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Review for Seminars in Immunology

Outer Membrane Vesicle vaccines

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Abstract

Outer Membrane Vesicles (OMV) have received increased attention in recent years as a vaccine platform against bacterial pathogens. OMV from *Neisseria meningitidis* serogroup B have been extensively explored. Following the success of the MeNZB OMV vaccine in controlling an outbreak of *N. meningitidis* B in New Zealand, additional research and development resulted in the licensure of the OMV-containing four-component 4CMenB vaccine, Bexsero. This provided broader protection against multiple meningococcal B strains. Advances in the field of genetic engineering have permitted further improvements in the platform resulting in increased yields, reduced endotoxicity and decoration with homologous and heterologous antigens to enhance immunogenicity and provide broader protection. The OMV vaccine platform has been extended to many other pathogens.

In this review, we discuss the progress in the development of OMV vaccine delivery platform, high-lighting successful applications, together with potential challenges and gaps.

Highlights

- OMV constitute an emerging platform for vaccine development.
- Simplicity of manufacture make OMV an attractive technology for affordable vaccines.
- More clinical studies will help elucidation of OMV mechanism of action.
- Genetic engineering permits OMV modification resulting in enhanced applicability.

Keywords: Outer Membrane Vesicles, OMV, GMMA, vaccines, bacterial diseases.

Abbreviations

OMV	Outer Membrane Vesicle
nOMV	native Outer Membrane Vesicle
dOMV	detergent-extracted Outer Membrane Vesicle
mdOMV	mutant-derived Outer Membrane Vesicle
glyOMV	glycoengineered Outer Membrane Vesicle
GMMA	Generalized Modules for Membrane Antigens
LPS	Lipopolysaccharide
TLR	Toll Like Receptor
PAMP	Pathogen-Associated Molecular Pattern
PRR	Pattern Recognition Receptor
IFN	interferon
MHC	major histocompatibility complex
APC	antigen presenting cells
DC	dendritic cell

1. Introduction

In 2020, despite advances in medicine, there remains a huge global burden of infectious diseases. 91% of infectious disease deaths are in low and middle-income countries [1], where, amongst other problems, implementation of existing vaccines can be challenging [2]. Vaccination is one of the most cost-effective health interventions globally *(site MacLennan and Saul PNAS)*. Combined with other approaches, such as clean water, improved hygiene, health education and better nutrition, vaccination has prevented millions of deaths, debilitating illnesses and disabilities, especially among children under 5 years of age [3-5]. In the absence of commercial incentives, simple affordable technologies represent an attractive route for vaccine development [6]. The increasing number and global distribution of pathogens resistant to antimicrobial drugs is a major global health challenge, compromising the ability to cure a wide range of infectious diseases that were once straightforward to treat with antibiotics [7, 8]. Novel platforms can accelerate vaccine development for many unmet medical needs including antimicrobial-resistant bacteria [9, 10].

Outer Membrane Vesicles (OMV) have received increasing attention in recent years as a platform for vaccine delivery against bacterial pathogens [11]. OMV were first explored for vaccine development against *Neisseria meningitidis* serogroup B disease. After the success of vaccination with the MeNZB OMV-based vaccine in controlling an outbreak of *N. meningitidis* B in New Zealand, additional research and development resulted in the licensure of Bexsero, with broader protection against multiple meningococcal B strains [12]. In this review, we discuss progress in the development of OMV vaccine delivery platforms. We highlight successful applications of the different forms of this technology for the generation of safe and immunogenic vaccines, together with potential gaps and challenges.

2. OMV biology

OMV are naturally released during the growth of Gram-negative bacteria [13]. Natural OMV release has been associated with several biological functions [14]. Chatterjee and Das discovered OMV in 1967 while studying the cell-wall structure of *Vibrio cholera in vitro* [15]. Since then, OMV formation has been observed in an increasing number of Gram-negative bacteria [16, 17]. The presence of OMV was observed in patients with meningococcal infection, suggesting a role for OMV in bacteria pathogenesis [18]. Many studies subsequently revealed roles for OMV in enhancing the survival of bacteria in harsh environments, and delivering virulence factors and DNA to host cells [16, 17]. For a number of bacteria, OMV participate in the formation of biofilms and thereby increase survival in hosts [19] or in soil [20]. It appears that OMV production helps bacterial defence against antibiotics, e.g. by diluting the damaging effect of gentamicin [21]. The possibility of gene transfer between bacteria by OMV has been reported for various bacteria, [22] including the transfer of antibiotic resistance [23]. OMV could however simply result as a by-product of an imbalance between cell growth and outer membrane synthesis, resulting in release of excess membrane material as OMV [24].

3. OMV as vaccines

OMV, naturally released from Gram-negative bacteria, mimic the outside of bacteria, resembling a pathogen but, as they are non-living, lack the ability to cause associated disease. OMV are primarily comprised of bacterial outer membrane constituents and thus contain key antigenic components required to elicit a protective immune response. For this reason, soon after their discovery, OMV were proposed as a vaccine platform.

Spontaneous release of vesicles from bacteria results in the formation of native OMV (nOMV), but for many species this occurs at levels too low for application to vaccine manufacture. To overcome low yields, vesicle-like aggregates of insoluble outer membrane proteins have been chemically

extracted from whole bacteria using detergents (e.g. deoxycholate). The process results in detergent-extracted OMV (dOMV). As well as improving production, the use of detergents also reduces LPS content and hence OMV endotoxicity. However, the approach also leads to the loss of important protective lipoprotein antigens, compromised vesicle integrity, and contamination of the resulting preparation with cytoplasmic proteins [25, 26] (Table 1).

More recently, bacteria have been genetically manipulated to increase blebbing, producing mutant-derived OMV (mdOMV) also called GMMA (Generalized Modules for Membrane Antigens) (Table 1). The production of extracellular vesicles can be increased greatly by the disruption of genes involved in crosslinking the outer membrane and peptidoglycan layer in the periplasm. The common genetic target for disruption, leading to high-yield production of mdOMV, is *tolR* of the Tol-Pal system which is present in most Gram-negative bacteria [27-30]. However, the Tol-Pal system is not ubiquitous. Furthermore, deletion of the Tol-Pal system in some *E. coli* strains leads to a defect in polymerization of LPS O-antigen [31], a key target of protective immunity.

