Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

CHAPTER ONE

The Role of Self-Assembling Lipid Molecules in Vaccination

Luis Martinez-Gil*,[1], Peter H. Goff[†], Gene S. Tan[‡]
*ERI BioTecMed, University of Valencia, Burjassot, Valencia, Spain
[†]Icahn School of Medicine at Mount Sinai, New York, NY, United States
[‡]J. Craig Venter Institute, La Jolla, CA, United States
[1]Corresponding author: e-mail address: luis.martinez-gil@uv.es

Contents

Abstract

The advent of vaccines represents one of the most significant advances in medical history. The protection provided by vaccines has greatly contributed in reducing the number of cases of infections and most notably to the eradication of small pox. A large number of new technologies and approaches in vaccine development are currently being investigated with the goal of providing the basis for the next generation of prophylactics against an ever-expanding list of emerging and reemerging pathogens. In this chapter, we will focus on the role of lipids and lipid self-assembling vesicles in new and promising vaccination approaches. We will start by describing how lipids can induce activation of the innate immune system and focus on some

Advances in Biomembranes and Lipid Self-Assembly, Volume 27
ISSN 2451-9634
https://doi.org/10.1016/bs.abl.2017.12.001

lipid-derived vaccine adjuvants. Next, we will review current lipid-based self-assembling particles used as vaccine platforms, specifically liposomes and virus-like particles, and how virus-like particles have facilitated research of highly pathogenic viruses such as Ebola.

1. INTRODUCTION

The increase in life expectancy in the last century can arguably be linked to the prevention and treatment of infections [1]. In this regard, together with antibiotics, vaccines have played a fundamental role in decreasing morbidity and mortality in the human population. In the last few decades, vaccines have contributed to the abatement and eradication of many diseases (Table 1). One notable success is in the eradication of small pox in 1977 and in the significant reduction of cases of poliomyelitis globally from more than 350,000 cases per year in the late 1980s to just 37 cases in

Table 1 Impact of Vaccination

Disease	Region	# of Cases (Year)	# of Cases (2016)	% of Reduction
Small pox	Europe	n.d.	0	100[a]
	Global	1,500,000[a] (1967)	0	100
Diphtheria	Europe	618 (1980)	47	92.4
	Global	97,511 (1980)	7097	92.7
Pertussis	Europe	90,546 (1980)	54,028	40.3
	Global	1,982,355 (1980)	139,535	93
Tetanus	Europe	1715 (1980)	124	92.8
	Global	114,251 (1980)	13,502	88.2
Poliomyelitis	Europe	n.d.	0	100[a]
	Global	350,000[a] (1988)	37	99.9[a]
Measles	Europe	851,849 (1980)	4175	99.5
	Global	4,211,431 (1980)	186,605	99.9
Rubella	Europe	621,039 (2000)	359	99.9
	Global	670,894 (2000)	22,361	96.7

[a]Estimated values.
n.d., not available data.
Data obtained from WHO.

2016 (Table 1). Moreover, immunization programs that target a group of just 10 vaccine-preventable diseases in low- and middle-income countries have saved over $350 billion in direct costs and probably more in indirect costs, highlighting the economic contribution of public health initiatives [2]. These, among many other results, exemplify the past successes and contributions of vaccines. Nonetheless, vaccines will also revolutionize the future of public health because the number of vaccinable illnesses (including cancer) and the age groups that can benefit from them is expected to grow tremendously [3]. Proof of their promising future is that several initiatives, including the Bill and Melinda Gates Foundation, have been stimulating the research and applicability of vaccines to better capture the full potential of what they may deliver.

The objective of a vaccine is to establish immunological memory by educating the adaptive immune system to respond to and infection and prevent. However, new approaches are also investigating the possibility of using vaccines as therapeutics against certain diseases such as cancer. Vaccines are composed of an antigen and, in most cases, an adjuvant [4]. Antigens are the components derived from the pathogen (or disease) to be prevented or treated, which are recognized by the immune system for the production of specific antibodies. Adjuvants are included (when needed) to increase and modulate the production of antibodies. More precisely, according to the European Medicines Agency, an adjuvant is *a component that potentiates the immune responses to antigen and/or modulates it toward the desired immune response*. Finally, in some cases, effective delivery of the antigen and the adjuvant requires the addition of a vehicle or a vector. It is worth mentioning that the efficiency of a vaccine cannot be estimated by the knowledge accumulated studying each of its components (antigen, vehicle, and adjuvant) separately. Therefore, individual characterization of every vaccine formulation is required.

Toward the development of new and better vaccines, biomembranes and lipids play a fundamental role. Not only are they excellent platforms for the delivery of the antigen into the target cell or its proper presentation to the immune system (vehicle/vector) but also have been shown to be excellent adjuvants. However, the role of lipids in the immune response has historically been ignored or, at its best, relegated to a second level when compared to the importance given to proteins and nucleic acids. Nonetheless, advances in immunology and vaccinology are demonstrating that not only lipids are as potent as proteins and nucleic acids in mounting an immune response, but they also have the ability to modulate the response triggered by the antigen.

This modulation of the immune system can be driven actively, by stimulation of cellular receptors from the innate immune system [5], or passively by influencing on how the stimuli are presented to the immune cells.

