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Review paper

Recent advances in bacterial outer membrane vesicles: Effects on the immune system, mechanisms and their usage for tumor treatment

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ABSTRACT

Tumor treatment remains a significant medical challenge, with many traditional therapies causing notable side effects. Recent research has led to the development of immunotherapy, which offers numerous advantages. Bacteria inherently possess motility, allowing them to preferentially colonize tumors and modulate the tumor immune microenvironment, thus influencing the efficacy of immunotherapy. Bacterial outer membrane vesicles (OMVs) secreted by gram-negative bacteria are nanoscale lipid bilayer structures rich in bacterial antigens, pathogen-associated molecular patterns (PAMPs), various proteins, and vesicle structures. These features allow OMVs to stimulate immune system activation, generate immune responses, and serve as efficient drug delivery vehicles. This dual capability enhances the effectiveness of immunotherapy combined with chemotherapy or phototherapy, thereby improving anticancer drug efficacy. Current research has concentrated on engineering OMVs to enhance production yield, minimize cytotoxicity, and improve the safety and efficacy of treatments. Consequently, OMVs hold great promise for applications in tumor immunotherapy, tumor vaccine development, and drug delivery. This article provides an overview of the structural composition and immune mechanisms of OMVs, details various OMVs modification strategies, and reviews the progress in using OMVs for tumor treatment and their anti-tumor mechanisms. Additionally, it discusses the challenges faced in translating OMV-based anti-tumor therapies into clinical practice, aiming to provide a comprehensive understanding of OMVs' potential for in-depth research and clinical application.

1. Introduction

Cancer is one of the diseases with the highest mortality rate today, and overcoming it will greatly promote the extension of human life expectancy. Due to the high heterogeneity of tumors coupled with their propensity for recurrence and metastasis, the treatment has always been a challenge in the medical field. Several clinical approaches are currently being employed for the treatment of cancer, (such as surgery [1–3], radiation therapy [4–6], chemotherapy [7,8], and targeted therapy [9–11]), and each have their own advantages and disadvantages. Despite substantial efforts in global basic research, drug development, and clinical trials are conducted globally every year to improve cancer treatment, the inherent challenge of anti-tumor drugs causing toxicity to normal tissues remains an unavoidable concern. It has been noted that bacteria have natural invasiveness, tumor targeting ability, and cytotoxicity, leading to their extensive use in the biomedical field, and some strains have even progressed to the clinical trial stage [12–14]. However, the development and application of bacterial therapy as therapeutic drugs face limitations due to factors such as the unclear mechanism of action, unsuitability of plasmids for expressing therapeutic mammalian proteins in clinical research, challenges in controlling effective doses, and the potential toxicity to normal tissues [15]. In recent years, as research on the utilization of bacteria for anti-tumor therapy has advanced, researchers have found that outer membrane vesicles (OMVs) produced by Gram-negative bacteria exhibit anti-tumor effects similar to their source bacteria. Moreover, due to the lack of bacterial reproductive and replication ability, OMVs

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demonstrate enhanced biosafety compared to bacteria when applied to tumor therapy [16]. OMVs are spherical bilayer membrane nanostructures formed through programmed spontaneous secretion from the extracellular membrane of Gram-negative bacteria, typically having a diameter ranging from 20 to 250 nm. The discovery of OMVs dates back to 1967 when Chatterjee et al. [17] first reported their observation during the study of the cell wall structure of Vibrio cholerae *in vitro*. Subsequent decades of research have revealed that almost all Gram-negative bacteria, certain Gram-positive bacteria, and some archaea have the capacity to secrete OMVs during natural growth, as shown in Table 1 [18–38].

Many studies have shown that OMVs are a communication tool between bacteria and host cells, and bacteria regulate cell function by secreting OMVs to deliver active substances to the host [39,40]. Based on the unique structure and function of OMVs, they can be designed for the treatment of various diseases. OMVs derived from bacterial outer membranes contain the same antigens as parent bacteria and various pathogen-associated molecular patterns (PAMPs) [41–43]. Antigen proteins and PAMPs are taken up by cells and presented to antigen-presenting cells (APCs), stimulating the body to produce antigen-specific immune responses [44]. In addition, the presence of multiple antigenic determinant enables OMVs to regulate or enhance the body's antigen-specific immune response. In addition, OMVs are nanoscale vesicles with enhanced permeability and retention (EPR) effects, making them easier to transmit and penetrate tissues through the bloodstream [45]. They can quickly penetrate tumor tissue and accumulate at the tumor site. The various antigenic proteins and PAMPs they contain can simultaneously trigger robust anti-tumor immune responses, highlighting their potential for anti-tumor therapy [46]. As a result, natural OMVs or engineered OMVs can be explored for the development of vaccines [41,47–49]. Moreover, direct modification of OMVs or genetic engineering modification of parental bacteria can yield OMVs modified with targeted ligand. This allows for cell-specific targeting and enrichment, mitigating potential side effects on normal tissues during treatment [50]. The vesicle structure of OMVs can be developed as drug delivery carriers, and drugs can be covalently attached to the surface of OMVs or encapsulated inside OMVs, which can increase drug utilization and reduce toxic side effects, as shown in Fig. 1 [51]. Overall, the biological and physicochemical properties of OMVs render them highly promising for tumor treatment.

2. Structure and immune system activating mechanism of OMVs

2.1. Structure of OMVs

OMVs are spherical nanocapsules composed of various immuno-stimulatory components in a nanoscale lipid bilayer, typically with a particle size of 20–250 nm [52]. They mainly contain cellular components such as bacterial outer membrane and periplasmic, encompassing membrane lipids, membrane proteins, lipopolysaccharides (LPS), peptidoglycans, secreted proteins, and virulence factors. LPS are the most important component of the OMVs bilayer membrane; the pivotal component of the OMVs bilayer membrane is LPS, and it may also contain intracellular elements such as intracellular proteins, DNA, RNA, enzymes, metabolites, and signaling molecules, most of which are PAMPs and pathogen specific antigens, which can activate the host's innate and adaptive immune responses [53–56]. The lipids of OMVs primarily consist of phospholipids from the outer membrane of Gram-negative bacteria, and LPS sourced from the cell wall of Gram-negative bacteria, serving as a vital component of PAMPs [57]. Most of the proteins in OMVs are virulence-associated proteins, as well as some pore proteins and transmembrane channel proteins that transport sugars, amino acids, and ions [51]. Due to their unique structure and biological properties, OMVs are widely recognized by scientists as one of the immune stimulators for candidate vaccines and drug delivery vehicle [58–60].

Table 1
Bacteria that have been reported to produce outer membrane vesicles (OMVs).