E. coli mdOMV have been over-produced by deletion of *nlpI* leading to changes in peptidoglycan dynamics [32]. Disruption of the VacJ/Yrb ABC (ATP-binding cassette) transport system has been used to increase mdOMV production by *Haemophilus influenzae*, *V. cholerae* and *E. coli*. This is thought to occur through the accumulation of phospholipids in the outer leaflet of the outer membrane [33, 34]. Deletion of Lpp [35], MltA (*gna33*) [25], RmpM [36, 37], OmpT [38], PagL [39], enterobacterial common antigen [33, 40], DegP [13] and Virk [41] also result in over-blebbing of the bacterial outer membrane. Mutations in genes responsible for increasing the membrane curvature can also lead to dmOMV production [42].

Purification of naturally-released OMV is straightforward: a first tangential flow filtration (TFF) allows separation of culture supernatant containing OMV from whole bacteria, while a second TFF retains OMV while removing soluble proteins and other low molecular weight impurities [43, 44].

Recently continuous-mode production of *Neisseria meningitidis* OMV has been investigated as a means of increasing nOMV yield and reducing production costs [45].

Additional mutations can be introduced into OMV-producing bacterial strains in order to reduce OMV endotoxicity (Table 1). This is often through the modification of lipid A structure. Lipid A, a component of LPS, is a phosphorylated and acylated diglucosamine recognized by Toll like receptor (TLR) 4 and myeloid differentiation factor 2 (MD-2) on host innate immune cells [46]. The interaction of lipid A and the TLR4/MD-2 complex results in an inflammatory response which can lead to fever and potentially death. Alterations in the number of acyl chains and phosphate groups of lipid A leads to structural rearrangements [46-48] decreasing ligand affinity for the TLR4/MD-2 complex and subsequently reducing the inflammatory response [49, 50]. The number of acyl chains can be genetically manipulated by the deletion of the acyltransferases involved in the late biosynthesis and secondary acylation of lipid A. In *E. coli*, *Shigella* and *Salmonella* these enzymes are encoded by *msbB* (*lpxM*), *htrB* (*lpxL*) and *pagP*, which respectively add a myristoyl, lauroyl or palmitoyl group to the lipid A [51-54]. In *Neisseria meningitidis*, secondary acylation of lipid A is performed by *lpxL*1 and *lpxL*2 [55-61].

mdOMV can be further manipulated for overexpression of homologous or heterologous protein antigens which can serve as vaccine antigens, in order to increase the breadth and magnitude of the immune response induced, resulting in increased protection against different strains of the same bacterial species or against multiple species (Table 1). In this review, OMV derived from bacterial strains genetically mutated to increase blebbing and/or reduce endotoxicity and/or overexpress desired antigen and/or delete unwanted antigens are considered to be mdOMV.

Figure 1 visualizes the formation of the different types of OMV mentioned above together with the nomenclature used.

3.1 OMV as vaccines against Gram-negative bacteria

3.1.1 OMV-based vaccines against meningococcal pathogens

A dOMV based vaccine (VA-MENGOC-BC, Finlay Institute, Havana, Cuba) was first licensed for use in Cuba against *Neisseria meningitidis* serogroup B (MenB) in 1987 [62]. Subsequently dOMV have been successfully employed as vaccines to prevent meningococcal group B outbreaks, in Norway [63, 64] and New Zealand (MeNZB) [65, 66] with dOMV from NZ98/254, a New Zealand outbreak-specific strain. OMV are prepared from manufacturing strains that match circulating strains causing epidemic meningococcal group B disease. The efficacy of these vaccines has been estimated at 83% in Cuba, 57% to 87% in Norway and at least 70% in New Zealand [62, 66-68]. The main immunogenic protein in *N. meningitidis* dOMV is PorA, which is highly variable between strains [69]. As a result, the immune response elicited by dOMV vaccines is mostly strain-specific and so a monovalent dOMV vaccine is inadequate in more common endemic situations with multiple circulating meningococcal B strains.

A novel multivalent vaccine (Hexamen) was developed in the Netherlands based on dOMV-derived from genetically-engineered strains containing genes encoding six subtypes of PorA. In a clinical phase I study, a single dose of 15 μg of each of the individual PorA induced a four-fold increase in bactericidal antibodies against all six isogenic strains in approximately half of volunteers [70, 71]. The MenPF-1 vaccine against MenB, containing dOMV overexpressing the ferric enterobactin receptor FetA, has been shown to be safe, well tolerated and able to induce high bactericidal titers in a phase I clinical trial [72]. A multi-component meningococcal B vaccine, 4CMenB (Bexsero)[73] was approved for human use by the EMA and the FDA in 2013. It added three surface-exposed recombinant proteins to MeNZB dOMV. These proteins are a fusion protein containing NHBA, a fragment of NadA and a fusion protein containing fHbp. The antigens were identified through reverse vaccinology [74]. 4CMenB is conservatively estimated to provide 66-91 % coverage against meningococcal serogroup B strains worldwide [12].

At the Norwegian Institute of Public Health, dOMV against *N. meningitidis* serogroup A and W have been evaluated, showing equal or higher serum bactericidal assay (SBA) and opsonophagocytosis assay (OPA) titers compared to licensed unconjugated and conjugated polysaccharide meningococcal

vaccines in mice [75]. High SBA titers against both MenA and MenW vaccine strains were detected after only one dose of dOMV, and the titers further increased after a second dose. At the Finlay Institute, the same process for production of dOMV against MenB has been used to obtain dOMV from *N. meningitidis* serogroup A (dOMVA), serogroup W (dOMVW), and serogroup X (dOMVX). The same extraction process has since been applied to obtain OMV on a research scale from *Vibrio cholerae* (dOMVC), *Bordetella pertussis* (dOMVBP), *Mycobacterium smegmatis* (dOMVSM), and *Bacillus Calmette–Guérin* (dOMVBCG). All these dOMV have been evaluated for specific antibody induction and, using functional bactericidal assays and bacterial challenge studies in mice, have been shown to have protective potential [76].