Direct activation of the innate immune system not only elicits a cellular antiviral response, but it also participates in maturation and trafficking of dendritic cells (DCs), T cell cross-priming, and stimulation of humoral immunity or isotype switching [6]. These are all characteristics that are highly desirable in a vaccine adjuvant and thus interferon (IFN) and other cytokine products of the activation of the innate immune system and IFN-inducing molecules, including lipid and lipid derivatives, have been extensively explored as vaccine adjuvants [7–9]. Proof of the potency of lipids as stimulants of the immune system is the septic shock caused by the hyperactivation of the immune system when bacteria Lipid A (TLR4 agonist, see below) is released into the bloodstream.

In this chapter, we explore how lipids stimulate the immune system and how this knowledge is leading to a new line of vaccines and vaccine adjuvants. We will start by describing the innate immune response to lipids, focusing on the activation and signaling of the TLR4, a cell membrane-bound receptor of immunostimulatory lipids. Next, we will introduce the role of lipids and lipid derivatives as vaccine adjuvants. Finally, we will explore different types of lipid-based vesicles, particularly liposomes and virus-like particles (VLPs) as vaccine vectors.

2. IMMUNE RESPONSE TO LIPIDS

2.1 Innate Immune Response to Lipids

Innate cellular immunity is by definition the cellular response that occurs immediately after an infection occurs. *Innate immunity*, in contrast to *adaptive immunity*, elicits nonspecific responses to any pathogen and is not influenced by prior contact with the infectious agent. The primary component of such a broad-spectrum reactive system is in the identification of the stimuli that triggers the response, in this case, the pathogens invading the cell. The identification of pathogens relies on specialized cellular receptors. The variability of infectious agents that can potentially infect a cell is enormous. Furthermore, some of these pathogens are, mostly due to their genomic nature (RNA) and elevated replication rates, fast-evolving organisms capable of changing during the lifetime of any individual, thus expanding the variability of the stimuli. In this scenario, how can cells encode receptors to detect all these pathogens?

The innate immune system relies on the identification of molecular patterns associated with infectious agents rather than in the identification of each pathogen individually. These patterns (known as pathogen-associated molecular patterns (PAMPs)) are commonly found in microbes but absent in the host organisms and consequently can be recognized as foreign entities against which the cell must react. This method of not-self-recognition allows the identification of many pathogens using a relatively small repertoire of receptors; however, its specificity toward an individual pathogen is poor. The receptors charged with this fundamental task are called pattern recognition receptors (PRRs). There are several classes of PRRs: Toll-like receptors (TLRs), C-type lectin receptors (CTLRs), NOD-like receptors (NLRs), or RIG-I-like receptors (RLRs) among others. The activation of each one depends not only on the type of stimulus but also on its location within the cell. For instance, extracellular bacteria will activate receptors on the plasma membrane, while intracellular replicating viruses will, most likely, activate soluble receptors in the cytosol. As a matter of fact, in most cases, pathogens can activate several PRRs at the same time. The combined information obtained from the activation of multiple PRRs is what helps the immune system to compensate for the poor specificity of PPRs and tailor its response to the type of pathogen invading the cell.

Table 2 summarizes the localization of each human TLR family member (Data source UniProt [10]), the stimuli engaging the receptor, and its subcellular location.

A quick look at the TLR family in humans reveals the breadth of stimuli that activate the innate immune system but also the importance of lipids or lipid derivatives as immunostimulants. Precisely, TLR2 and TLR4 are stimulated with glycolipids, while TLR1 and TLR6 are activated upon interaction with lipoproteins. In the next section, we will explore the peculiarities of TLR4 activation and signaling as an example of a lipid-activated PRR.

2.2 Lipid-Stimulated PRRs (TLR4)

Lipopolysaccharide or LPS is the principal component of the cell wall of Gram-negative bacteria (such as *Escherichia coli*) (approximately, there are 10^6 Lipid A residues and 10^7 glycerophospholipids in a single *E. coli* cell [11]). Structurally, LPS can be divided into three parts (Fig. 1) [12]:

I. The O-antigen. The O-antigen or O-polysaccharide constitutes the outermost region of the LPS. It is the largest and most variable domain

Table 2 The Human TLR Family

Receptor	Ligand/s	Location
TLR1	Multiple triacyl lipopeptides	Cell surface
TLR2	Multiple glycolipids	Cell surface
	Multiple lipoproteins	
	Zymosan (β-glucan)	
TLR3	Double-stranded RNA, poly I:C	Endosome
TLR4	Lipopolysaccharide	Cell surface Endosome
TLR5	Bacterial flagellin	Cell surface
TLR6	Multiple diacyl lipopeptides	Cell surface
TLR7	Imidazoquinoline	Endosome
	Loxoribine (a guanosine analog)	Lysosome
	Single-stranded RNA	Phagosome
TLR8	Single-stranded viral RNA	Endosome
	Phagocytized bacterial RNA	
TLR9	Unmethylated CpG oligodeoxynucleotide DNA	Endosome Lysosome
TLR10	Unknown	Cell surface

and is comprised of a repetitive glycan chain. The O-antigen can be recognized by the adaptive immune system and antibodies can be generated against it. However, due to the variability of this domain, the antibodies raised against the O-antigen will be species specific and at times strain specific.

II. The core oligosaccharide. The core oligosaccharide links the O-antigen with the hydrophobic section of the LPS, the Lipid A. It is constituted by two parts. The *outer core* (also known as O-core), composed of hexose residues (in most cases D-glucose, D-mannose, or D-galactose) linked to the *inner core* constituted by oligosaccharides, usually heptose (Hep) and 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo).