Bacterial species	Type	Refs.
Escherichia coli	Gram-negative	[18,19]
Helicobacter pylori	Gram-negative	[20–22]
Salmonella typhimurium	Gram-negative	[23,24]
Pseudomonas aeruginosa	Gram-negative	[25,26]
Borrelia burgdorferi	Gram-negative	[27]
Neisseria gonorrhoeae	Gram-negative	[28]
Shigella flexneri	Gram-negative	[29]
Staphylococcus aureus	Gram-positive	[30–33]
Bacillus subtilis	Gram-positive	[33–35]
Bacillus anthracis	Gram-positive	[33,35]
Bacillus cereus	Gram-positive	[33–35]
Mycobacterium ulcerans	Gram-positive	[33,36]
Archaea	Archaea	[37,38]

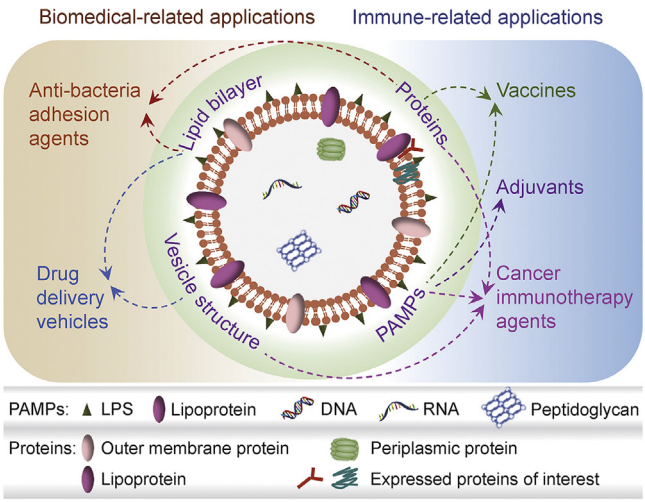

Fig. 1. The composition of outer membrane vesicles (OMVs) and its applications in the biomedical. Reprint with permission from Ref. [51]. PAMPs: pathogen-associated molecular patterns; LPS: lipopolysaccharides.

2.2. Immune mechanism of OMVs

The normal immune system of the body is capable of recognizing and eliminating tumor cells. However, tumor cells can disrupt this inherent anti-tumor immunity through immune escape mechanism, making it impossible to control tumor growth. Immunotherapy for tumors mainly involves reactivating the body's anti-tumor immunity to effectively eliminate tumor cells [61]. Gram-negative bacteria continuously release OMVs during their natural growth process, which contains a large amount of substances from the outer membrane and periplasm of the parent bacteria. These key antigen components within OMVs antigen components can induce protective immune responses. The high immunogenicity of OMVs makes it an excellent preparation for stimulating natural immunity and serving as a vaccine carrier [62]. The abundant PAMPs in OMVs will trigger a series of downstream immune responses, thereby activating the immune system.

OMVs can regulate different immune cells and regulate the body's immune system, as shown in Table 2 [63–71]. For neutrophils, they play an important role in the immune response induced

Table 2
The immune mechanism of outer membrane vesicles (OMVs) on different immune cells.

Related immune cells	Cellular function	Immune mechanism	Refs.
Vascular endothelial cell	Presenting antigens to T cells, expressing adhesion factors and cytokines, and participating in immune responses	Releasing IL-8/CXCL1 to recruit neutrophils	[63]
Neutrophils	Phagocytosis of pathogens and tissue-specific immunity	Releasing pro-inflammatory cytokines (such as TNF-α, IL-1β) and chemokines	[64]
Macrophage	Immune regulation, phagocytosis, and antigen presentation	Inducing polarization of macrophages from M2 to M1 Inducing pro-inflammatory response and promoting macrophage secretion of IL-6, IL-8, TNF-α, and IL-1β Triggering inflammatory reactions	[65–68]
Dendritic cells	Initiating, regulating, and maintaining immune responses	Inducing DC to secrete pro-inflammatory factors TNF-α and IL-12 Promoting the expression of MHC-II and CD86 in DC Activating DC enhances the immune response of B cells and T cells Activating antigen-specific T cells	[69–71]

IL: interleukin; CXCL1: C-X-C motif chemokine ligand 1; TNF-α: tumor necrosis factor α; MHC: major histocompatibility complex.

by OMVs. Under the stimulation of OMVs, vascular endothelial cells can recruit neutrophils by releasing interleukin (IL)-8/C-X-C motif chemokine ligand 1 (CXCL1) in a Toll like receptor 4 (TLR4) and NF-κB dependent manner [63]. In addition, neutrophils can be stimulated by OMVs to release pro-inflammatory cytokines (such as tumor necrosis factor α (TNF-α), IL-1β) and chemokines, leading to inflammation [64]. These studies indicate that OMVs carrying a large amount of PAMPs can recruit and activate neutrophils to induce immune responses. For macrophages, their surface Toll like receptor 2 (TLR2) receptors can be activated by OMVs to induce effective pro-inflammatory responses [56,65,66]. After being engulfed by macrophages, OMVs can activate the intracellular pattern recognition receptors (PRRs) receptors of macrophages, triggering an inflammatory response. It has been found that bacterial OMVs from different sources can activate the inflammatory vesicle signaling pathway in macrophages and induce the release of inflammatory factors [66–68]. For dendritic cells (DCs), they are the central cells that initiate, regulate, and maintain immune responses, and can activate initial T cells. Research has found that OMVs can induce the secretion of pro-inflammatory factors TNF-α and IL-12 in DCs, while promoting the high expression of antigen presenting molecule major histocompatibility complex (MHC)-II and co-stimulatory molecule CD86 in DCs. In addition, OMVs can enhance the antigen-specific immune response of B and T cells by activating DCs [69], and the activation of OMVs on DC cells mainly depends on the activation of the TLR4 pathway in DC cells [70]. PAMPs on OMVs can also activate TLR, Nod like receptors (NLR), and C-type lectin receptors (CLR). These receptors can modulate the release of cytokines, elevate the expression of co stimulatory molecules (such as CD40 and CD80), and increase the upregulation of DC adhesion to integrins (such as CD11c) during antigen presentation, activating both innate and acquired immune systems [56,71].

2.3. The advantages and disadvantages of OMVs

Due to their adaptability and flexibility, OMVs have been proven to be an interesting drug delivery method for treating cancer and infectious diseases [72–74]. The potential mechanism by which OMVs trigger immune responses is to protect them from the host's innate immune system by using various components and virulence factors, allowing them to cross the mucosal barrier, spread and reproduce in distant organs [75,76]. The composition of OMVs makes them an important factor in activating the host's innate and acquired immune response pathways. Currently, all experimental evidence shows that the innate immune response to OMVs is the result of the combined action of PAMPs and recognition of LPS, which can effectively activate the body's immune system and exert anti-tumor effects [77–80]. In addition to the powerful immune regulatory molecule LPS, OMVs also contain OM pore proteins and other important innate immune activation ligands. Lipoprotein and OM proteins are a class of bioactive molecules that can activate immune cells and induce leukocyte migration [81].

Due to the lack of bacterial reproduction and replication ability, OMVs exhibit better biosafety than bacteria. In addition, OMVs are nanoscale vesicles that are easier to transmit and penetrate tissues through the bloodstream, thus quickly reaching tumor tissue and exerting anti-tumor effects [82]. Besides, OMVs have strong tolerance to various bioengineering modifications, such as ultrasound, filtration compression, and electroporation. [55,83,84]. Through modification, their tumor targeting can be enhanced, and efficient transportation of tumor therapeutic drugs can be achieved. The administration method of OMVs is not singular, and different administration methods can better meet the treatment of different types of tumors. Overall, the biological and physicochemical properties of OMVs can be improved. Its characteristics make it have great potential in tumor treatment.

The most abundant and effective immune stimulating component in OMVs is LPS. However, it is worth noting that LPS can activate macrophages, endothelial cells, and other inflammatory factors, leading to host damage and even septic shock [51,85–87]. Many studies have shown that TLR4complexes directly transmit stimulation signals from LPS into cells, triggering host inflammatory responses and leading to bacterial infections [80,88,89]. OMVs, as carriers of LPS, can activate inflammatory responses and cause damage to host tissues. Therefore, the reason why OMVs have not been widely used is closely related to their own toxicity. Hence, it is urgent to develop effective detoxification strategies to improve the safety of OMVs and expand their application in tumor treatment. At the same time, it is necessary to consider the relationship between immunotherapy and the tumor microenvironment, so that OMVs can better exert immune efficacy.

