axis-targeted checkpoint inhibitors, OMVs also exhibit significant potential for development in targeting macrophage-associated immune checkpoints such as SIRP$\alpha$. SIRP$\alpha$ is a prototypical immune checkpoint molecule that functions as an immunoregulatory receptor on the cell surface. It is predominantly expressed on various myeloid cells, including macrophages, dendritic cells, and monocytes, and its functions serves exactly as an immune checkpoint molecule similar to PD-1/PD-L1 and CTLA-4. It can specifically recognize ligands on tumor cells and promote immune evasion. Unlike PD-1/PD-L1 and CTLA-4, SIRP$\alpha$ binds to CD47 on tumor cells, and upon engagement, tumor cells emit a "don't eat me" signal, inhibiting macrophage-mediated phagocytosis of cancer cells[166]. Based on this principle, researchers have developed an SIRP$\alpha$-Fc fusion protein to competitively bind CD47. This process blocks the inhibitory signaling that prevents macrophage phagocytosis of tumor cells[145].

Moreover, unlike the PD-1/PD-L1 axis, the CD47−SIRP$\alpha$ pathway is not a target of the adaptive immune response. Given its predominant expression on myeloid cells, it is evident that the CD47−SIRP$\alpha$ interaction is rooted in the innate immune system, thereby offering unique therapeutic potential. By combining the GM-CSF-mediated trained immunity strategy with the SIRP$\alpha$-Fc blockade approach, researchers successfully endowed OMVs with the ability to modulate immune responses both in the bone marrow and within the tumor microenvironment. For the first time developed and validated a novel OMV-based trained immunity-related vaccine, termed TIrV (OMV-SIRP$\alpha$@CaP/GM-CSF)[145]. This novel TIrV vaccine trains the immune system in two stages. The first is centrally trained immunity, where immune memory is passed from hematopoietic stem and progenitor cells in the bone marrow to peripheral monocytes. The second is peripheral trained immunity, where monocytes pass this trained state to local innate immune cells, such as macrophages. Tumor-associated macrophages (TAMs), a major group of immune-suppressive cells in the tumor microenvironment, mainly come from infiltrating monocytes and are considered important targets for trained immunity[149,150]. However, current central and peripheral training strategies have limited the ability to reach or activate macrophages inside the TME, due to strong suppressive signals in tumors. Therefore, local and precise targeting of TAMs is viewed as a more effective and focused antitumor strategy.

The CD47−SIRP$\alpha$ pathway is highly expressed and often overexpressed in many solid tumors. This pathway blocks the ability of macrophages to engulf tumor cells by sending a "Don't eat me" signal through SIRP$\alpha$. In recent years, researchers have developed CD47-blocking antibodies or SIRP$\alpha$-Fc fusion proteins to shut down this pathway. This allows TAMs to regain their ability to phagocytose tumor cells. In addition to promoting direct engulfment of tumor cells, blocking CD47−SIRP$\alpha$ can also enhance immune responses through antibody-dependent cellular phagocytosis (ADCP) mediated by the Fc region. The newly developed TIrV combines GM-CSF, which promotes immune training in the bone marrow, with SIRP$\alpha$-Fc, which activates macrophages in the tumor. This creates a "top-down" immune strategy that links the bone marrow and TME, greatly enhancing the antitumor immune response. It not only boosts the phagocytic ability of TAMs but works together with T-cell activation as well, representing a promising approach that bridges innate immunity and adaptive immune checkpoint therapy[145].

In recent years, researchers have found that in addition to the high expression of PD-L1 and CD47 in the tumor microenvironment, several emerging targets such as TIM-3, LAG-3, and TIGIT also deserve attention[167]. These targets are co-inhibitory receptors involved in immune regulation[168]. They function to suppress the ability of certain immune cells, including NK cells, dendritic cells, macrophages, and especially T cells, to recognize and kill tumor cells. Although no immune checkpoint therapies using OMVs as delivery carriers have been developed for these targets so far, they still hold strong potential for future combination therapies. When it comes to T cells, if they are repeatedly exposed to tumor antigens in the tumor microenvironment, they initially mount an active response and can recognize tumor antigens effectively. However, due to the continuous presentation of antigens by tumor cells, T cells and other immune cells are subjected to prolonged, chronic stimulation[169]. This leads to the persistent activation of immune checkpoint pathways such as TIM-3, LAG-3, and TIGIT[170]. In addition, a variety of immunosuppressive factors, including cytokines like TGF-$\beta$ and IL-10, cells like TAMs and Tregs, may further suppress the antitumor function of immune cells like T cells.