III. Lipid A. Lipid A is the lipid-like domain of the LPS and the actual PAMP recognized by TLR4. It is composed of two phosphorylated glucosamides acylated by six lipid chains of variable length (Fig. 1).

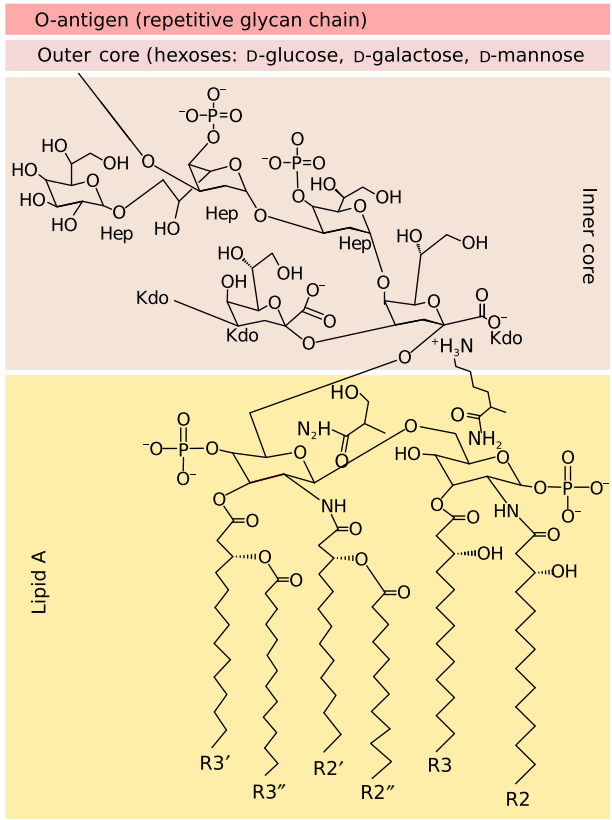

Fig. 1 Schematic representation of *E. coli* LPS. The *outer core*, *inner core*, and *Lipid A* sections of the LPS molecule are highlighted with *light red*, *light brown*, and *yellow*, respectively. Heptose (Hep), 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo). The acyl chains of Lipid A have been labeled (R2, R3, R2′, R3′, R2″, and R3″) according to the literature.

As we just mentioned, TLRs are engaged in a wide variety of stimuli, including lipids and lipoproteins (Table 2). Particularly, TLR4 is activated by bacterial LPS (Fig. 1). TLR4 has a modular structure composed of an extracellular sensing domain and an intracellular signaling domain connected through a single transmembrane segment that anchors the protein into the endosome or the plasma membrane (Fig. 2). In the cytosolic side, responsible for the signaling cascade initiation, there is a Toll/IL-1 receptor (TIR) domain [13]. The extracellular domain, responsible for the sensing of the ligand, belongs to the leucine-rich repeats (LRRs) family. LRRs are 20–29-residue sequence repeats present in a variety of proteins with diverse functions involved in protein interactions including protein–protein

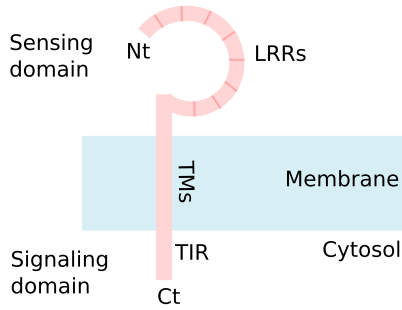

Fig. 2 TLR schematic representation. Schematic representation of a TLR. The position of the leucine-rich repeats (LRRs) and the Toll/IL-1 receptor (TIR) domains are indicated. *Ct*, carboxy terminus; *Nt*, amino terminus.

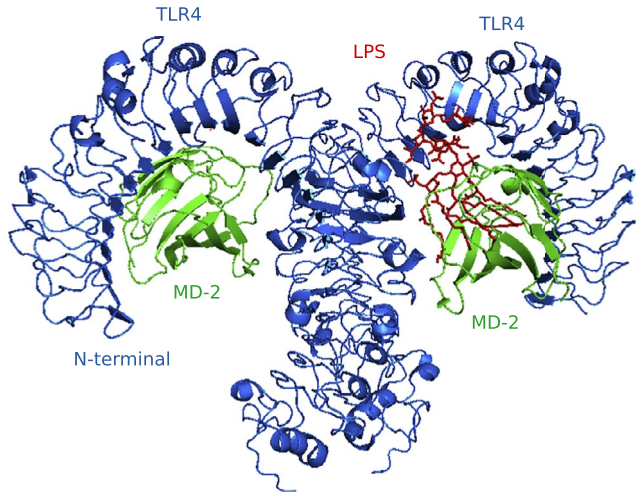

Fig. 3 TLR4/MD-2/LPS complex. Schematic representation of the TLR4/MD-2/LPS dimer complex structure. Note that only the outer TIR domain is represented. The TLR4 is depicted in *blue*, the MD-2 in *green*, and the LPS in *red*. For simplicity reasons, only one molecule of LPS is depicted.

interactions and protein–ligand interactions [14]. Currently, we have a deep knowledge of the structural details of the TLR4 activation (Fig. 3). In fact, several structures of the TLR4/Adaptor/LPS complex and the TIR domain have been resolved separately (e.g., PDB codes: 3FXI [15] and 5UZB [16], respectively). Furthermore, the structure of the TLR4 transmembrane domain was recently solved by nuclear magnetic resonance (PDB code: 5NAM [17]) completing the atomistic modular description of the entire receptor.