Comparative proteomic analysis has revealed differences in the protein composition of dOMV and nOMV [25, 26]. Detergent treatment leads to the loss of lipoproteins, some of which are targets of broadly-protective antibody responses, such as fHbp and NHBA. For this reason, recent research has focused on the development of vaccines based on nOMV which are naturally released from bacteria. MenB nOMV elicited both a mucosal immune response and a systemic bactericidal antibody response after intranasal immunization in mice [77]. Vaccination of healthy volunteers demonstrated the ability of nOMV to induce serum antibodies with strong bactericidal activity [78].

More recent work has focused on the use of mdOMV as candidate vaccines against meningococcus. mdOMV produced at high yield, avoiding the use of detergent, are deficient in capsular polysaccharide and contain genetically-modified LPS to reduce reactogenicity [57, 61, 79, 80]. Genetically-detoxified mdOMV confer protection against MenB nasopharyngeal colonization [81] and induce broad serum bactericidal antibody responses against multiple *N. meningitidis* serogroups in mice [82-89], rabbits [90, 91] and monkeys [92].

Koeberling et al. developed an mdOMV-based vaccine derived from an African *N. meningitidis* W strain with deleted capsule locus, *lpxL1* and *gna33* and overexpressed fHbp v.1, which is expressed by the majority of serogroup A and X isolates. This candidate vaccine has potential as an affordable

vaccine with broad coverage against meningococcal strains from all main serogroups currently causing meningococcal meningitis in sub-Saharan Africa [61].

The Walter Reed Army Institute of Research developed an mdOMV vaccine containing modified LPS secondary to the deletion of *lpxL1*, enhanced expression of fHbp variant 1, stabilized expression of OpcA and expression of a second PorA. The vaccine induced antibodies with serum bactericidal activity and had acceptable reactogenicity in a phase I clinical study [79]. Table 2 summarizes *Neisseria meningitidis* type B OMV vaccines tested so far in clinical trials.

Following widespread use in New Zealand in 2004-2008 of the dOMV-based vaccine MeNZB to control an outbreak of *N. meningitidis* B, reduced rates of gonorrhoea were retrospectively detected among sexual health clinic patients, with an estimated vaccine effectiveness against gonorrhoea of 31% (95% CI 21-39) [93]. Subsequently efficacy against *N. gonorrhoea* was found with other dOMV-based vaccines against *N. meningitidis* B in Cuba and Canada [94]. As recently reported [95, 96], these findings suggest that the use of meningococcal or gonococcal OMV could represent a rational approach for the development of a vaccine against *N. gonorrhoea*.

3.1.2 OMV-based vaccines against non-meningococcal pathogens

OMV-based candidate vaccines are being developed at the preclinical level against many other pathogens (Table 2), and have demonstrated ability to induce humoral and cellular immune responses [24].

Liu et al. showed that nOMV from flagellin-deficient *S.* Typhimurium induced strong mucosal and systemic responses and provided effective protection against challenge with diverse *Salmonella* serovars. Cross-protection was thought to be the result of antibodies against outer membrane proteins shared by different *Salmonella* serovars [97]. At the University of Navarra, nOMV from *S. flexneri* 2a were able to protect mice against shigellosis when administered by the nasal or conjunctival route. Enhanced protection was achieved after intradermal immunization when these nOMV were encapsulated in poly(anhydride) nanoparticles [98]. Mitra et al. produced nOMV from

different *Shigella* strains that mixed together could confer significant passive protection in neonatal mice [99]. Mucosal immunization with nOMV-derived from *V. cholerae* induced long-term protective immune responses against this gastrointestinal pathogen [100-103]. Mixing of genetically-detoxified mdOMV from enterotoxigenic *E. coli* and *V. cholerae* resulted in protection against both pathogens [53]. Roier et al. created nOMV mixtures of nontypeable *H. influenzae* (NTHi) strains and intranasal immunization with these NTHi nOMV resulted in a robust humoral and mucosal immune response [104]. *B. pertussis* nOMV candidate vaccines have been shown to raise efficiently nOMV-specific antibodies in mice to a level comparable with that obtained with the current approved whole cell *B. pertussis* vaccine [105-107]. *B. pertussis* nOMV were found to be more effective against a current circulating isolate than the whole-cell vaccine and able to induce long-lasting immunity [107]. Mice vaccinated with nOMV from *Francisella* were protected against subsequent challenge [108].

The emergence of multidrug-resistant bacterial isolates has highlighted the need to develop alternative strategies for managing *P. aeruginosa* infections. Naturally secreted *P. aeruginosa* nOMV trigger significant inflammatory responses via the TLR4 signaling pathway and protect mice against pseudomonal lung infection [109]. The humoral immune response appears to be critical for OMV-mediated protection [110]. More recently, the nOMV-based approach has been tried against *Klebsiella pneumoniae* with extracellular vesicles found to induce protection in a preclinical animal model with a mechanism dependent both on humoral and cellular immunity [111]. nOMV from carbapenem-resistant *Klebsiella pneumoniae*, combined with size-controlled BSA nanoparticles to obtain uniform and stable vaccines through hydrophobic interaction, induced high specific antibody titers in mice. The survival rate of the mice infected with a lethal dose of carbapenem-resistant *Klebsiella pneumoniae* was significantly increased after nOMV immunization [112].

mdOMV released from Gram-negative bacteria mutated to increase blebbing and reduce endotoxicity through de-acylation of the lipid A moiety, also called GMMA, have been developed as vaccine candidates against *Shigella* [43] and non-typhoidal *Salmonella* [113, 114]. *S.* Typhimurium and *S.* Enteritidis GMMA induced high anti-O-antigen-specific IgG responses comparable to those induced by corresponding CRM_{197} glycoconjugates formulated with alum. Immunization with these mdOMV/GMMA resulted in a more diverse IgG subclass profile and enhanced bactericidal activity than the equivalent glycoconjugates which mainly induced IgG1 [115]. Immunization with the mdOMV also reduced bacterial colonization of mice subsequently infected with *Salmonella* strains. More recently a similar comparison in mice was performed for *Shigella flexneri* 6 showing that mdOMV and glycoconjugate elicited similar levels of persistent anti-O-antigen IgG with bactericidal activity, when formulated on Alhydrogel [116].