3. Application research of OMVs in tumor treatment

3.1. The role of OMVs in immunotherapy

Based on its unique immunological and structural characteristics, the anti-tumor effect of OMVs is receiving increasing attention. The PAMPs enriched on the surface of OMVs makes it easy for neutrophils in the blood to phagocytose. Due to the inflammatory targeting ability of neutrophils, OMVs can utilize neutrophils to target inflammatory tumor tissue. It is worth noting that after reaching inflammatory tumors, OMVs can be released during the

formation of neutrophil extracellular traps (NETs) without affecting their activity, which endows OMVs with natural tumor targeting ability [90]. In addition, OMVs can induce tumor associated macrophages (TAMs) to polarize towards pro-inflammatory M1 macrophages, as shown in Fig. 2 [91]. OMVs can recruit various immune cells, including natural killer (NK) cells and T cells, which can reverse the immunosuppressive microenvironment in the tumor area and induce the production of interferon-γ (IFN-γ) in the tumor area to mediate tumor cell apoptosis [92]. OMVs with nanoscale structures can freely circulate to lymph nodes, and the PAMPs enriched on the surface of OMVs make them easy for DC cells to uptake and retain lymph nodes, inducing Th1 type immune responses [93,94]. Cheng et al. [84] found that OMVs demonstrate substantial accumulation in lymph node in mice, which may be attributed to the cell-independent drainage facilitated by their small size and the cell-dependent drainage by virtue of their natural "foreigner" status, which activates effective anti-tumor immune responses. Qing et al. [95] reported that OMVs isolated from four different bacteria downregulated the frequency of immunosuppressive cells (such as dendritic cells and M2-like macrophages) in tumors, significantly increasing the infiltration of $CD8^+$ T cells in tumors. According to Kim and coworkers' study [92] on the anti-tumor effects of OMVs naturally produced by Gram-negative bacteria such as *Escherichia coli* and *Salmonella*, the results showed that OMVs can target tumor tissue and accumulate in tumor tissue. They possess a remarkable capability to induce anti-tumor immune response and cure tumors in tumor-bearing mice, without causing significant adverse reactions. After intravenous administration, OMVs can specifically target and accumulate in tumor tissue, subsequently inducing the production of anti-tumor cytokines C-X-C motif chemokine ligand 10 (CXCL10) and IFN-γ. However, mice lacking IFN-γ cannot induce OMVs mediated immune responses, underscoring the dependency of this anti-tumor effect on IFN-γ. Due to the presence of LPS in the structure of OMVs, they may lead to fatal sepsis. Safety is a crucial consideration for tumor treatment. Therefore, comprehensive safety assessments of OMVs should be carried out to ensure their secure clinical application in the future.

3.2. Modified OMVs for tumor treatment

As nanovesicles secreted by bacteria, OMVs have the characteristic of being easy to modify, which makes them have great application prospects in anti-tumor research. Modifying OMVs can not only enhance their tumor targeting and reduce systemic toxicity, but also endow them with specific functions as needed.

3.2.1. Genetically engineered OMVs

Functional modification of parental bacteria through genetic engineering, metabolic engineering, or parental bacterial membrane engineering can alter the function of their secreted OMVs. The genetic engineering strategies of OMVs are mostly based on the transgenic expression of proteins or fusion proteins in bacteria. These heterologous proteins are either encapsulated in OMVs as periplasmic proteins or expressed on the outer membrane leaves of OMVs by connecting with outer membrane proteins. OMVs can achieve multiple functions through genetic engineering modification, as shown in Table 3 [96–103]. Cheng et al. [84] induced specific anti-tumor immune responses by presenting antigens specifically to the surface of OMVs. By fusing with cytolysin A (ClyA) protein, tumor antigens are displayed on the surface of OMVs. The results showed that the antigen peptide displayed by ClyA protein on the surface of OMVs successfully activated the immune response of the mouse tumor model, promoted the maturation of DCs and the presentation of corresponding antigens, and suppressed the lung metastasis of melanoma expressing corresponding antigens. OMVs modified with different protein traps can simultaneously

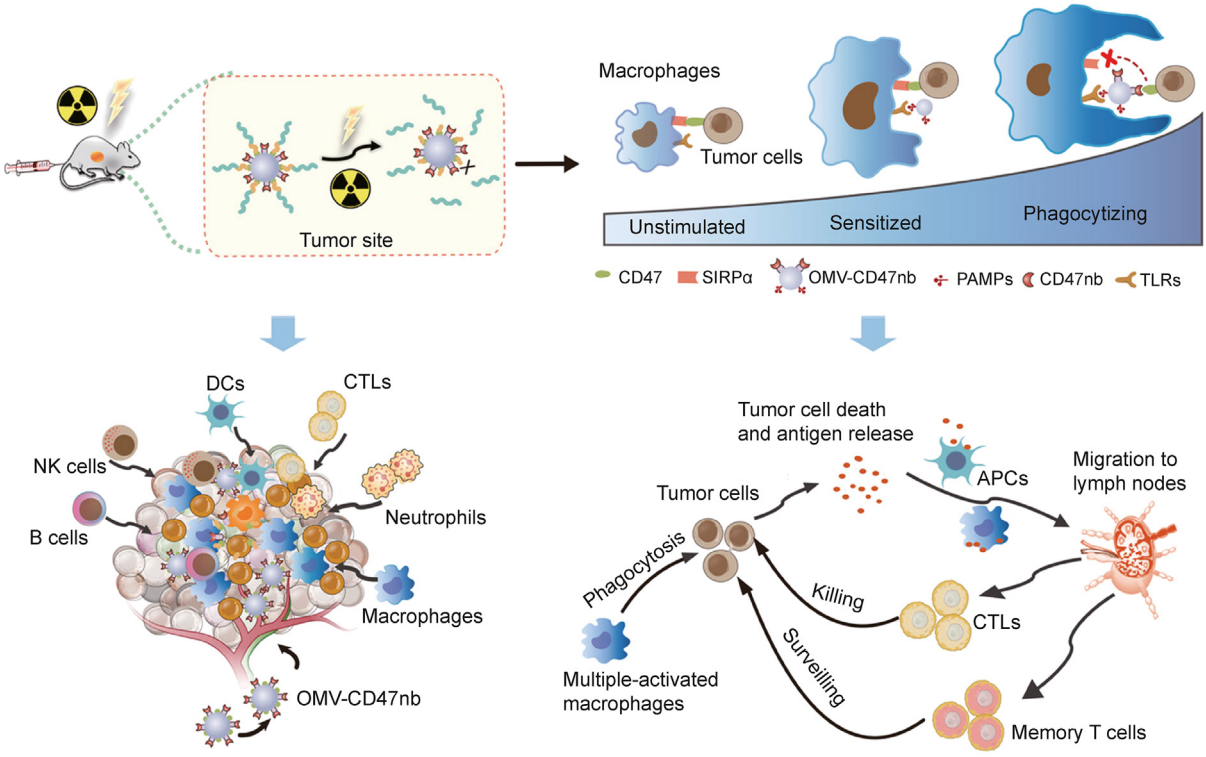

Fig. 2. The radiation-triggered, controlled release behavior of outer membrane vesicles (OMV)-CD47 nanobody (CD47nb) and multiple-activation mechanisms of tumor associated macrophages (TAMs). Reprint with permission from Ref. [91]. SIRP: signal regulatory protein; PAMPs: pathogen-associated molecular patterns; TLRs: Toll like receptors; DCs: dendritic cells; CTLs: cytotoxic T lymphocytes; NK cells: natural killer cells; APCs: antigen-presenting cells.

Table 3
Modification of outer membrane vesicles (OMVs) through different approaches and their effect.