The recognition process of the Lipid A starts with the LPS extraction from the bacterial membrane by two accessory proteins, LPS-binding protein and CD14 [18]. Next, the actual binding of Lipid A to TLR4 occurs, a process that requires the adapter protein MD-2 (also known as lymphocyte antigen 96) [19]. In fact, it is thought that it is the MD-2 protein that directly recognizes and binds the lipophilic part of the LPS. MD-2 has a β-cup fold structure composed of two antiparallel β sheets that form a hydrophobic pocket where the LPS is bound, mainly, through hydrophobic interactions (Fig. 3). Binding of LPS to MD-2 drives the formation of a dimeric symmetrical complex LPS/MD-2/TLR4 with a 2:2:2 stoichiometry. This ligand-dependent dimerization initiates the intracellular signal cascade and eventually triggers the cellular response.

Binding of the LPS to the TLR4 does not disturb the structural shape of the receptor as it retains its characteristic horseshoe shape [20] (Fig. 3). The interactions sustaining the dimer are both hydrophobic and hydrophilic in nature. While most (all but one) hydrocarbon chains of Lipid A are buried in the hydrophobic pocket of MD-2, the R2 acyl chain is exposed and left to interact with one of the TLR4 monomers. Furthermore, the two phosphate groups of Lipid A interact with polar residues in MD-2 and both copies of TLR4. These hydrophobic and hydrophilic interactions together with the hydrogen bonds and ionic interactions between MD-2 and TLR4 constitute the core of the dimerization interface. For a complete description of the Lipid A/MD-2/TLR4 interactions, visit Refs. [15,21].

2.3 TLR4 Signaling

Binding of Lipid A triggers the dimerization of TLR4 and the activation of a signaling cascade that ends in the generation of an appropriated cellular response. Two main pathways, depending on the selective use of different adaptor combinations [22,23], can be triggered after TLR4 activation: the TLR4/MyD88/NF-κB and the TLR4/TRIF/IRF3 pathways (Fig. 4). The myeloid differentiation primary response protein MyD88 (MyD88) pathway starts from the TLR4 complex located on the plasma membrane, while TRIF/IRF3 cascade begins after complex internalization into endosomes. Thus, it is the membrane location of the adaptors which in turn defines the activated pathway. The localization signaling response adds a new level of complexity to the system and increases the specificity of the response, facilitating the mounting of a specific response to the invading pathogen.

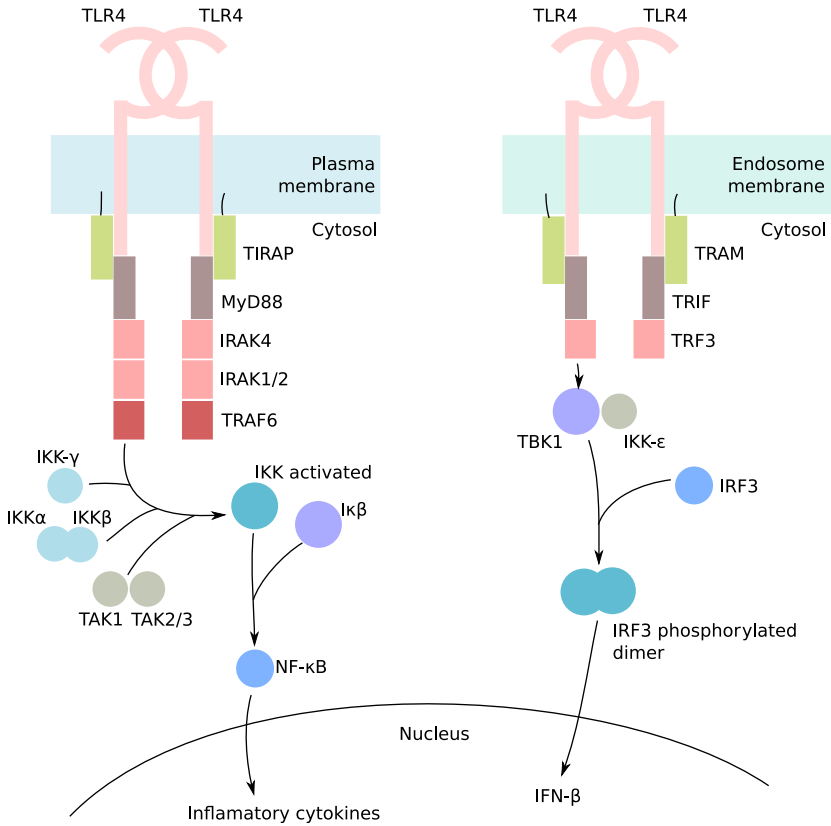

Fig. 4 TLR4 signaling pathway. Once TLR4 is activated, it dimerizes and triggers a signaling cascade through a membrane adaptor. Localization of this adaptor is key in the cellular response to the stimulus. Both TIRAP/NF-κB (*left*) and TRAM/IRF3 (*right*) pathways are depicted. The plasma and the endosomal membranes are represented in *light blue* and *green*, respectively.