Over time, after prolonged immune suppression and persistent efforts to attack tumor cells, T cells may become exhausted[171]. These exhausted T cells lose their ability to effectively recognize and kill tumor cells. Exhausted T cells can be divided into two types: early exhausted T cells and terminally exhausted T cells[169]. Early exhausted T cells may regain function when treated with immune checkpoint inhibitors such as anti-PD-1 or anti-CTLA-4

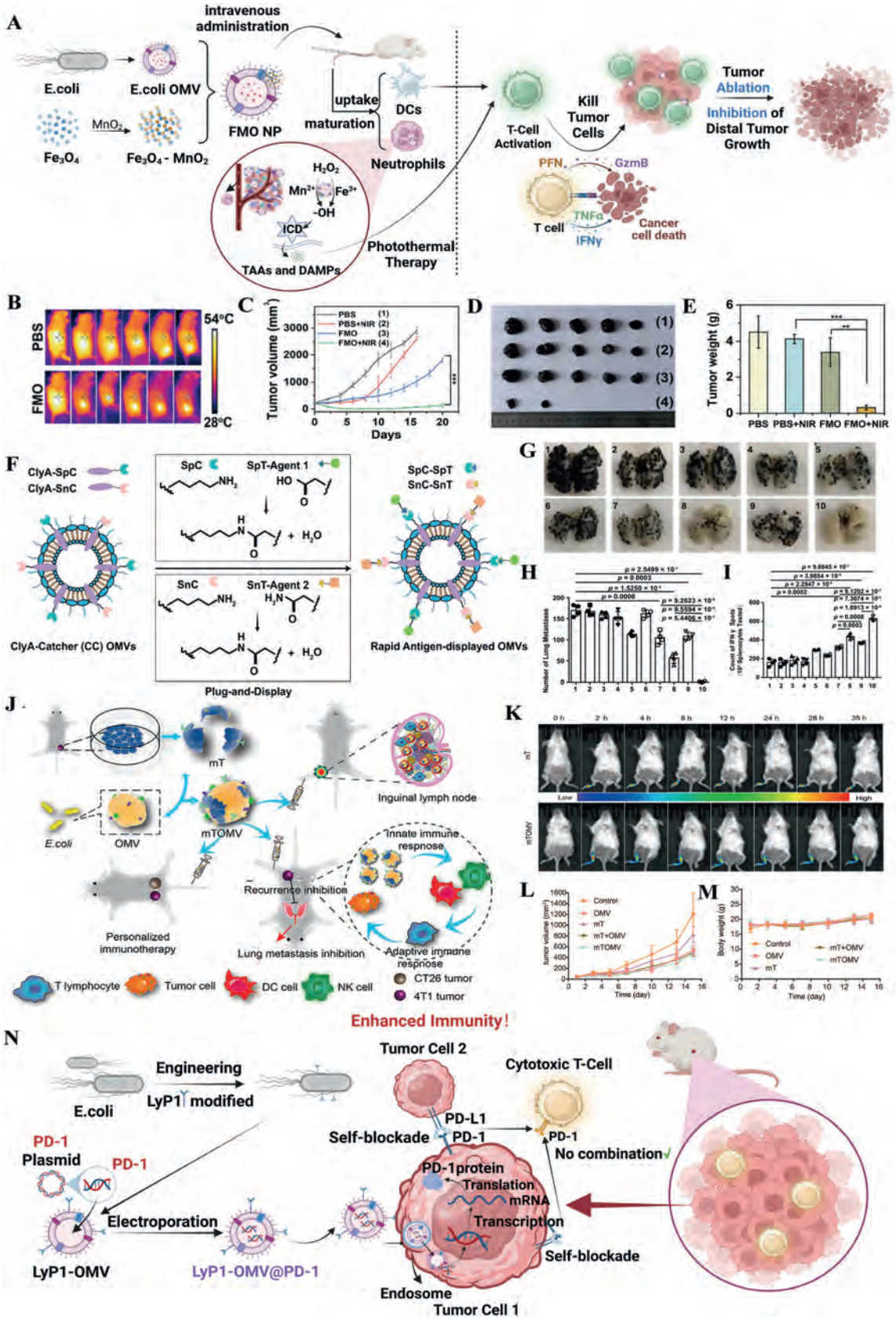



**Figure 7** Graph A−M upon show the multi-functions of OMV in programming adaptive immunity, while graph N shows an exemplification of OMV strategies for immune checkpoint blockade therapies. Bioengineered $Fe_3O_4$-$MnO_2$ (FMO) nanoparticle platforms that can be delivered to target TME *via* neutrophil-mediation. The FMOs can enhance T-cell activation, kill tumor cells and suppress tumor metastasis. (A) Graphical