A *Shigella sonnei* mdOMV vaccine (1790GAHB) has been tested in clinical studies, and found to be well tolerated and to elicit anti-LPS O-antigen-specific antibodies in healthy European adults and in adults in Kenya where shigellosis is endemic [117, 118]. The vaccine was also able to boost this antibody response in previously primed adults [119]. The same technology is being applied to the development of a 4-component vaccine consisting of mdOMV from *S. sonnei* and three of the most prevalent *S. flexneri* serotypes, *S. flexneri* 1b, 2a and 3a.

3.2 OMV as carrier for heterologous antigens

An additional feature of OMV is their ability to be decorated with specific proteins or polysaccharides from heterologous pathogens.

Kesty and Kuhen were the first to demonstrate the possibility of incorporating heterologously-expressed outer membrane and periplasmic proteins into mdOMV produced by laboratory and pathogenic *E. coli* strains [120]. Periplasmic expression of such recombinant antigens is relatively simple and results in the accumulation in the lumen of mdOMV [121-123]. Expression of heterologous antigens on the mdOMV surface can be achieved by fusing a target protein to

membrane-associated proteins. For example, heterologous proteins have been exported to the outer membrane surface fused to the β-barrel domain of autotransporters (e.g. Hbp, AIDA), toxins (e.g. ClyA) and outer membrane proteins (e.g. OmpA) [124-129].

Engineered *E. coli* mdOMV displaying green fluorescent protein (GFP), fused with bacterial hemolysin ClyA, elicited stronger anti-GFP antibody titers in immunized mice compared to GFP alone [128]. Mice immunized intranasally with S. Typhimurium mdOMV engineered to express the pneumococcal protein PspA in the lumen, induced serum antibody responses against PspA, in addition to responses against *Salmonella* LPS and outer membrane proteins. No detectable antibody responses to PspA were developed in mice immunized with an equivalent dose of recombinant PspA [130]. Immunization with *E. coli* mdOMV expressing *A. baumannii* Omp22 rapidly generated significantly higher Omp22-specific antibodies than immunization with higher amounts of recombinant Omp22 protein formulated with alum [131].

Other examples of OMV expressing heterologous proteins have been reported, demonstrating that OMV often induce humoral immune responses far more effectively than recombinant antigenic proteins formulated with alum [61, 124, 132]. *V. cholerae* OMV when tested as delivery vehicles for ETEC antigens CFA/I and FliC induced a detectable immune response against both organisms [53]. In another study, intranasal immunization with *V. cholerae* mdOMV expressing *E. coli* alkaline phosphatase PhoA induced a small but significant specific immune response against PhoA [121]. Proteins from Group A Streptococcus (GAS) (e.g. Slo, SpyCEP, SpyAD) and Group B Streptococcus (GBS) (e.g. SAM_1372) were fused to the *E. coli* OmpA leader sequence and expressed in the lumen of *E. coli* mdOMV. These mdOMV induced high functional antibody titers in mice against the recombinant form of the proteins. Furthermore, immunization with Slo-OMV and SpyCEP-OMV protected mice against lethal challenge with GAS [122].

The hemoglobin protease (Hbp) autotransporter of *E. coli* has been used to display two *S. pneumoniae* protein antigens on *Salmonella* mdOMV. Intranasal immunization with *Salmonella* mdOMV

displaying high levels of these antigens at the surface induced strong protection in a murine model of pneumococcal colonization, without the need for a mucosal adjuvant [129]. The Hbp platform was also used to display *Mycobacterium tuberculosis* antigens on the surface of *E. coli* and *Salmonella* Typhimurium mdOMV [125].

Recently the feasibility of displaying heterologous glycans on mdOMV has been demonstrated, resulting in glycoengineered OMV (glyOMV) [133]. This approach combines glycan biosynthesis with OMV formation in *E. coli* laboratory strains, where carbohydrates are directly linked to lipid A [134-136]. Using this platform, the O-antigen from *Francisella tularensis* has been displayed on mdOMV from a hyperblebbing *E. coli* strain. Immunization with these glyOMV resulted in sustained O-antigen-specific IgG production in mice, 2–3-fold higher than IgG levels elicited by the native LPS, and protected the animals against lethal challenge with *F. tularensi* [134]. A protective mucosal immune response was generated with subcutaneous administration of these glyOMV. glyOMV have also been tested as vaccine candidates against *S. pneumoniae* serotype 14 in mice and *Campylobacter jejuni* in chickens [136]. *S. pneumoniae* serotype 14 glyOMV induced IgG and opsonophagocytic activity comparable to those induced by Pneumococcal Conjugate Vaccine PCV13. This approach has also been investigated for the development of a vaccine candidate against a prominent uropathogenic *E. coli* (UPEC) serotype, O25b [133].

Heterologous antigens can also be attached post-OMV production, for example by expressing SpyTags on mdOMV that are then coupled to SpyCatcher fused to any protein [137, 138]. This approach utilizes the SpyCatcher domain from a *Streptococcus pyogenes* surface protein, which recognizes a cognate 13-amino-acid peptide (SpyTag). After recognition, a covalent isopeptide bond is formed between the side chains of a lysine in SpyCatcher and an aspartate in SpyTag [139]. Other different conjugation strategies have been proposed for linkage of heterologous proteins or polysaccharides to the mdOMV surface. It has been shown that mdOMV decorated with heterologous polysaccharide or protein antigens promote enhanced immunogenicity compared to

traditional formulations (e.g. recombinant proteins and glycoconjugate vaccines), without negative impact to the anti-mdOMV immune response [140-142]. *Brucella* LPS [143] and capsular polysaccharides from serogroup C meningococci [144] have been conjugated successfully to *Neisseria meningitidis* serogroup B nOMV.