In the MyD88 pathway, the Toll/Interleukin-1 Receptor domain-containing Adapter Protein (TIRAP) adaptor binds the TLR4–TLR4 dimer through the TIR domains (Figs. 2 and 4). Localization of the TIRAP to the plasma membrane is mediated by a phosphatidylinositol 4,5-bisphosphate-binding domain at the amino terminus of the protein before the TIR domain [24]. The TIRAP/TLR4 complex is then recognized by MyD88 and subsequently by the interleukin-1 receptor-associated kinases 2 and 4 (IRAK2 and IRAK4) (Fig. 4). Next, TNF receptor-associated factor 6 (TRAF6) is recruited and induces the activation of inhibitor of nuclear factor kappa-B kinase (IKK) and transforming growth factor-β-activated

kinase 1 (TAK1) [25] and the release of NF-κB. TAK1 will activate various mitogen-activated protein kinases, and together with NF-κB, they induce the production of proinflammatory cytokines [26] (Fig. 4).

In the TLR4/TRIF/IRF3 pathway, the first adaptor molecule in the signaling cascade is known as TRIF-related adaptor molecule (TRAM) [27]. TRAM is cotranslationally modified by the addition of a myristoyl group. This fatty acyl chain facilitates the localization of the otherwise soluble protein to the endosomal membrane where its association is further stabilized by electrostatic interactions with phospholipids. Once TRAM is bound to TLR4, it is capable of recruiting TRIF which, through a phosphorylation cascade, will activate the interferon regulatory factor 3 (IRF3) [28]. Then, phosphorylated IRF3 dimerizes and translocates to the nucleus to initiate the transcription of type I interferon [29] (Fig. 4).

3. LIPIDS, LIPID DERIVATIVES, AND LIPID MIMETICS AS VACCINE ADJUVANTS

Adjuvants (from the Latin adjuvare—to help) are by definition substances incorporated into the vaccine to enhance the immune response toward the antigen. On their own, they are capable of stimulating the immune system (triggering the production of cytokines) but are unable to provide a long-lasting protection against any pathogen. Adjuvants have been deliberately added to vaccines since the 1920s when alum (aluminum salt) was accidentally identified to increase antibody responses and vaccine efficiency. Since then, much has been discovered about how these adjuvants activate innate immune responses in both nonimmune and immune cells. Adjuvant activation of DCs is particularly relevant, as this leads to enhance antigen uptake, migration to lymph nodes, and priming of T and B cells and thus to increased vaccine efficiency.

Interest in vaccine adjuvants has recently increased significantly for several reasons. Public health authorities together with vaccine manufacturers have established ambitious goals for enhancing present vaccines, which by definition is the primary function of adjuvants. The incorporation of an adjuvant to a vaccine preparation not only enhances its potency but might also reduce the necessary dose, increase the breadth of the antibody response, and reduce the production time and cost. Additionally, many new vaccine candidates are currently being investigated. In many cases, in these new vaccines, the antigen is not sufficiently immunogenic and could benefit from the supplementation with an adjuvant to produce an efficacious vaccine.

Collectively, new adjuvants could improve existing vaccines and boost the generation of new vaccines.

Despite the enormous efforts on adjuvant development, there are only a handful of approved adjuvants. Table 3 summarizes the approved adjuvants in the United States. Strikingly, most of the approved adjuvants are either TLR4 agonists or oil-in-water emulsions, in other words, lipid-based adjuvants. Furthermore, many of the adjuvants under investigation are either lipid derivatives or lipid mimetics that can be easily engineered into lipid-based vaccines. In the next section, we will focus on TLR4 adjuvants as examples of lipid-based adjuvants.

3.1 TLR4-Directed Vaccine Adjuvants Beyond Lipid A

The inherent toxicity of LPS, as well as its Lipid A subunit (associated with its high potency stimulating the immune system), poses a safety risk when considering it for use as a vaccine adjuvant. However, the chemical complexity of LPS suggested that modifications might be made in order to "detoxify" the compound. In other words, a desirable TLR4 adjuvant should robustly activate the innate immune system to drive a diverse cellular and humoral response against a given pathogen without being too reactogenic or causing systemic side effects.

Mutant strains of bacteria with deficiencies at various steps of LPS synthesis were isolated and ultimately the LPS assembly pathway (Raetz pathway) was defined, initially in *E. coli*; however, a similar synthetic pathway exists in other Gram-negative bacteria [30]. These bacterial mutants served as important reagents that were enriched for various Lipid A and LPS

Table 3 FDA-Approved Adjuvants

Adjuvant (Components)	Vaccine
Aluminum salts	DTaP, HBV, pneumococcal, and more
AS04[a] (Alum + MPL)	HPV (Cervarix[a])
AS03[a] (α-tocopherol, squalene, and polysorbate 80 in an oil-in-water emulsion oil in water emulsion)	Influenza H5N1
MF59[b] (squalene in an oil-in-water emulsion oil in water emulsion)	Trivalent influenza vaccine

[a]Cervarix, AS03, and AS04 are manufactured by GlaxoSmithKline.
[b]MF50 is manufactured by Novartis.
US Food and Drug Administration (http://www.fda.gov).