Type of modification	Strategy of modification	Effect of modification	Refs.
Genetic engineering	Knockout or modification of genes such as lpxL1, LpxM, lpxL, lpxM, pagP, lpxP, Lpp, relA, spot, and eptA	Increasing the production of OMVs or reduce the toxicity of OMVs	[96–99]
Genetic engineering	HPV16E7 was presented on OMVs	Enhanced anti-tumor effect	[100]
Genetic engineering	Loading 154 amino acid BFGF onto OMVs	Enhanced anti-tumor effect	[101]
Genetic engineering	Expression of targeted peptide LyP1	Improved tumor targeting ability of OMVs	[102]
Genetic engineering	Decorating OMVs with EGFRv III and B16-M30	Protecting mice from tumor attack	[103]
Membrane fusion	Fusion of melanoma cell membrane with attenuated *Salmonella* OMVs	Homogeneous-target and immune activation	[104]
Membrane fusion	*E. coli* OMVs fuse with B16-F10 cancer cell membrane	Homogeneous-target and immune activation	[105]
Membrane fusion	Fusion of *E. coli* OMVs with tumor cell membranes	Homogeneous-target and immune activation	[106]
Membrane fusion	Fusion of thylakoid membranes with OMVs	PDT effects and tumor specific immune response	[107]
Membrane fusion	Fusion of *E. coli* OMVs with tumor cell membranes	Homogeneous-target and immune activation	[108]
Chemical modification	Chemical coupling of Mal-PEG4-NHS onto the surface of OMVs	Capturing tumor antigens and inducing strong anti-tumor immune responses	[109]
Chemical modification	Chemical coupling of 6-Mal-PLGVR-NHS-AD (cL-AD) onto the surface of OMVs	Reducing toxicity Enhancing anti-tumor effects	[110]
Chemical modification	Build “shielding shells”, such as constructed metal phenolic “prison”	Reducing toxicity Enhancing enrichment Specific release	[111]

HPV16E7: human papillomavirus type 16 early protein E7; BFGF: basic fibroblast growth factor; EGFRv III: epidermal growth factor receptor variant III; PDT: photodynamic therapy; Mal: maleimide; PEG4: polyethylene glycol 4; NHS: N-hydroxysuccinimide.

display multiple different tumor antigens, thereby triggering a synergistic anti-tumor immune response. LPS are one of the main outer membrane components of Gram-negative bacteria, which can cause fever, inflammation, and septic shock by activating TLR4 [96]. Therefore, reducing LPS levels may be important in limiting inflammatory responses. Studies have shown that genetic modifications targeting genes such as lpxL1, LpxM, lpxL, LpxM, pagP, lpxP, and eptA can reduce LPS toxicity while maintaining the immune activity of OMVs [96–99]. Therefore, genetic engineering is a promising detoxification technique. Due to the persistent infection of cervical cancer caused by high-risk genotype human papillomavirus (HPV), Wang et al. [100] used gene recombination technology to embed the early protein gene of HPV 16 into engineered OMVs, successfully inducing co transformation of HPV-16 oncoproteins E6, E7, and c-Ha ras oncogenes into TC-1 transplanted tumor-bearing mice, inducing specific cellular immune responses and inhibiting tumor growth. In order to address the technical barriers of oral tumor vaccines and utilize the biological characteristics of gut symbiotic bacteria, Yue et al. [59] designed an oral tumor vaccine derived from genetically engineered bacteria. They fused the tumor antigen (Ag) and Fc fragments of mouse immunoglobulin G (IgG; mFc) to the surface protein ClyA of OMVs, and OMVs expressing ClyA-Ag-mFc on the surface showed significant anti-tumor effects as tumor vaccines. Huang et al. [101] used gene recombination technology to load 154 amino acid basic fibroblast growth factor (BFGF) onto OMVs of *E. coli*. OMVs were used to induce host production of anti BFGF autoantibodies, which inhibited tumor angiogenesis, induced tumor cell apoptosis, reversed tumor immune barrier, and promoted tumor specific cytotoxic T lymphocytes (CTLs), ultimately leading to tumor regression. Pan et al. [102] successfully developed a LyP1 peptide modified OMVs loaded with programmed death 1 (PD-1) plasmid to induce tumor cell self-expression of PD-1, as shown in Fig. 3 [102]. Nanocarriers can be effectively internalized by tumor cells through LyP1 peptide mediated targeting, exhibiting 1.5-fold tumor accumulation compared to nontargeted OMVs. The self-expressed PD-1 was bound to programmed death ligand 1 (PD-L1) expressed by both autologous and neighboring tumor cells to block the PD-1/PD-L1 pathway, resulting in significant anti-tumor effects of nanocarriers on various tumors. Li et al. [78] modified OMVs by insertion of the ectodomain of PD-1. This genetic modification does not compromise the capability of OMVs to induce immune activation. Furthermore, OMV-PD1 demonstrates robust accumulation at the tumor site, coupled with effective binding between the vesicles and PD-L1 on tumor cells, ultimately block the inhibitory axis of PD-1/PD-L1 by consuming tumor cell PD-L1, thereby enhancing the anti-tumor effect of the drug. By combining immune activation and checkpoint inhibition, engineered OMVs drive the accumulation of effector T cells in tumors, leading to strong inhibition of tumor growth. Grandi et al. [103] used the 14 amino acid B cell epitope of human epidermal growth factor receptor variant III (EGFRv III) and the $CD4^+$ T cell neo-epitope (B16-M30) derived from the *kif18b* gene mutation to decorate OMVs, either alone or in combination, to completely protect mice from tumor attack. Immunization is accompanied by high titers of anti EGFRv III antibodies, induction of M30 specific T cells, and infiltration of $CD4^+$ and $CD8^+$ T cells at the tumor site, which inhibits the tumorigenicity of melanoma cell lines in mice.

Although genetic engineering has shown the potential of OMVs in tumor treatment, there are still some challenging issues when it comes to clinical use. In addition, the secretion of OMVs largely depends on the growth conditions of bacteria, so when genetically engineered OMVs are scaled up on a large scale, their heterogeneity may lead to reproducibility issues and the preparation consistency cannot be guaranteed. Therefore, developing new methods to produce high-yield and high-purity OMVs is of great significance for clinical applications.

3.2.2. Membrane fusion modified OMVs

Physical modification of OMVs can be achieved through membrane fusion. It has been proven to be an effective strategy to modify tumor antigens on the surface of OMVs to induce anti-tumor immune responses. The vaccine with tumor antigen obtained by fusing tumor cell membranes with OMVs through membrane fusion technology can trigger a stronger immune response than a single antigen, as shown in Table 3 [104–108]. Chen et al. [104] fused melanoma cytomembrane vesicles (CMVs) with attenuated *Salmonella* OMVs to construct an eukaryotic-