antibodies. In contrast, terminally exhausted T cells remain nonfunctional even after treatment. This is one of the main reasons why many patients show a poor response to PD-1 or CTLA-4 inhibitors, with their disease continuing to progress despite treatment. To address this issue, researchers have proposed combining classical checkpoint inhibitors with therapies that target "secondary immune checkpoints" such as TIM-3, LAG-3, and TIGIT[167,168]. LAG-3 is induced early during T-cell activation and remains highly expressed under chronic antigen stimulation[172]. It is often co-expressed with PD-1 on tumor-infiltrating T cells and serves as a typical marker of exhaustion. By binding to ligands like MHC-II and FGL1, it suppresses T-cell proliferation. Blocking LAG-3 has been shown to partially restore T cell activity, and clinical trials combining LAG-3 with PD-1 antibodies have demonstrated therapeutic benefits[173]. TIM-3 is more closely associated with terminal T-cell exhaustion. It is co-expressed with PD-1 on $CD8^+$ T cells, Tregs, and dendritic cells under chronic stimulation. Through binding to ligands such as Galectin-9, TIM-3 contributes to T cell apoptosis and immune suppression[174]. Blocking TIM-3 signaling may help recover some cytotoxic activity and shows potential for use in combination therapies, especially in cases resistant to standard immune checkpoint inhibitors[175]. TIGIT primarily functions by competitively binding CD155 (PVR), which inhibits the activation and function of T cells and natural killer cells. It is also highly expressed on Tregs, where it enhances immunosuppressive effects. TIGIT is often co-expressed with PD-1 and LAG-3, forming a complex inhibitory network[176]. Blocking TIGIT has shown promise in treating patients who do not respond to or have developed resistance to immune checkpoint therapies.

These three checkpoints not only mark T cell exhaustion but are also becoming key combination targets in overcoming resistance to anti-PD-1 therapy[168]. A crucial step in achieving effective checkpoint blockade is to ensure precise delivery of antibodies or inhibitors to the tumor site. Taking TIM-3 as an example, conventional antibodies often fail to accumulate effectively in tumors, resulting in widespread systemic blockade of TIM-3[175]. This can trigger autoimmune side effects and worsen T-cell exhaustion. Therefore, a delivery strategy that ensures local concentration is urgently needed. OMVs offer a promising solution due to their natural tendency to accumulate in tumors[13]. By engineering OMVs to carry RNA fragments or antibodies targeting immune checkpoints, a local delivery platform can be constructed to achieve precise inhibition. In addition to delivering blocking agents, OMVs contain PAMPs that can further activate innate immune cells, such as macrophages and dendritic cells[140,142]. This dual action, blocking the immune brakes while pressing the immune accelerator, may significantly enhance the effectiveness of checkpoint inhibitor therapies.

Consequently, as an important class of immune checkpoint blockade delivery carriers, OMVs have significant advantages in activating various immune cells, implementing immune checkpoint blockade strategies, and maintaining immune homeostasis. Combined with their high plasticity for surface engineering, OMVs constitute a critical foundation for the development of next-generation vaccines and immunotherapeutic strategies.

## 6. Clinical translation challenges

### 6.1. *Safety issues*

OMVs originate from Gram-negative bacteria, the outer membrane of which is rich in LPS, the main component of endotoxins. When OMVs enter the body, LPS is released into the cytoplasm, activating pathways such as the TLR4 receptor, triggering a strong innate immune response, and releasing proinflammatory cytokines such as IL-6, TNF-$\alpha$ and TNF-$\beta$[177,178]. It also induces cell pyroptosis through pathways mediated by caspase-11 and the NLRP3 inflammasome[179]. LPS is highly toxic to organisms, and intravenous injection of OMVs containing LPS can lead to severe systemic inflammatory responses, such as cytokine storms, which in turn can cause severe tissue damage, organ failure and even symptoms such as sepsis or toxic shock syndrome[177,180]. According to the literature, the median lethal dose (LD50) of intraperitoneal injection of LPS is approximately 300 μg per mouse[181]. Therefore, the dose of OMVs must be strictly controlled during administration to reduce the incidence of severe adverse reactions. Studies have shown that unmodified OMVs can cause significant toxicity at high doses. For example, mice injected intravenously with 200 μg of OMVs experienced significant weight loss and tissue damage such as liver inflammatory cell infiltration, renal interstitial fibrosis, and colonic mucosal structural disorder, while injection of 200−400 μg of OMVs caused the death of half of the mice[180]. In tumor-bearing mice, administration of $10^{12}$ particles of *E. coli* OMVs led to the death of most mice; $5.0 \times 10^{11}$ particles of OMVs caused the death of half of the mice, while administration of $2.5 \times 10^{11}$ particles of OMVs allowed all mice to survive completely[19]. Therefore, removing LPS from OMVs can reduce side effects, improve safety in biomedical applications, and help avoid non-specific damage to target cells by OMVs. Various strategies have been adopted to reduce the endotoxic effects of OMVs. By using surface modification techniques, such as coating