4. OMV and Immunity

Multiple animal studies suggest that OMV are more immunogenic than traditional vaccine formulations consisting of adjuvanted recombinant proteins or glycoconjugate vaccines [145] (Table 3). The following factors could contribute to this enhanced immunity: 1) size of OMV (in the range of 20-250 nm) facilitates their entry into lymph nodes (LNs) through direct lymphatic drainage and phagocytosis and subsequent carriage by antigen presenting cells (APCs) [146, 147]; 2) OMV co-deliver several types of Pathogen-Associated Molecular Patterns (PAMPs) [11, 148]; 3) OMV present multiple copies of antigens to the immune system in their natural conformation and orientation in the context of a bacterial outer membrane (Figure 2).

Salmonella dOMV have been found to induce the expression of CD86 and MHC class II molecules on dendritic cells and the production of TNF-α and IL-12, and promote the development of protective B cell and T cell response *in vivo* [149]. As verified by adoptive transfer and gene-knockout studies, the protective effect of *E. coli* OMV immunization has been shown to be primarily driven by the stimulation of T cell immunity, especially by production of IFN-γ and IL-17 from T cells. Investigating the bacterial-killing ability of macrophages, IFN-γ and IL-17 production were found to be the main factors promoting bacterial clearance [150].

Lapinet et al. verified that *N. meningitidis* dOMV can stimulate human neutrophils, resulting in the production of TNF-α and IL-1β and upregulation of CXCL8, CCL3 and CCL4 [151]. Jäger et al. showed that *Legionella pneumophila* nOMV can generate pro-inflammatory cytokines from macrophages [152]. Luirink et al. prepared *S.* Typhimurium mdOMV expressing Ag85B, an antigen of *Mycobacterium tuberculosis*, and showed that delivery of the mdOMV to dendritic cells *in vitro*

primed Ag85B-specific T cell hybridomas to secrete IL-2 [125]. mdOMV can interact with other host cells including epithelial cells, resulting in the production of cytokines and chemokines that activate the pro-inflammatory response. nOMV from *P. aeruginosa* elicited IL-8 secretion from lung epithelial cells contributing to the inflammation response [153].

As mentioned previously, OMV contain PAMPs that can interact with the corresponding Pattern Recognition Receptors (PRRs) on APCs resulting in their activation, which is required for a strong adaptive immune response [145]. A major role in the recognition of OMV PAMPs has been attributed to TLR4, which is involved in the detection of the lipid A moiety of LPS [154]. However, OMV also contain other PAMPs. TLR2 is involved in the recognition of bacterial lipoproteins [155]; TLR5 is the receptor that recognizes flagellin, which may be present in OMV preparations from flagellated bacteria [156, 157]. TLR9 is involved in the recognition of unmethylated CpG motifs that are prevalent in bacterial DNA [153, 158, 159], and TLR13 recognizes bacterial ribosomal RNA [160]. TLR9 and TLR13 have a minor role in the immune response to OMV, since their cognate molecules are rarely found in OMV preparations.

Few studies have been performed so far to elucidate the specific contribution of TLR activation to the immune response induced by OMV. The role of TLR2 and TLR4 has been investigated in the induction of immune responses in mice after immunization with *N. meningitidis* dOMV [161]. TRIF-deficient and TLR4-deficient mice showed reduced IgG antibodies and lower levels of bactericidal antibodies following immunization, whereas the immune response was not impaired in TLR2-deficient mice, suggesting that TLR4 but not TLR2 stimulation is important for the immunogenicity of the *N. meningitidis* dOMV. It has also been shown that induction of IL-10 and type I interferon-dependent, antigen-specific and IFNγ-secreting CD8+ cytotoxic T lymphocyte responses in mice by *N. meningitidis* nOMV requires LPS. The inclusion of exogenous PAMPs as immune stimulators (TLR3, TLR4, TLR7, and TLR9 agonists) was required to generate a robust immune response [162-164].

Using Human Embryonic Kidney cells stably transfected to express specific TLRs, *Shigella* [165], *Salmonella* [166] and *Neisseria meningitidis* [61] mdOMV have been shown to activate TLR2 efficiently. Removal of acyl chains from the LPS of these mdOMV has been used as a strategy to reduce endotoxicity while maintaining sufficient PAMP/PRR-mediated immune stimulation. By blocking the targets of TLRs in human PBMC, alone or in combination, the relative contribution of TLR2, TLR4 and TLR5 to induce a pro-inflammatory response to *Shigella* [165] and *Salmonella* [166] dmOMV with modified lipid A has been examined. These experiments demonstrate that TLR4 stimulation is drastically reduced and remaining pro-inflammatory responses are mostly mediated by TLR2 activation. The relative contribution of PRRs other than TLR2, TLR4 and TLR5 appears to be marginal. However, studies with LPS-deficient mdOMV from *Neisseria* demonstrated the ability of non-LPS PAMPs (TLR3, TLR7, and TLR9 agonists) to enhance immune responses to vesicles [163, 164].

The adjuvant activity of OMVs has been investigated by mixing them with model antigens. A combination of keyhole limpet hemocyanin (KLH) and mdOMV induced a significantly stronger KLH-specific antibody response and KLH-specific T cell activation compared to KLH alone. The same finding was obtained by mixing mdOMV with ovalbumin (OVA) and an OVA-derived peptide as models [167]. OMV have also been shown to provide adjuvant activity to hepatitis B virus surface antigen (HBsAg), with production of robust HBsAg-specific IgG and IgA response [168]. In contrast, a physical mixture of GFP with *E. coli* mdOMV did not result in an anti-GFP IgG response in mice, while expression of GFP on mdOMV resulted in similar response to GFP adjuvanted with alum [128]. Additional studies have shown that chemical conjugation of antigens to mdOMV results in improved immunogenicity compared to when the antigen is simply mixed with mdOMV [140-142]. These findings warrant further investigation and could help to understand the main features of the immune response to OMV-based vaccines.

When the immune response of Borrelial surface-exposed lipoprotein OspA in *Neisseria meningitidis* mdOMV [169] was compared with that of the luminal-expressed OspA in the same

mdOMV in mice, only surface-exposed OspA was able to elicit an OspA-specific antibody response. In contrast, several studies have demonstrated the ability of antigens expressed in the lumen of mdOMV to induce a significant immune response. Therefore the principles remain unclear as to whether localizing a heterologous antigen on the OMV surface results in an enhanced immune response compared with luminal expression [121-123].