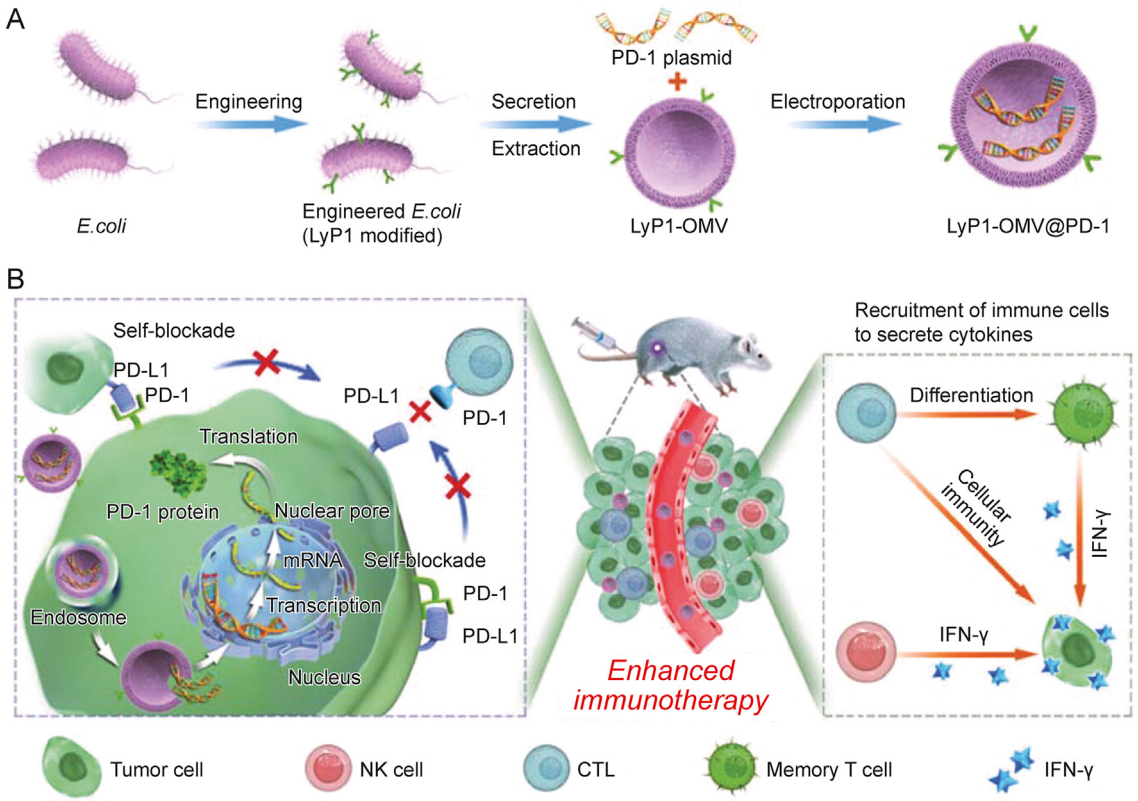

Fig. 3. The application of genetically engineered outer membrane vesicles (OMVs) in tumor treatment. (A) Genetically engineered OMVs loaded with programmed death 1 (PD-1) plasmid. (B) Genetically engineered OMVs induce tumor cell self-expression of PD-1. Reprint with permission from Ref. [102]. PD-L1: programmed death ligand 1; mRNA: messenger RNA; CTL: cytotoxic T lymphocyte; NK cell: natural killer cell; IFN-γ: interferon-γ.

prokaryotic vesicle (EPV) nanoplatform. The fused EPV exhibited strong ability to stimulate the immune system and induce tumor specific immune responses. After inoculation with EPV, it can stimulate the immune system and trigger anti-tumor immune responses, protecting mice from melanoma attacks, indicating that this EPV has great potential as a cancer prevention vaccine. Wang et al. [105] fused OMVs with B16-F10 cancer cell (CC) membranes to form OMV-CC hybrid membranes, which also enhanced the body's anti-tumor immune response. Li et al. [106] developed an effective vesicular cancer nanovaccine by fusing OMVs and tumor cell membranes (TCM), namely BTs, as shown in Fig. 4 [106]. Bacterial derived pathogenic adjuvants in BTs can achieve DC targeting, promote complete maturation of DC and antigen presentation of DC. The TCM in BTs assembles various endogenous tumor antigens, which can generate multi antigenic anti-tumor immunity. This fusion membrane enables efficient delivery of antigens and adjuvants to the same DC, achieving good anti-tumor therapeutic effects. These hybrid vesicles obtained through membrane fusion technology inherit and integrate the immune functions of two maternal components, effectively activating DC immune responses and inducing strong anti-tumor specific immunity based on CTLs, demonstrating excellent tumor prevention and treatment effects.

3.2.3. Chemically modified OMVs

Chemical modification of OMVs can be achieved by forming chemical bonds between functional groups and OMVs membrane components, as shown in Table 3 [109–111]. Li et al. [109] used the reaction antigen between amino groups and active N-hydroxysuccinimide (NHS) esters on the surface proteins of OMVs to graft maleimide (Mal)-polyethylene glycol (PEG)4 NHS onto the surface of OMVs to obtain OMV-Mal, which can form stable thioether bonds with proteins, as shown in Fig. 5 [109]. Post photothermal therapy (PTT), due to the characteristics of lymph node drainage and immune recognition of OMVs, locally injected OMV-Mal can capture a large amount of tumor releasing proteins, including a large amount of tumor antigens, and increase the uptake of tumor antigens by DC, which will induce a strong anti-tumor immune response. For the similar strategy, Li et al. [110] selected the matrix metalloproteinase responsive peptide 6-Mal-PLGVR-NHS (cL) and linked the serum albumin loaded with doxorubicin (SA-DOX, AD) to the NHS of cL through an amide reaction. The exposed thiol group of OMVs binds to the Mal at the other end of the peptide after treatment, forming the final product OMV-cL-AD. This strategy can inhibit OMVs, reduce toxicity, and enhance anti-tumor effects through chemical immunotherapy The SA on the surface of OMVs significantly enhances the accumulation of OMV-cL-AD formulation at the tumor site. Then, the shielding layer on OMV-cL-AD is disrupted in the tumor microenvironment, leading to the release of both OMVs and AD, thus enabling synergistic tumor therapy with immunotherapy and chemotherapy. Their work indicates that adding a shielding layer to OMVs through chemical modification methods and drug loading is a promising immunochemotherapy strategy.

It has been found that it can reduce inflammation during systemic circulation by using a temporary “shielding shell” to design OMVs, and this shell can be designed to dissolve in an acidic microenvironment to release the core. Based on this idea, Qing et al. [95] incubated OMVs with calcium chloride ($CaCl_2$) and utilized the acidic residues on the surface of OMVs to chelate with Ca^{2+}, providing “nucleation sites” to promote calcium phosphate (CAP) nucleation and mineralization, ultimately covering the surface of OMVs. The biological toxicity of biomineralized OMVs is