abstract showing the preparation of FMO NPs and their therapeutic mechanism. (B) Infrared thermographic images of mice in the PBS + NIR and FMO + NIR groups. (C) Tumor volume changes in mice during treatment. (D) Representative images of B16F10 primary tumors. (E) Average tumor weights on the 20th day[121]. Adapted with permission from Ref. 121. Copyright © 2023 American Chemical Society. (F) Schematic illustration of the (CC) OMVs system for antigen display. SpyCatcher (SpC) and SnoopCatcher (SnC) were expressed as fusion proteins with ClyA (ClyA-Catcher, CC) on the OMVs surface. SpyTag (SpT) or SnoopTag (SnT)-labeled antigens bind to CC OMVs through isopeptide bond formation between the tag and the catcher. (G) Lungs collected on experimental Day 17 at the end of the treatment period and photographed. (H) Countertumor nodules. (I) Quantitative data derived from the ELISPOT assays that shows IFN-$\gamma$ secretion by splenocytes isolated from the various treatment groups[92]. Adapted with permission from Ref. 92. Copyright © 2021 Springer Nature. (J) Schematic illustration of the hybrid vesicles from the bacterial outer membrane and tumor cell membrane to enhance the innate immune response for personalized immunotherapy. (K) *In vivo* fluorescence images at various time points after intraplantar injection into the right hind paw of mice with Cy 5.5 labeled mT and mTOMV, respectively. (L) Relative volumes of CT26 tumors ($n = 7$). (M) Body weight of mice during the experimental period ($n = 7$)[163]. Adapted with permission from Ref. 163. Copyright © 2021 American Chemical Society. (N) The engineered delivery system LyP1-OMV@PD-1 induced PD-1 expression on tumor cells, which bound to endogenous PD-L1, triggered self-blockade between tumor cells and improved antitumor immune responses. Schematics in A, N were created with BioRender.com.

with calcium phosphate (CaP) shells, a temporary "shielding layer" can be formed on the surface of OMVs, reducing their immunogenicity and inflammatory response in the systemic circulation. When OMVs reach the target, the calcium phosphate shell degrades in the acidic tumor microenvironment, releasing OMVs and achieving targeted delivery[22]. However, the biomineralization process of calcium phosphate shells is difficult to precisely control, resulting in significant heterogeneity in the size and structure of the modified OMVs. Additionally, the pH of the tumor microenvironment may vary depending on the location and stage, leading to an unpredictable release of calcium phosphate shells. Furthermore, the implementation of surface modification techniques requires complex processes, increases production costs, and limits clinical translation. By modifying the surface of OMVs with PEG, their toxicity can be significantly reduced. The modified OMVs, even at a dose of 800 μg exhibit good biocompatibility[180]. Although PEG modification has advantages in the surface modification of OMVs, it also has some limitations, such as the potential for antibody-dependent clearance effects with long-term use, leading to systemic inflammation[182]. PEG-modified OMVs may have insufficient responsiveness and release efficiency in specific environments (such as the tumor microenvironment). To reduce the risks related to LPS, researchers are actively exploring the use of genetic engineering technology to modify bacteria in order to reduce or remove the LPS content in OMVs. For example, the use of genetic engineering technology to remove or modify the structure of LPS (*e.g.*, knocking out *msbB* (*lpxM* ) and *pagP* genes to leave only the pentaacylated form of LPS) is also considered a viable method for reducing the toxicity of OMVs[183]. In addition, the production of OMVs using strains with low endotoxin toxicity is also regarded as a promising research direction[184].

To reduce the endotoxin activity of OMV, sodium deoxycholate was used to replace the outer membrane LPS during OMV preparation, resulting in dOMV yields 3.48 times higher than OMVs, significantly improving the safety of the formulation[185]. Nevertheless, the process of preparing dOMV using sodium deoxycholate is relatively complex, which not only increases production costs but also consumes more time. During processing, lysis of bacterial cells causes cytoplasmic protein contaminants to mix into dOMVs, which may increase the potential toxicity risk. In addition, the processed dOMVs exhibit aggregation phenomena, which cause the loss of negatively charged surface antigens, and this can also affect the immunogenicity and function of dOMVs[185,186]. By dispersing OMVs in hydrogels to regulate their release rate, systemic inflammatory storms can be avoided, significantly improving their safety[187]. The purity of OMVs also affects their toxicity. By optimizing the separation and purification process of OMVs, the content of LPS can be effectively reduced. For instance, the use of Optiprep density gradient medium (10%−60%) for OMVs isolation can effectively distinguish OMVs from other membrane fragments, thereby obtaining high-purity OMV samples and significantly reducing LPS contamination and its toxicity[188]. Although researchers have explored various methods to reduce the toxicity of OMVs and enhance their safety, the clinical application safety of OMVs requires further demonstration through extensive research and experimentation. Moreover, further optimization of the preparation process and modification strategies for OMVs remain a focus of future research.