5. Future directions and considerations

The success of dOMV-based vaccines against *N. meningitidis* serogroup B have encouraged the application of the OMV platform for the development of other vaccines (Table 2). Over the years, use of detergents to increase OMV yields to levels useful for vaccine manufacture has been superseded by genetic engineering. OMV naturally released from bacteria can be very easily purified, supporting the development of affordable vaccines [6]. Furthermore, OMV naturally released from bacteria are more representative of the outer surface of bacteria. Multiple antigens are presented in their natural orientation and conformation, a format found to confer enhanced and broader protection in most scenarios in mice compared with dOMV [82-92], where treatment with detergents destroy bacterial membranes and leads to the loss of important protective antigens, particularly lipoproteins and LPS (Table 1).

The inherent characteristics of OMV support their potential to induce strong immune responses (Figure 2) [11, 24]. However, we still only understand a limited amount about the mode of action of OMV in mice and even less so in humans. More studies are required to better explain how OMV work, and such studies will improve the design of OMV-based vaccines. Clinical studies will be fundamental to corroborate preclinical findings. Even though the OMV platform has been applied to many pathogens at the preclinical level, only a few candidate OMV vaccines have to date reached the clinical stage. This is likely related to the relative lack of familiarity with the OMV platform compared with more traditional vaccine technologies, to regulatory issues involved in novel vaccine

development and manufacturing, and to the analytical complexity of OMV-based vaccines compared to more defined subunit vaccines.

OMV have already proven utility as a flexible vaccine platform. As reported, there are many examples of their use for the display of both protein and polysaccharide antigens. Decoration of OMV with antigens from a pathogen different from the one the OMV derives opens the possibility of using OMV for the development of multicomponent vaccines that confer protection against multiple pathogen species.

However, genetic manipulation, not only for antigen expression but also for overblebbing and reduction of reactogenicity, requires expertise and time in order to identify successful strategies that do not impact other features of OMV that are critical for immunogenicity. For example, mutations introduced to increase blebbing or to modify lipid A structure can result in reduced levels of LPS O-antigen, a key target of protective immunity, or in compensatory mutations that maintain numbers of lipid A acyl chains and consequently endotoxicity [31, 113]. As yet, there are no proven universal OMV development strategies, and optimal approaches need to be identified for each specific antigen and each different OMV-producing bacterium. Expression of high specific antigen levels on OMV can be difficult. Inducible promoters are often usedat the preclinical stage, but are not compatible with production processes at large scale. Development of a generic OMV with known expression systems will facilitate expression of heterologous antigen [44]. In such a scenario, OMV are not employed to target a specific disease per se but as vehicles for specifically expressed antigens. In addition, chemical conjugation has been proposed as a valuable approach for OMV decoration with protein and polysaccharide antigens. Conjugation can be tool used rapidly to generate novel OMV-antigen constructs and verify their ability to induce a good immune response in animal models. With this approach, antigens density and orientation can now be modulated with relative ease. Once optimal design has been identified, genetic expression can then be adopted to simplify large scale manufacture.

More recently OMV have been explored as cancer immunotherapy agents, drug delivery vehicles and as carrier for viral antigens, further expanding the boundaries of the platform. Some examples have been reported showing ability of mdOMV to accumulate in tumor tissues, induce an antitumor immune response and finally eradicate the tumors, after intravenous injection in mice [11]. Decoration of mdOMV with targeting ligands can facilitate the accumulation of drugs at specific target locations. Passive diffusion and *in vivo* loading have been proposed to encapsulate drugs into OMV [170, 171]. Decoration of mdOMV with viral antigens has been shown to protect against viral infections in animals [172].

In conclusion, OMV are and will continue to be a promising technology for vaccine development. They are immunogenic and their ease of production makes them particularly suitable for low-cost vaccine manufacture. The option of using genetic manipulation of parental OMV-producing bacterial strains in order to increase OMV release, upregulate homologous and/or heterologous antigens expression and downregulate unwanted antigens makes this platform particularly flexible. There is the opportunity to develop OMV to protection not only against the Gram-negative bacteria from which they derive, but also against Gram-positive and viral pathogens and good potential to develop affordable multivalent OMV vaccines.

Conflict of interest

This work was undertaken at the request of and sponsored by GlaxoSmithKline Biologicals SA. GSK Vaccines Institute for Global Health Srl is an affiliate of GlaxoSmithKline Biologicals SA. Francesca Micoli is an employee of the GSK group of companies. Calman MacLennan declares no conflict of interest.
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Table 1. Main features of dOMV and mdOMV compared with nOMV, highlighting advantages and disadvantages of different types of OMV.

	nOMV	**dOMV**	**mdOMV**
Production	Naturally released from Gram-negative bacteria	Detergent treatment promotes OMV release from whole bacteria	Natural released from genetically-modified bacteria
Manufacturability	Low yield (depending on bacterial genus/species and nOMV-production strain)	High yield	High yield
	Simple and economic purification	Possible batch to batch variation due to heterogeneity of dOMV	Simple and economic purification
			Potential difficulty with identifying target genes for mutation
			Generation of optimal mdOMV-producing strain can be problematic due to unwanted secondary mutations
Safety	High potential reactogenicity due to unmodified PAMPs, particularly LPS	Detergent extraction reduces LPS/lipoprotein content and hence OMV endotoxicity	Lipid A is genetically detoxified, toxins and unwanted antigens can be deleted
Immunogenicity	Self-adjuvanticity due to PAMPs	Reduced levels of PAMPs	Self-adjuvanticity due to retained PAMPs, depending on extent of PAMP removal/modification
	Low expression levels of relevant protective antigens	Potential loss of important protective antigens (e.g. lipoproteins, LPS)	Potential for broad protection by over-expression of species-conserved antigens
	Often strain-specific rather than species-specific protection	Often strain-specific rather than species-specific protection	Potential for expression of specific homologous and heterologous antigens (both proteins and polysaccharides) resulting in broad cross-strain and cross-species protection