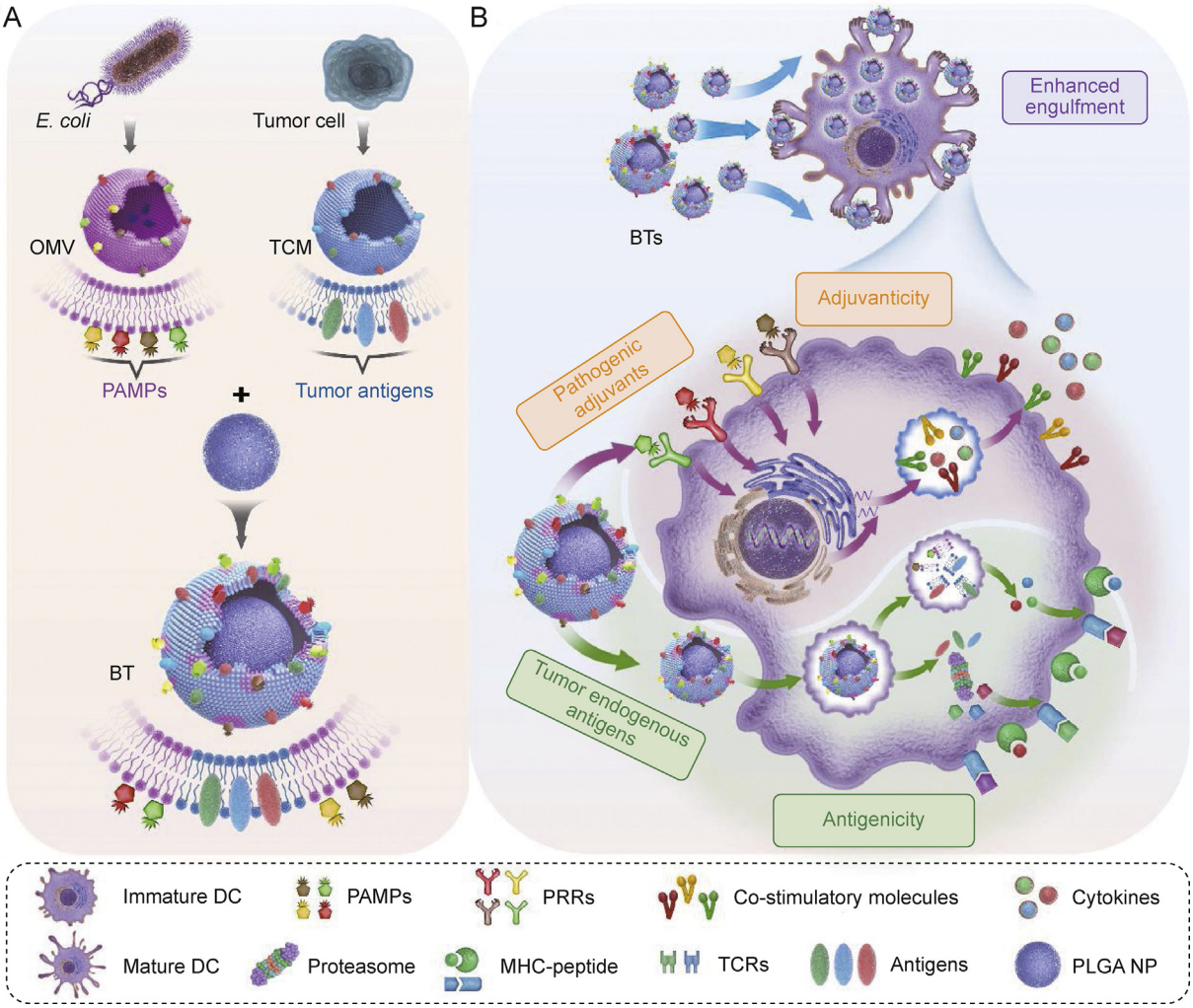

Fig. 4. The preparation and immune induction of hybrid membrane nanovaccines. (A) Preparation of hybrid membrane nanovaccines. (B) Nanovaccines induce immune response. Reprint with permission from Ref. [106]. OMV: outer membrane vesicle; TCM: tumor cell membranes; DC: dendritic cell; PAMPs: pathogen-associated molecular patterns; PRRs: pattern recognition receptors; MHC: major histocompatibility complex; TCRs: T cell receptors; PLGA: poly(lactic-co-glycolic acid); NP: nanoparticle.

significantly reduced, and they can dissolve and release OMVs in the acidic microenvironment of tumors to inhibit tumor growth. Some antigens can fuse with proteins from OMVs to form chimeric proteins on the OMVs membrane. Cheng et al. [84] utilized ClyA fusion to capture the protein SpyCatcher/SnoopCatcher, and the specific antigen protein binds to the tag protein SpyTag/SnoopTag through peptide bonds, allowing various tumor antigens to quickly and simultaneously display on the surface of OMVs by binding to the protein tag. This OMVs based vaccine platform suppresses tumor metastasis and growth by presenting specific antigens on the surface of OMVs, and induces long-term immune memory, thereby triggering specific anti-tumor immune responses. In addition, there have been reports of fusing CD47 nanobody (CD47nb) with the surface protein ClyA (ClyA CD47nb) on OMVs (OMV CD47nb) and introducing azide (N3) groups into the glycoconjugates on the bacterial outer membrane. Next, through a click chemical reaction between N3 and dibenzocyclooctyne (DBCO) groups, OMV-CD47nb is coated with DBCO modified PEG/Se to form PEG/Se@OMV-CD47nb. The PEG/Se layer modification not only reduces the immunogenicity of OMV-CD47nb, thereby significantly increasing the safe dose of intravenous injection. As an immunostimulant, OMV-CD47nb also reshapes tumor microenvironment (TME) by inducing M1 polarized TAMs and recruiting multiple immune cells. The phagocytic effect of TAMs mediated by OMV-CD47nb on tumor cells induces the release of tumor antigens, which are further processed by APCs and presented to draining lymph nodes, subsequently triggering T cell-mediated anti-tumor immunity [91]. Nie et al. [111] constructed metal phenolic “prison” (MPP) engineering OMVs through coordination between metal ions (Fe III) and polyphenols (TA). MPP can reduce the toxicity of OMVs and increase their accumulation in tumors, while also providing additional chemodynamic therapy (CDT) effects. In the low pH and high ATP environment of the tumor site, MPP dissociates, releasing the internal OMVs and initiating a robust tumor-specific immune response. Consequently, this strategy not only significantly suppresses the primary tumor but also inhibits the abscopal tumor through the formation of anti-tumor immune memory. In addition, combining immune checkpoint blockade can further enhance chemodynamic immunotherapy, providing a new approach to promote the application of OMVs. Peng et al. [112] used palmitic acid to combine RWrNMGGGGIVRRADRAAVP (RGP) and arginyl-glycyl-aspartic acid (RGD) with OMV to form RGP-OMV and RGD-OMV, respectively. Indocyanine green (ICG) is loaded onto three types of OMVs through fusion effects and electrostatic interactions with RGD/RGP, forming ICG-OMV, ICG-RGD-OMV, and ICG-RGP-OMV, respectively. ICG-RGP-OMVs can penetrate the stratum