As potential carriers for biological therapy and drug delivery, evaluating the safety of OMVs is of vital importance. Studies have shown that the safety of OMVs is closely related to their dosage. Specifically, high doses of OMVs have shown significant toxicity in mouse experiments, causing the death of more than half of the experimental animals. In contrast, low doses of OMVs showed better tolerance[19]. This finding emphasizes the need for precise dose control when using OMVs to avoid potential toxic reactions. By optimizing the dose and frequency of administration, toxicity can be minimized, while ensuring efficacy. In addition to the dose, the safety assessment of OMVs also needs to focus on LPS. As the main inflammatory component of OMVs, LPS may induce systemic immune responses and toxicity. Reports indicate that the LPS content in OMVs is approximately 42.53 ng/mg[125], suggesting that when designing vaccines or treating diseases in clinical translation, the safety of LPS in OMVs must be fully considered.

As a drug delivery and therapeutic carrier, the safety of OMVs is influenced by various factors such as dose, endotoxin content, cytotoxicity, and immunogenicity. To promote the clinical application of OMVs, researchers are improving the safety of OMVs through dose optimization, genetic engineering modification, surface modification, and long-term experimental verification, laying a solid foundation for their clinical applications.

### 6.2. *Industrialization and cost challenges*

Gram-negative bacteria secrete a small number of OMVs under natural growth conditions. Wild-type strains, such as *E. coli*, naturally secrete OMVs at a yield of approximately 0.85 mg/L. There are differences in size and composition, which make quality control difficult, affect their homogeneity and therapeutic effects, thus making it difficult to meet the needs of large-scale clinical applications[189,190]. To improve the yield and quality of OMVs, researchers have been exploring multiple directions and striving for breakthroughs. When using basic media, such as LB medium, the yield of OMVs is usually low, especially in the absence of specific nutrients. By adding inducers (such as LysP53) and optimizing the preparation process, the yield of OMVs can be increased, but the induction conditions (including concentration and duration) and process parameters must be strictly controlled to ensure stability[186]. Over addition of inducers can inhibit the secretion of OMVs, and certain induction methods may lead to an increase in endotoxin content, thereby increasing the difficulty of subsequent purification[191]. Therefore, the optimization of these conditions requires precise control, which would otherwise limit the yield. Moreover, for clinical translation, a standardized inducer addition process is essential to ensure batch-to-batch reproducibility, and precise technical control of the induction conditions during large-scale fermentation is costly and difficult to achieve[192]. Currently, genetic engineering technology has matured, and researchers are gradually focusing on genetic engineering techniques to obtain strains with high OMV production. Kim et al.[21] used genetic engineering to knock out the *msbB* gene encoding lipid A acyltransferase in *E. coli*, which not only reduces LPS toxicity but also induces high OMV production[21]. However, genetically engineering bacteria to improve OMV production is complex and time-consuming, and different strains require different modification strategies, limiting their clinical translation applications[186]. Providing conditional stimuli during bacterial cultivation can also increase OMV production. Studies have shown that antibiotic treatment can cause hypervesiculation in *A. baumannii*, resulting in an OMV yield of 1.3−1.5 times that of the untreated group[189]. In addition, conditions such as high temperature, hypoxia, and nutrient deficiency can also stimulate bacteria