Table 2. *Neisseria meningitidis* type B OMV vaccines tested in clinical trials and key vaccines currently in development against non-meningococcal pathogens (n.b. clinical trials with non-meningococcal vaccines have to date been limited to the 1790GAHB *S. sonnei* vaccine)

Pathogen	Vaccine	Type OMV	Developer	References
N. meningitidis serogroup B	VA-MENGOC-BC	dOMV	Finlay Institute, Havana, Cuba	**[62]**
	MeNZB	dOMV	Novartis Vaccines, Italy, and National Institute of Public Health, Norway	[66]
	-	dOMV	National Institute of Public Health, Norway	[67]
	Hexamen	dOMV	Laboratory of Vaccine Development and Immune Mechanisms, RIVM, The Netherlands	[71]
	MenPF-1	dOMV	Oxford Vaccine Group, University of Oxford, UK	[72]
	4CMenB	dOMV	Novartis Vaccines and Diagnostics, Italy	[12]
	-	nOMV	Walter Reed Army Institute of Research (WRAIR), USA	[78]
	-	mdOMV	Walter Reed Army Institute of Research (WRAIR), USA	[79]
N. gonorrhoea	*Ng* outer membrane vesicles (OMVs) with IL-12	mdOMV	University at Buffalo, USA	[96]
EXPEC	-	mdOMV	Washington University	[133]
K. pneumoniae	Bacteria-derived extracellular vesicles	nOMV	AEON Medix	[111]
nontyphoidal *Salmonella*	Bivalent NTS GMMA	mdOMV	GSK Vaccines Institute for Global Health, Italy	[115]
Shigella	*S. sonnei* GMMA (1790GAHB)	mdOMV	GSK Vaccines Institute for Global Health, Italy	[118]
	4-component *Shigella* GMMA	mdOMV	GSK Vaccines Institute for Global Health, Italy	
Mycobacterium tubercolosis	Tubercolosis vaccine	mdOMV	Abera Bioscience, Sweden	[125]

V. cholerae	-	nOMV	Tufts University School of Medicine, Boston, USA	[100, 102]
	-	nOMV	Pasteur Institute Tehran, Iran	[101]
	-	nOMV	Shahed University, Tehran, Iran	[103]
nontypeable *H. influenzae*	-	nOMV	University of Graz, Austria	[104]

Table 3. Animal studies showing that OMV-based candidate vaccines are more immunogenic compared with equivalent classical vaccine formulations.

OMV based candidate vaccine	**Classical vaccine**	**References**
PorA on *N. meningitidis* mdOMV	*N. meningitidis* PorA protein	[173]
N. meningitidis mdOMV over-expressing fHbp	*N. meningitidis* fHbp protein	[61, 174]
Salmonella Typhimurium mdOMV expressing PspA	Pneumococcal PspA protein	[130]
E. coli mdOMV expressing Omp22	*A. baumannii* Omp22 protein	[131]
E. coli mdOMV expressing HtrA	*Chlamydia* HtrA protein	[124]
E. coli mdOMV expressing different heterologous antigens	Corresponding protein antigens	[132]
B. pertussis nOMV	*B. pertussis* whole cell and acellular vaccines	[105]
S. Typhimurium and *S.* Enteritidis mdOMV	*S.* Typhimurium and *S.* Enteritidis O-antigen CRM_{197} glycoconjugates	[115]
E. coli mdOMV displaying O-antigen from *F. tularensis*	*F. tularensis* LPS	[175]
A+W meningococcal dOMV	Meningococcal conjugate- and polysaccharide vaccines	[75]

Legend Figures

Figure 1. OMV formation. Native OMV are naturally released from Gram-negative bacteria (nOMV). OMV yield can be increased by detergent-extraction resulting in detergent-extracted OMV (dOMV). Increasingly, OMV can be derived from bacteria genetically manipulated to destabilise the linkage of the outer membrane with the inner membrane and peptidoglycan layer in the periplasm, thereby upregulating OMV release. Additional mutations can be introduced to reduce lipid A endotoxicity and/or enhance expression of specific protein or polysaccharide antigens. All OMV derived from genetically-manipulated bacteria are here indicated as mdOMV. OMV mutated for expressing heterologous polysaccharides are also called glycoengineered OMV (glyOMV).

Figure 2. Key factors proposed to contribute to OMV-induced immune response. The particulate nature of OMV is thought to increase uptake by antigen-presenting cells (APCs). APCs activation occurs through recognition of multiple PAMPs on OMVs by Toll-like receptors (TLRs). B cell activation is facilitated by the repetitive surface organization of OMV (through cross-linking of B cell receptors (BCR)) and by the 20–250 nm size of OMV, which allows them direct access to the lymphatic system. T cell–B cell collaboration is essential for the generation of high affinity antibody-producing plasma cells and memory B cells.