precursors. Later, scientists began working with mutant bacteria from a variety of species including *Bordetella pertussis* in order to optimize immunogenicity and minimize reactogenicity of whole-cell vaccines adjuvanted with an endogenous Lipid A derivative [31]. Chemical modification of LPS extracts in the 1970s began generating new species of Lipid A molecules [30]. Since researchers found that different Lipid A variants had varying degrees of potency and endotoxicity, academic labs and pharmaceutical companies have pursued detoxified Lipid A molecules as adjuvants for viral and bacterial vaccines. Eventually, it was recognized that TLR4 was the receptor for LPS and Lipid A derivatives and that differential TLR4/MD-2 signaling via either the TRIF or MyD88 adaptor proteins generated phenotypically different responses to various TLR4 ligands in a species-specific manner. In addition to LPS derivatives, small molecule agonists of TLR4 are under development as vaccine adjuvants [7]. Over the past 10 years, novel purification schemes and synthetic strategies have proliferated in search of Lipid A-derived and nonlipid small molecule TLR4-directed adjuvants to usher in a new era of chemically well-defined, subunit vaccines. These novel adjuvants are needed to address challenging pathogens such as HIV, pandemic influenza viruses, malaria, dengue, and emerging viral pathogens like the Zika virus where robust humoral and cellular immunity is required to protect the host.

3.2 Lipid A Derivatives as Vaccine Adjuvants

Monophosphoryl Lipid A (MPLA) is a heterogeneous mixture of chemically modified Lipid A molecules derived from the LPS of *Salmonella minnesota* Re595 [32]. Clinical grade MPLA, termed MPL adjuvant, was FDA approved for use in humans as a component of AS04 (Adjuvant System 04) (Table 3), a combination of MPL and alum, to adjuvant the Cervarix vaccine against human papilloma virus strains 16 and 18 to combat cervical cancer and other cancers [33,34]. AS04 is also licensed for use in Europe as a component of the hepatitis B virus vaccine, Fendrix [35]. As such, the MPL adjuvant was the first FDA approved vaccine adjuvant since 1930 when aluminum salts become part of vaccine preparation. While MPLA is both safe and efficacious in humans in two distinct viral vaccine formulations, the heterogeneous composition is suboptimal. MPLA is a mixture of tetra-, penta-, and hexa-acylated Lipid A molecules with a single 4′ phosphoryl group and no polysaccharide moiety, and it is generated by chemical or enzymatic hydrolysis of purified LPS [30,32]. On human TLR4, the hexa-acylated Lipid A derivatives tend to be the most

potent agonists [30,36], while the tetra-acylated Lipid A component of MPLA has been shown to have human TLR4 antagonist activity [11,15,37]. Notably, the 4′ monophosphate of Lipid A is retained for MPLA activity; however, removal of the other phosphate groups from Lipid A is necessary to obtain MPLAs favorable safety profile compared to LPS [38].

A better understanding of TLR4 signaling has led to a proliferation of rationally designed TLR4 agonists. In 1998, TLR4 was identified as the receptor for LPS [39], and it was subsequently demonstrated that TLR4 could signal through both TRIF and MyD88 as discussed elsewhere in greater detail (see Fig. 4 for a schematic representation of the TLR4 signaling pathway). The recognition that MPLA signals primarily through the TRIF adaptor protein spurred the development of TRIF-selective ligands as vaccine adjuvants [39,40]. In parallel, activation of MyD88 stimulates transcription factor NF-κB and mitogen-activated protein kinases to induce primarily inflammatory cytokines such as IL-6 and TNFα [41]. Activation of TRIF leads to the downstream activation of NF-κB and IRF3, via unique intracellular signaling relative to MyD88, and induces both type I interferons in addition to inflammatory cytokines [41]. Therefore, the endotoxicity of LPS is dependent upon proinflammatory cytokine activation via MyD88, and mice deficient in MyD88 are resistant to LPS-induced septic shock [42]. In contrast, the induction of type I interferons by TRIF signaling is responsible for the favorable adjuvant qualities of Lipid A including the induction of a Th1-type immune response observed with MPLA [38,40].

With this understanding, several TLR4/MD-2 complex ligands have been rationally designed as viral and bacterial vaccine adjuvants. Some noteworthy preclinical and clinical synthetic Lipid A analogs include glucopyranosyl lipid adjuvant (GLA) [43], aminoalkyl glucosaminide 4-phosphates (AGPs) [44], and E6020, which has no disaccharide backbone [45]. Structure–activity relationship studies with AGPs demonstrated that a relatively small stereochemical change in Lipid A structure, affecting how charged groups on the hydrophilic portion of Lipid A interact with TLR4/MD-2, may substantially affect downstream MyD88, while TRIF signaling is minimally affected [46]. The ability for medicinal chemists to modulate MyD88 and TRIF signaling is a tremendous opportunity to optimize TLR4-directed adjuvants. GLA has advanced to clinical trial and demonstrated both safety and immunogenicity for a fully synthetic Lipid A analog in humans [47]. Given regulatory and safety concerns, wholly synthetic molecules with well-defined mechanisms of action may represent the future of TLR4-based adjuvants coming into clinical use.