to secrete more OMVs due to stress, the stability and repeatability of these methods still need to be verified by a large number of experiments[193], and stress conditions may cause changes in the lipid composition of OMVs, thereby affecting their stability and interaction with host cells[118]. In addition, OMVs formed under stressful environments usually contain stress response proteins or toxic factors, which have stronger immune activation capabilities and can more effectively activate DCs and promote the production of cytokines. Some OMVs produced under specific stress conditions may exhibit high cytotoxicity, especially at high concentrations, which may pose safety risks for treatment[186,194]. Furthermore, dynamic changes in pH during bacterial cultivation are important factors. If the pH value deviates from the ideal range (such as pH 7.2), it inhibits bacterial metabolic activity and limits the production of OMVs[195]. At the same time, the control of cultivation time also has a significant impact on OMV yield. OMVs are mainly produced during the exponential growth phase. Too short a cultivation time cannot accumulate enough OMVs, and too long a cultivation time may cause bacteria to enter the stationary phase, reducing OMV yield[191,195]. In summary, the production of OMVs can be effectively increased through induction, genetic engineering modification, and conditional stress stimulation. However, these methods still face many limitations and challenges, which restrict the potential of OMVs in clinical applications to some extent. The preparation of OMV involves multiple steps, including bacterial culture, separation, purification, and characterization of OMVs. Currently, commonly used separation methods include ultracentrifugation, density gradient centrifugation, and membrane filtration technology. Ultracentrifugation is the most widely used technique for OMV purification. This technique utilizes centrifugal forces ranging from 60,000 to 300,000$\times g$ to precipitate and separate uniformly sized OMVs, thereby achieving high-purity separation. Its wide applicability makes it a traditional and classic method for extracting OMVs[16]. Density gradient centrifugation establishes a continuous sucrose or OptiPrep gradient, effectively separating OMVs from other cellular components based on density differences and can improve purification efficiency when combined with ultracentrifugation[16,196]. However, the time and financial costs associated with these methods are relatively high, which, to some extent, limits their widespread application in the field of industrialization[193]. Compared to ultracentrifugation and other techniques, membrane filtration technology has the advantages of simple operation and short process time, can effectively maintain the integrity of OMVs, reduce losses due to aggregation or uneven size, and is suitable for the purification process after large-scale fermentation production, making it suitable for scale-up production. However, membrane filtration technology requires precise control of the pore size and transmembrane pressure. In addition, the adsorption or aggregation effect of the OMV membrane may cause filter membrane blockage, and it is difficult to separate contaminants with a size similar to OMVs, limiting the purity of OMVs[197]. Other separation and purification methods, such as using the ExoBacteria™ OMV Isolation Kit to extract and purify OMVs, simplify the process, but the yield is low[193]. Bacterial cell lysis may occur during the extraction of OMVs using detergents (such as sodium deoxycholate), causing contamination by cytoplasmic proteins and reducing the purity of OMVs, which may result in the loss of negatively charged surface antigens, thereby affecting the function of OMVs[186]. Therefore, the existing OMV extraction and purification methods face core issues, including low yield, complex processes, expensive equipment, high heterogeneity, and endotoxin risk. These problems directly affect the stability, safety, and repeatability of OMV products and pose multiple obstacles to clinical translation.

The quality control of OMVs is also a focal point of research. Generally, the morphology and size distribution of OMVs are evaluated using TEM and SEM. In addition, the presence of specific antigens or proteins in OMVs can be verified through Western Blotting. However, there are numerous types of bacteria, and there are significant differences between OMVs secreted by different strains[186]. Interestingly, even the same strain showed significant differences in the composition of the secreted OMVs under different culture conditions. For example, compared to OMVs secreted without antibiotic treatment, OMVs induced by the antibiotic levofloxacin in drug-resistant *A. baumannii* showed 361 upregulated and 361 downregulated proteins, causing significant changes in protein composition[189]. LysP53 stimulates the production of OMVs (LOMVs) after interacting with *A. baumannii*, *E. coli*, and *Salmonella*. Compared to naturally produced OMVs (nOMVs), LOMVs contain a significantly increased number of cytoplasmic and cytoplasmic membrane proteins but fewer periplasmic and extracellular proteins, showing better homogeneity, higher protein yield, lower endotoxin content, and lower cytotoxicity[186]. In addition, there were differences in the quality of OMVs secreted by the same strain obtained using different extraction methods. Treatment of strains with sodium deoxycholate can yield more OMVs (dOMVs), but it causes the loss of negatively charged surface antigens, resulting in differences in protein components compared to natural OMVs (nOMVs)[185,186]. Therefore, even the OMV components secreted by the same bacterial strain can exhibit significant variation due to the influence of various conditions. Currently, there is no effective method to precisely control the secretion amount and consistency of OMVs, which limits their large-scale application.

### 6.3. *Challenges of vaccine development*

Currently, vaccines based on OMV technology have made significant progress in the field of clinical development, with some candidate vaccines approved or undergoing clinical trials. 4CMenB (trade name: Bexsero®) is a multi-component protein vaccine developed by GlaxoSmithKline (GSK) that specifically targets group *B meningococcal* (*MenB*) bacteria. The vaccine contains three recombinant protein antigens—Neisseria adhesin A, Neisserial heparin-binding antigen, Factor H-binding protein, and detoxified OMVs (dOMV, mainly containing PorA P1.4 antigen) from the MenB epidemic strain in New Zealand (NZ98/254)[198]. It has successfully completed extensive clinical trials, confirming its immunogenicity and safety profile, and was first approved for marketing in the EU in 2013, becoming a milestone in OMV vaccine development[199]. The product utilizes the inherent adjuvanticity of OMVs, displaying natural outer membrane antigens and PAMPs, triggering a strong protective immune response without the need for large amounts of exogenous adjuvants, and plays a key role in curbing the incidence of meningococcal infections in populations of different age groups in various countries[199]. Given that multivalent vaccines can provide more robust and broad-spectrum anti-infection protection, the MenZB vaccine, which targets a single strain, has been replaced by the more comprehensive multi-antigen vaccine 4CMenB owing to its limited protection range and shorter duration of protection[117]. Interestingly, clinical trials have proven that the 4CMenB vaccine can also provide a certain degree of protection against infections