Figure

Figure 1

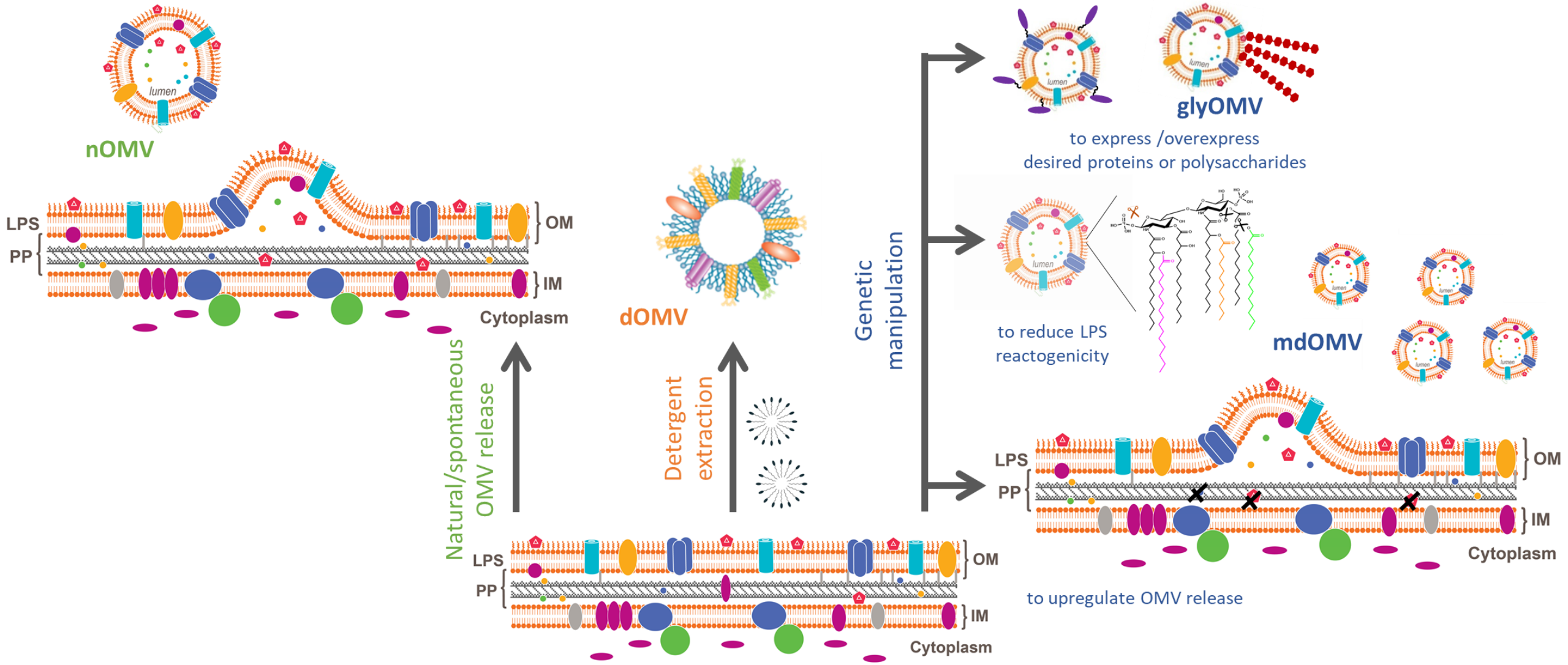
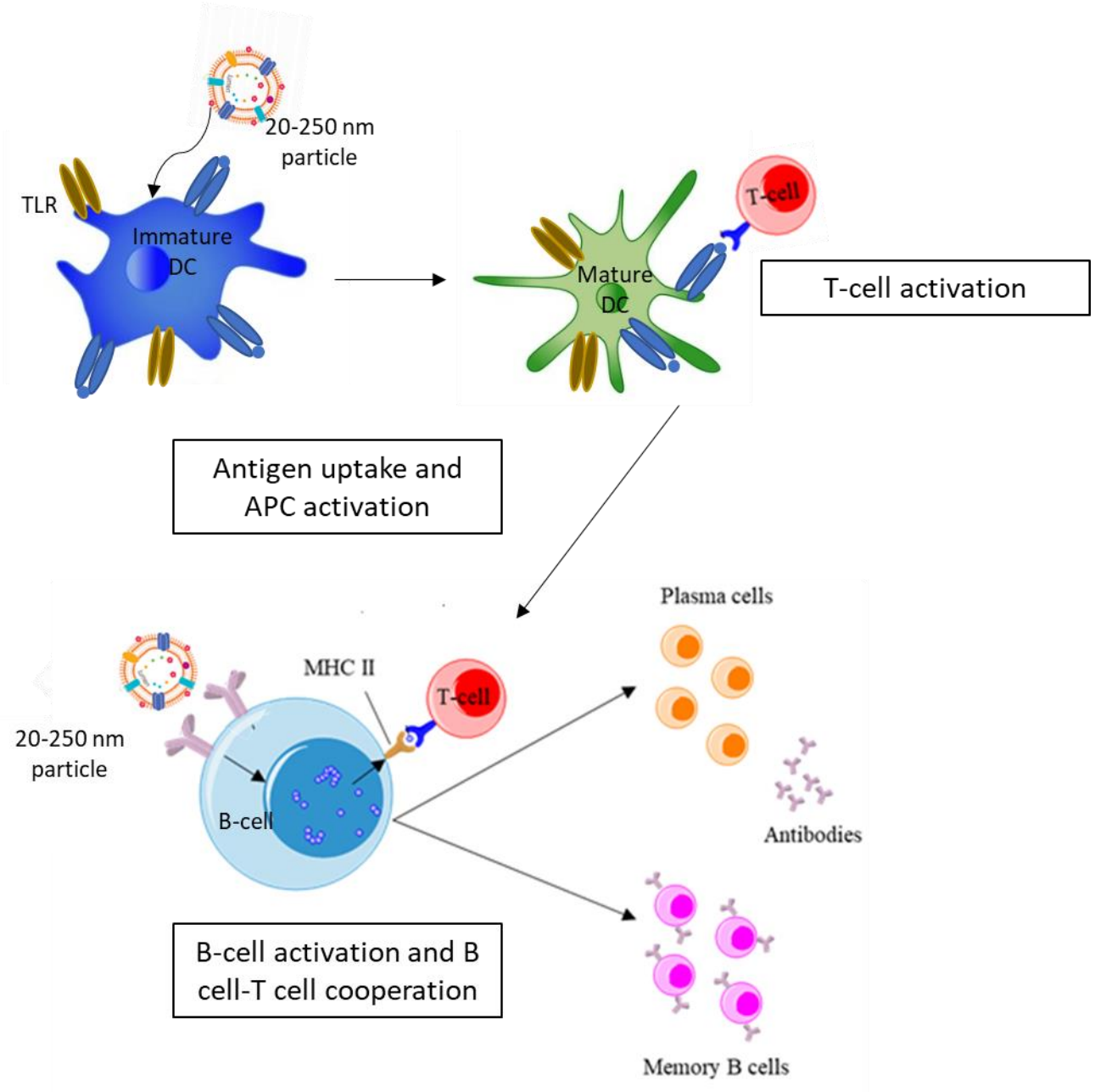


Figure 2


20-250 nm particle
TLR
Immature DC
Mature DC
T-cell
T-cell activation
Antigen uptake and APC activation
Plasma cells
MHC II
T-cell
20-250 nm particle
B-cell
Antibodies
B-cell activation and B cell-T cell cooperation
Memory B cells