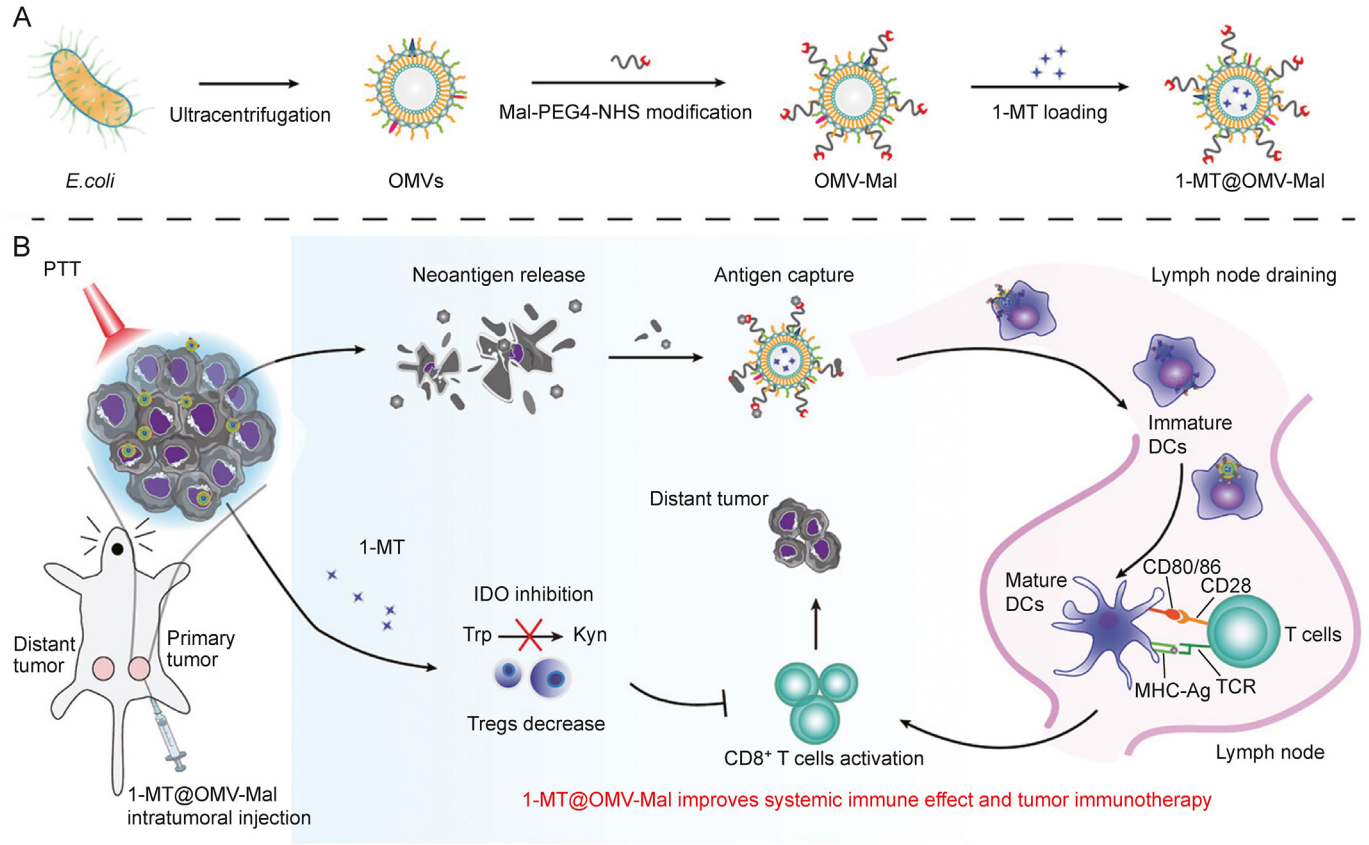

Fig. 5. Synthesis of chemically modified outer membrane vesicles (OMVs) and their antitumor immune mechanism. (A) The synthesis process of chemically modified OMVs. (B) The mechanism of antitumor immune responses elicited by chemically modified OMVs after photothermal therapy (PTT). Reprint with permission from Ref. [109]. 1-MT: 1-methyl-tryptophan; IDO: indoleamine 2, 3-dioxygenase; Trp: tryptophan; Kyn: kynurenine; DCs: dendritic cells; MHC: major histocompatibility complex; TCR: T cell receptor.

corneum and accumulate in melanoma. Near infrared stimulation not only induces the photothermal and photodynamic responses of ICG-RGP-OMV to primary melanoma spheroids, leading to rapid clearance of the primary tumor, but also stimulates OMV to release tumor necrosis factor related apoptosis-inducing ligand (TRAIL), thereby activating cell apoptosis in disseminated tumor masses and ultimately eradicating melanoma.

The above are the three most promising strategies for modifying OMVs, each with its own advantages and disadvantages, which require different choices based on different application scenarios. Overall, endowing OMVs with specific functions through different modification methods is beneficial for their practical applications and clinical translation.

3.3. OMVs as carriers for integrated tumor therapy

3.3.1. Combined immunotherapy and chemotherapy

OMVs have many advantages as tumor drug delivery carriers, making them a research hotspot. OMVs have a natural vesicle structure that can be used for drug loading, and their small size allows them to passively accumulate in tumors through the EPR effect, which helps to deliver drugs to tumors and improve anti-tumor effects [58]. Moreover, OMVs with special targeted ligand modifications can be obtained through genetic engineering. These targeting groups endow OMVs with targeting functions, allowing drugs to be delivered to specific regions and enriched for more efficient targeted therapy [78]. In traditional chemotherapy, drugs are prone to failure without protection, while the vesicle structure of OMVs can protect drugs from degradation and denaturation before reaching the target site, reduce drug release in non-targeted areas, thereby reducing side effects and improving treatment efficacy [113]. OMVs as drug carriers can overcome the drawbacks of current chemotherapy, such as severe and persistent adverse reactions, low specificity, rapid clearance of chemotherapy drugs in the body, short circulation half-life, and the need for higher doses of drugs to achieve therapeutic effects [114]. Importantly, OMVs have immunogenicity and can induce immune responses. When combined with drugs, they can be used for multifunctional combination therapy of tumors to achieve better therapeutic effects [115].

There have been many reports proving that OMVs as carriers of chemotherapy drugs have superior anti-cancer effects and biosafety, as shown in Table 4 [116–121]. Chen et al. [116] proposed a bioengineering method to encapsulate attenuated *Salmonella* typhimurium OMVs onto polymer micelles loaded with the cancer chemotherapy drug tegafur, a prodrug of fluorouracil (5-fluorouracil (5-FU)), as shown in Fig. 6 [116]. The OMVs were also modified with polyethylene glycol and tumor targeting ligands to increase their stability and tumor targeting ability. These designed engineered OMVs can play a dual role in chemotherapy and immune regulation, making tumor cells sensitive to CTLs, and directly kill tumor cells. Systemic injection of these OMVs not only provides effective protective immunity against the occurrence of melanoma, but also significantly inhibits tumor growth *in vivo*, prolongs the survival rate of melanoma mice, and effectively inhibits tumor metastasis to the lungs. Studies have demonstrated that the anti-tumor drug doxorubicin (DOX) is transported through OMVs to treat tumors, reducing its side effects in experimental mice. Kuerban et al. [117] obtained naturally released vesicles from attenuated Klebsiella pneumoniae

Table 4
Outer membrane vesicles (OMVs) as carriers for integrated tumor therapy.

OMVs function	Loaded material	Enhanced treatment	Refs.
Drug carrier/immunomodulator	Tigafur	Chemotherapy	[116]
Drug carrier/immunomodulator	DOX	Chemotherapy	[117,118]
Drug carrier/immunomodulator	5-FU	Chemotherapy	[119,120]
Drug carrier/immunomodulator	Paclitaxel	Chemotherapy	[121]
Photosensitizer carrier/immunomodulator	Ce6	PDT	[122]
Photosensitizer carrier/immunomodulator	Catalase-Ce6	PDT	[123]
Photosensitizer carrier/immunomodulator	IR780	PDT	[124]
Photosensitizer carrier/immunomodulator	IR780	PTT	[124]
Photosensitizer carrier/immunomodulator	Melanin	PTT	[125]
Photosensitizer carrier/immunomodulator	Fe_3O_4–MnO_2	PTT	[126]
Photosensitizer carrier/immunomodulator	CuS	PTT	[127]
Photosensitizer carrier/immunomodulator	Polydopamine	PTT	[128]

DOX: doxorubicin; 5-FU: 5-fluorouracil; Ce6: Chlorin e6; PDT: photodynamic therapy; PTT: photothermal therapy.

and prepared DOX-OMVs loaded with DOX. DOX encapsulated in OMVs acted faster than DOX encapsulated in liposomes. DOX-OMVs not only promoted the accumulation of chemotherapy drugs in tumor tissue, but also induced appropriate anti-tumor immune responses, thereby enhancing anti-tumor effects on non-small-cell lung carcinoma. Shi et al. [119] modified OMVs with mesoporous silica nanoparticles (MSNs) and loaded them with 5-FU to prepare OMVs-MSNs-5-FU, which combined the high drug loading capacity of the nanocarrier system and the intestinal adsorption of the biological carrier, significantly enhancing cytotoxicity and cellular uptake of tumor cells. Guo et al. [121] designed a co-delivery system of paclitaxel and small interfering RNA (siRNA) using OMVs as carriers, which combines three pathways: 1) pH responsive release of chemotherapy drugs, 2) tumor metabolism regulation, and 3) tumor immune regulation. In the triple negative breast cancer model, this OMVs has good effects on tumor related macrophage repolarization, tumor inhibition, tumor immune activation and TME remodeling, showcasing significant application potential.