3.3 Small Molecule TLR4 Adjuvants

While Lipid A derivatives, namely MPLA, have already enjoyed clinical success as vaccine adjuvants, the complexity of the molecules makes purification, chemical modification, and vaccine formulation challenging. Likely the result of the complexity of interactions between LPS and the TLR4 signaling apparatus [15], relatively few families of small molecule TLR4 agonists have been reported to date. High-throughput screens have been successful in producing candidates for vaccine adjuvants including distinct, nonlipid chemical entities such as substituted pyramid[5,4-*b*]indole derivatives [48,49], and α-aminoacyl amides synthesized via the Ugi reaction [50], and small molecule TLR4 ligands have demonstrated preclinical potential as vaccine adjuvants with potent immunostimulatory potential with limited reactogenicity in mouse [7].

Small molecules have an advantage over purified LPS derivatives such as MPLA in that they are a single, defined chemical species; however, research into small molecule TLR4 agonists is in its infancy, and it is as yet unclear whether these compounds will have any clinical impact.

3.4 Challenges With Animal Models for TLR4 Adjuvant Development

While the structural complexity of Lipid A provides scientists with ample opportunity for genetic and chemical modification to optimize adjuvancy over toxicity, the interactions between TLR4 agonists and the TLR4/MD-2 signaling apparatus are not straightforward and present unique challenges and opportunities in preclinical animal models. In vitro culture systems play an essential role in evaluating the potency or cytokine profile of a particular adjuvant, while animal models are needed to evaluate preclinical safety and efficacy. Not only is an intact immune system necessary to fully evaluate the protective efficacy of an adjuvanted vaccine, but the expression of TLR4 ligands is variable between different tissues and animals [51].

TLR4 ligands exhibit species-specific phenotypic variability in regard to their adjuvant and toxicity profiles [21]. In fact, a Lipid A precursor, tetraacylated lipid IVa, has been shown to be an agonist in murine cells but to antagonize human TLR4 [52]. Potent human TLR4 agonists, namely the Ugi compounds, have been described that have little or no activity in the range of preclinical models including mouse, rat, rabbit, ferret, and cotton rat cells [50]. Species-specific structural differences between the human and mouse TLR4/MD-2 complex have been identified which affect how the

receptor interacts with a given ligand [21]. Lipid IVa binds the murine TLR4/MD-2 receptor in a very similar pattern to LPS; however, when lipid IVa, an antagonist to human TLR4, was crystallized with human TLR4/MD-2, it bounds in a different conformational pattern when compared to LPS [21].

A number of strategies have been developed in order to circumvent this problem. Most simply, one may just screen for molecules that are active in both human and murine cells. Chemical, genetic, or enzymatic modifications of candidate compounds or Lipid A preparations may be employed to develop adjuvant candidates that signal more promiscuously. New animal models are being developed that more closely resemble human TLR4 signaling including a transgenic mouse model expressing human TLR4/MD-2 in mice deficient in murine TLR4/MD-2, which effectively reconstitutes LPS signaling [53]. Computational modeling to define the structural and chemical determinants of ligand–receptor interaction may also help to overcome the species barrier by driving rationally designed agonists active across a range of species [54].

4. LIPID-BASED SELF-ASSEMBLING VESICLE AS VACCINE PLATFORMS

4.1 Lipid-Based Nanovesicles as Vaccine Platforms

In the last few years, we have seen a large increase in the potential applications of nanoparticles for medical purposes. In fact, it is thought that the applications of nanotechnology will revolutionize the drug delivery methodologies in the not too distant future [55]. Vaccinology is not an exception and nanoparticles are expected to have an enormous impact on future vaccine development. Among all different types of nanoparticles, including inorganic nanoparticles [56], polymeric nanoparticles [57], carbon nanotubes [58], and nanogels [59], lipid-based nanoparticles (LBNs) have been the most extensively used. This preference for lipid-derived vesicles responds not only to their high delivery efficiency but also to their capacity for delivering a large variety of cargoes and their adaptability to the requirements of the specific treatment.

In the next section, we will focus on liposomes since they are the best known and most used self-assembling LBNs. Nonetheless, there are multiple variations of classic liposomes and some nonliposomal LBNs that are being investigated preclinically or in earlier phases of the development process.

Table 4 Lipid-Based Nanoparticles Used for Biomedical Applications

System	Composition	Advantages
Ninosomes[a]	Chol/nonionic surfactants	Cheaper and more stable than conventional liposomes
Transferosomes[a]	Phospholipids/surfactants	Increased penetrability (highly deformable)
Etosomes[a]	Phospholipids/alcohols	Increased penetrability (highly deformable)
Sphingosomes	Sphingolipids/Chol	Increased stability
Ufasomes	Fatty acid/surfactant	Cheaper, better entrapment efficiencies, and improved stability
Pharmacosomes	Cargo/phospholipids	Improved encapsulation of polar cargo
Quatsomes	Chol/cationic surfactants	Increased stability and homogeneity
Virosomes	Phospholipids/viral envelope proteins	Increased penetrability

[a]These formulations are considered variations of classical liposomes.

The best-known LBNs are summarized in Table 4; for an excellent and updated review of the properties, applications, and formulation of these advance technologies, see Ref. [60].

4.2 Liposomes as Vaccine Platforms

Liposomes were first utilized for medical purposes in the 1960s and rapidly their potential as vaccine delivery vehicles was explored [61,62] together with their adjuvants properties [63]. Currently (November 2017), there are 43 clinical trials registered at http://ClinicalTrials.gov (an open service of the US National Institute of Health, https://clinicaltrials.gov/) with the search terms *Liposome* AND *Vaccine* (Table 5), thus demonstrating the enormous potential of this type of formulation for future vaccine development.