caused by *N. gonorrhoeae*, which has antigenic homology with meningococcal bacteria[200]. In addition, numerous OMV-based vaccines are currently in the clinical development stage. Over the past few decades, serotype B meningococcal disease has been a major public health issue in Brazil. To address this challenge, the first indigenous OMV vaccine targeting the prevalent group B meningococcal in Brazil was developed. This vaccine covers 70% of the prevalent strains, and its main components include detergent-treated outer membrane vesicles and detoxified endotoxin (dLOS), supplemented with aluminum adjuvant, successfully achieving a balance between safety and immunogenicity. In the phase I clinical trial, 30 healthy adult volunteers were recruited and divided into three dose groups (12.5, 25, and 50 μg), each receiving three doses of the vaccine. The results showed that the vaccine could effectively induce an immune response, with adverse reactions mainly presenting as mild local reactions (such as pain and redness), and no serious adverse events occurred, demonstrating good safety[201]. As a new generation of gene-engineered membrane antigen platform, the development of generalized modules for membrane antigens (GMMA) vaccines has attracted much attention. The first GMMA vaccine to enter Phase IIa, 1790GAHB, is a novel vaccine against Shigella sonnei. This vaccine uses a detoxified strain that produces penta-acylated LPS, effectively reducing endotoxin activity and supporting intramuscular, intranasal, and intradermal injections. In animal model experiments, 1790GAHB demonstrated good tolerance, with no significant toxicity observed in the rabbit model. The results of the phase I clinical trial indicated that the vaccine had a dose-dependent immune response, and after booster vaccination in adults, the serum bactericidal antibody levels significantly increased[202]. Given that 1790GAHB only targets *S. sonnei*, a 4-valent GMMA vaccine (altSonflex1-2-3) was developed, covering *S. sonnei* and *S. flexneri* 1b, 2a, and 3a. Each dose contains 15 μg of O antigen. The safety and immunogenicity of this vaccine were evaluated in a healthy European adult population through intramuscular injection. The results showed that the safety data of altSonflex1-2-3 vaccination were consistent with those of previous GMMA vaccines (such as 1790GAHB), and no serious adverse events occurred. Furthermore, all four serotypes significantly induced antibody levels, demonstrating the broad-spectrum coverage ability of the multivalent GMMA vaccine[203]. The clinical success of these vaccines has provided a model for further OMV-based vaccine development, particularly for other challenging bacterial pathogens.

Despite the encouraging results of OMV vaccine development, most studies are still in the preclinical research stage. According to the latest data from The Clinical Trials Database (www.clinicaltrials.gov), which has currently listed approved OMV vaccines for clinical trials, mainly focuses on a limited number of disease areas. Specifically, most approved OMV formulations are only vaccines to prevent and treat meningococcal infections, with a small amount of clinical trial information on *N. gonorrhoeae* OMV vaccines. In addition, due to issues such as the natural toxicity of OMVs, production limitations, and difficulties in quality control and standardization, the development of vaccines is challenging, with most OMV vaccine projects stalled at preclinical or early clinical stages due to technical bottlenecks or commercial considerations. For example, the dysentery vaccine (1790GAHB), targeting *S. sonnei*, owing to its focus on a single antigen and a low dose in clinical trials, failed to elicit a sufficient protective immune response. In the controlled human infection model, the vaccine did not show significant protective effects; therefore, after completing Phase II clinical trials, it was not able to proceed[204]. The genome of *Yersinia pestis* is rich in insertion sequences (IS), which lead to frequent genetic recombination and deletion, thereby causing genetic instability and severely affecting the production efficiency of OMVs. A dose of up to 400 μg of OMVs is required to successfully encapsulate less than 10 μg of LcrV target antigen. This makes it difficult to induce an effective immune response, resulting in vaccine development process unable to advance smoothly to the clinical trial stage[205]. The glyOMV vaccine developed against *Streptococcus pneumoniae* type 14, while inducing IgG antibody levels in mice comparable to those of the PCV13 vaccine, has encountered bottlenecks in development due to technical limitations. The consistency of glycosylation modifications during scale-up production is difficult to ensure, leading to significant batch-to-batch differences[117]. Although OMVs as a novel vaccine platform have shown good potential in preclinical studies, they still face many challenges when transitioning to the clinical trial stage.