3.3.2. Combined immunotherapy and phototherapy

In addition to serving as a carrier for chemotherapy drugs to enhance anti-tumor effects, OMVs combined with PTT and PDT have received more attention, as shown in Table 4 [122–128]. Qing et al. [95] first neutralized the acidic tumor microenvironment by encapsulating the OMVs of *E. coli* (BL21) with CAP, leading to a high polarization of macrophages from M2 to M1, thereby improving anti-tumor efficacy. Then, active tumor targeting ligand folic acid (FA) and photosensitizer ICG were used to functionalize the nanoparticles to promote active targeting of tumors and photothermal induced immunogenic cell death. PTT and immunotherapy combined therapy enhanced the synergistic anti-tumor therapeutic effect. It was found that OMVs loaded with photosensitizer Chlorin e6 (Ce6) and chemotherapy drug DOX have good anti-tumor effects as a treatment platform. Through the synergistic treatment of photodynamic therapy (PDT), chemotherapy, and immunotherapy, triple negative breast tumors in mice were completely suppressed, and the occurrence of tumor metastasis was successfully prevented [122]. Wang et al. [105] fused OMVs and B16-F10 cell membranes derived from *E. coli* to obtain OMV-CC hybrid membranes, which were encapsulated onto hollow polydopamine (HPDA) NPs, as shown in Fig. 7 [105]. They utilized the advantages of OMVs immunotherapy and HPDA mediated PTT to enhance the anti-tumor efficacy against melanoma.

Li et al. [90] encapsulated polymer isoindigo derivative (PBIBDF)-bithiophene (BT), which was used as a photothermal transducer, in the core of a long-cycle micelle composed of PEG-b-poly(lactic-co-glycolic acid) (PLGA), and coated *E. coli* OMVs outside to prepare nano pathogens (NPNs). The combination of cisplatin loaded NPNs and PTT can effectively be recognized and internalized by neutrophils, and can exert strong anti-cancer effects. The combination of anti-tumor immune response and PTT effectively suppressed advanced tumor growth *in vivo*. Improving the tumor targeting effect of OMVs combined with phototherapy and chemotherapy can lead to superior synergistic therapeutic effects.

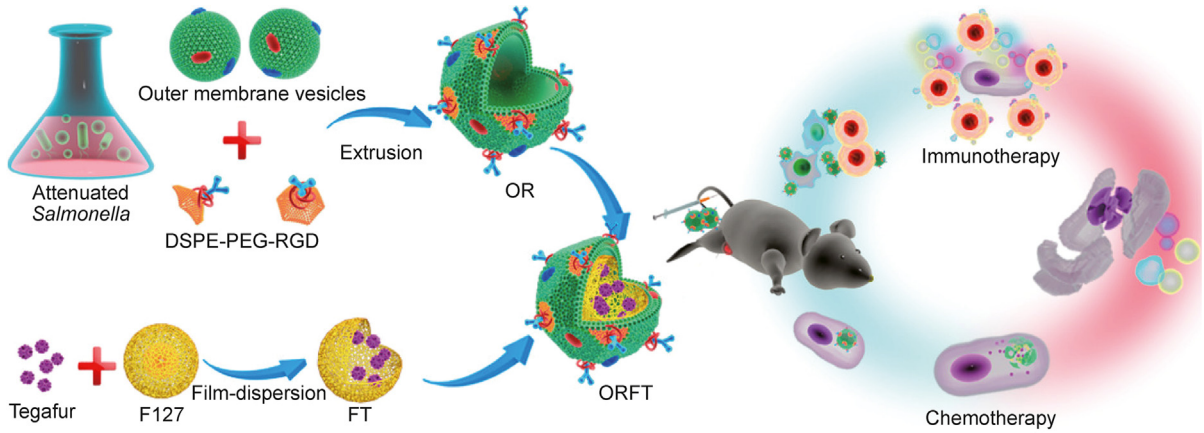

Fig. 6. Schematic illustration of the bioengineering process of functionalized outer membrane vesicle (OMV)-coated polymeric micelles and their synergistic therapy with chemotherapy and immunotherapy. Reprint with permission from Ref. [116]. DSPE-PEG: 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol)); OR: OMV-DSPE-PEG-arginyl-glycyl-aspartic acid (RGD); FT: tegafur-loaded micelles; ORFT: OR-coated F127 nanomicelles.

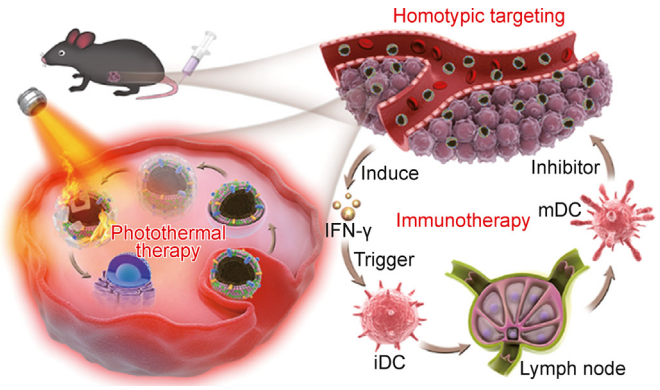

Fig. 7. Synergistic photothermal/immunotherapy of melanoma using hybrid membrane camouflaged nanoparticles. Reprint with permission from Ref. [105]. IFN-γ: interferon-γ; iDC: immature dendritic cell; mDC: mature dendritic cell.

In summary, OMVs can not only serve as carriers to deliver antitumor drugs such as photosensitizers and chemotherapy drugs to the tumor site, but also activate immune responses to enhance antitumor effects.

4. Conclusions and future perspectives

OMVs possess unique biological and physicochemical properties. Through the design of OMVs, such as genetic engineering, membrane modification, or membrane encapsulation, they can be developed into vaccines and drug delivery carriers. They have shown great potential in the field of tumor treatment, but there are still some clinical challenges that need to be overcome. OMVs are products of bacteria, and in complex human environments, OMVs may disrupt the microenvironment of target organs and lead to complications. The targeting effect of OMVs is variable, and the efficacy of different tumors during treatment may vary. The expression of immunotherapeutic agents for OMVs may vary, with low expression leading to poor efficacy, while high expression may lead to autoimmune diseases. In addition, OMVs still have certain toxicity as effective immune system activators. For example, LPS can cause strong inflammatory reactions, thereby regulating immune responses. However, excessive LPS can cause immunosuppressive reactions. Hence, future research should focus on the purification and detoxification of OMVs to strike a balance between therapeutic efficacy and safety in clinical trials.

Currently, various methods have been reported to optimize the production and reduce the toxicity of OMVs, but further in-depth research and exploration are needed. In the future, large-scale extraction of OMVs will be necessary to meet the growing demand for clinical applications. Additionally, there is limited research on reducing the toxicity of OMVs, and more efficient strategies need to be explored. With the diversity of bacterial sources, further research is required to determine whether OMVs from different bacteria have varying effects on different tumors. We believe that with the advancement and deeper exploration of biotechnology, OMVs have the potential to become ideal activators of anti-tumor immune responses and effective drug delivery platforms for tumor treatment, offering a cost-effective solution for clinical cancer therapy.

CRediT authorship contribution statement

Shuo Xiang: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Arshad Khan:** Investigation, Formal analysis, Conceptualization. **Qiufang Yao:** Writing – review & editing, Supervision, Funding acquisition. **Dong Wang:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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