## 7. Future development

OMVs have demonstrated tremendous application potential in the biomedical field, particularly in vaccine development, tumor therapy, and drug delivery, owing to their unique biological properties such as natural biocompatibility, functional diversity, and ease of modification. Looking ahead, the author believes that the development of OMV will primarily focus on the following aspects to establish an efficient and safe disease treatment platform. First, as a highly promising tumor therapy platform, OMVs are expected to facilitate the development of more tumor treatment strategies based on their characteristics. Through advanced genetic engineering and chemical modification techniques, OMV can be endowed with enhanced functionality and selectivity, enabling precise tumor targeting and controlled-release delivery. Furthermore, integrating OMV with therapeutic approaches such as photothermal therapy, antigen presentation, and drug delivery may lead to the creation of a multifunctional tumor treatment platform, thereby exerting more potent antitumor effects. Second, owing to its excellent adjuvant properties and heterologous antigen display capability, OMVs have become a research hotspot in vaccine development. However, current OMV administration methods are relatively limited, primarily relying on injections. In the future, by leveraging OMV's natural mucosal penetration ability novel delivery routes, such as oral and intranasal administration, could be explored and developed. This would not only improve vaccination efficiency but also significantly enhance administrative compliance and patient acceptance. Additionally, to further optimize OMV production, stability, and homogeneity, future research could focus on genetic engineering and screening bacteria to obtain high-yield OMV strains. Concurrently, standardized cultivation conditions should be established through extensive experimental optimization, fermentation processes should be standardized, and precise quality control techniques should be employed to evaluate and refine the OMV formulations. These efforts would lay the foundation for the standardized and large-scale production of OMV. To reduce the immunogenicity of OMVs and enhance biosafety, future studies could employ various modification strategies (*e.g.*, genetic engineering and chemical modification) to lower LPS content, thereby improving administration safety. This would not only expand OMV's applications in biomedicine but also provide patients with safer and more

effective treatment options. Finally, it is worth noting that OMV's applications in disease treatment will continue to diversify. Beyond traditional vaccine development and tumor therapy, OMVs are expected to play a significant role in the treatment of inflammatory diseases, detoxification, and other fields. For instance, preliminary success has been achieved with experimental OMV-based treatments for inflammatory bowel disease[109,127]. With ongoing research and technological advancements, OMV may find broader applications in disease models, such as infectious diseases, autoimmune disorders, and metabolic diseases.

## 8. Conclusions

Outer membrane vesicles have emerged as a versatile and biologically inspired nanoplatform with broad therapeutic potential. Derived from Gram-negative bacteria, OMVs inherently integrate cargo delivery and immune modulation within a single nanoscale structure, offering advantages that are difficult to replicate using synthetic nanocarriers. Their natural membrane composition, capacity for genetic and chemical engineering, and ability to interact efficiently with host cells position OMVs as promising tools for applications ranging from cancer therapy and vaccination to gene delivery and immune regulation.

Recent advances in OMV engineering have significantly expanded their functional repertoire. Through genetic modification of parental strains, surface functionalization, and hybrid nanomaterial integration, OMVs can be rationally designed to achieve improved safety, targeting specificity, and therapeutic efficacy. These strategies enable precise modulation of OMV immunogenicity and biodistribution, facilitating their use as intelligent delivery systems rather than passive carriers. In particular, the integration of drug delivery with immune activation highlights the unique potential of OMVs to address complex diseases that require multimodal therapeutic approaches.

Despite these encouraging developments, several challenges must be overcome to enable clinical translation. Safety concerns related to endotoxin activity, heterogeneity in vesicle composition, limitations in large-scale manufacturing, and the absence of standardized quality control frameworks remain significant barriers. Addressing these issues will require coordinated efforts across disciplines, including microbiology, immunology, pharmaceutical sciences, and regulatory science. Importantly, future progress will depend not only on technological innovation but also on a deeper mechanistic understanding of OMV−host interactions *in vivo*.

In summary, OMVs represent a dynamic and adaptable platform at the interface of biology and nanotechnology. Continued refinement of engineering strategies, combined with rigorous evaluation of safety and reproducibility, is expected to accelerate their transition from experimental systems to clinically relevant therapeutics. As research in this field matures, OMVs are poised to play an increasingly important role in the development of next-generation precision medicine.

## Acknowledgments

This work was supported by National Natural Science Foundation of China (Nos. 82504728 and 82373274) and Science and Technology Commission of Shanghai Municipality (Nos. 23S11901300 and 24430710500, China), Postdoctoral Fellowship Program of China Postdoctoral Science Foundation (No. GZC20241240).

## Author contributions

Jinlong Yang, Tang Wang, Yuekai Zheng wrote the original draft. Jinlong Yang, Jianping Qi, Zhongjian Chen, Yuning Wei, Juan Tao conceptualized and supervised the article. Jinlong Yang, Jianping Qi, Zhongjian Chen, Tang Wang, Yuekai Zheng conceptualized, supervised, wrote, revised the article, and visualized the figures.

## Declaration of generative AI and AI-assisted technologies

The authors used generative artificial intelligence tools for language polishing and grammatical refinement during manuscript preparation. The authors carefully reviewed and edited the content to ensure scientific accuracy and take full responsibility for the integrity of the work.

## Conflicts of interest

The authors declare no conflicts of interest.

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