Liposomes are composed predominantly of phospholipids, the most abundant lipids in biological membranes. Phospholipids are amphiphilic lipid molecules containing a polar head group made of a phosphate bound to an additional group (variable in nature). The head of the lipid is linked through a glycerol group to a hydrophobic tail constituted by two fatty acid chains. This amphiphilic structure facilitates self-assembly in an aqueous environment, resulting in vesicles with both a hydrophobic and a hydrophilic region ideal for the incorporation of different types of antigens.

Table 5 Liposome-Based Vaccines in Clinical Trials

Code	Description
NCT01326546	Therapeutic hepatitis B vaccine (synthesized peptide εPA-44) joint entecavir in treating chronic hepatitis B patients
NCT01673217	Decitabine, vaccine therapy, and pegylated liposomal doxorubicin hydrochloride in treating patients with recurrent ovarian epithelial cancer, fallopian tube cancer, or peritoneal cancer
NCT00197301	Liposomal-based intranasal influenza vaccine: safety and efficacy
NCT01095848	A phase 1 safety study of a cancer vaccine to treat HLA-A2-positive advanced stage ovarian, breast, and prostate cancer
NCT00876824	To study the effect of single infusions of amphotericin B lipid preparations in treatment of patients with Kala azar
NCT00922363	Trial on the safety of a new liposomal adjuvant system, CAF01, when given with the tuberculosis subunit vaccine AG85B-ESAT-6 as two injections with 2 months interval to healthy adult volunteers
NCT00960115	Study of tecemotide (L-BLP25) in participants with stage III unresectable non-small cell lung cancer (NSCLC) following primary chemoradiotherapy
NCT01094548	Study of tecemotide (L-BLP25) in subjects with slowly progressive multiple myeloma with no symptoms and who have had no chemotherapy
NCT00409188	Cancer vaccine study for unresectable stage III non-small cell lung cancer (START)
NCT01556789	Phase 1 study of ONT-10 in patients with solid tumors
NCT01978964	Phase 1b maintenance therapy study of ONT-10 in patients with solid tumors
NCT00020462	Vaccine therapy plus interleukin-2 in treating patients with stage III, stage IV, or recurrent follicular lymphoma
NCT00828009	BLP25 liposome vaccine and bevacizumab after chemotherapy and radiation therapy in treating patients with newly diagnosed non-small cell lung cancer that cannot be removed by surgery
NCT01015443	Cancer vaccine study for stage III, unresectable, non-small cell lung cancer (NSCLC) in the Asian population
NCT01976520	Vaccine therapy for treating patients with previously untreated chronic lymphocytic leukemia (CLL)
NCT00157196	Safety study of tecemotide (L-BLP25) in non-small cell lung cancer (NSCLC) subjects with unresectable stage III disease

Table 5 Liposome-Based Vaccines in Clinical Trials—cont'd

Code	Description
NCT01052142	Safety study of a liposomal vaccine to treat malignant melanoma
NCT02174978	Clinical trial with controlled human malaria infection to evaluate the safety and efficacy of the *Plasmodium falciparum* vaccine candidate with AS01B adjuvant system in malaria-naïve adults
NCT00915187	Safety and immunogenicity study of intramuscular CCS/C-adjuvanted influenza vaccine in elderly
NCT00157209	Phase 2b randomized controlled study of tecemotide (L-BLP25) for immunotherapy of NSCLC (non-small cell lung cancer)
NCT02647489	Safety and immunogenicity of the placental malaria vaccine candidate PAMVAC variously adjuvanted
NCT02927145	A challenge study to assess the safety, immunogenicity, and efficacy of a malaria vaccine candidate
NCT00004104	Vaccine therapy plus interleukin-2 with or without interferon alfa-2b in treating patients with stage III melanoma
NCT00925548	Stride—stimulating immune response in advanced breast cancer
NCT01423760	Common safety follow-up trial of tecemotide (L-BLP25)
NCT00923936	Pilot study of liposomal doxorubicin combined with bevacizumab followed by bevacizumab monotherapy in adults with advanced Kaposi's sarcoma
NCT01006187	Comparison of different methods for reducing pain from influenza vaccine injections
NCT02508376	Safety, tolerability, and immunogenicity of the vaccine candidates ID93 + AP10-602 and ID93 + GLA-SE administered intramuscularly in healthy adult subjects
NCT00952692	Study to assess DHER2 + AS15 cancer vaccine given in combination with lapatinib to patients with metastatic breast cancer
NCT01496131	Tecemotide (L-BLP25) in prostate cancer
NCT00093834	Vaccine therapy with or without cyclophosphamide and doxorubicin in women with stage IV breast cancer
NCT02942277	Safety and immunogenicity of Pfs25M-EPA/AS01 and Pfs230D1M-EPA/AS01 vaccines, transmission blocking vaccines against *Plasmodium falciparum*, at full and fractional dosing in adults in male

Continued

Table 5 Liposome-Based Vaccines in Clinical Trials—cont'd

Code	Description
NCT00052351	Vaccine therapy plus sargramostim and chemotherapy in treating women with stage II or stage III breast cancer
NCT00001042	Randomized, double-blind, placebo-controlled, clinical trial to compare the safety and immunogenicity of recombinant envelope protein rgp120/HIV-1SF2 combined with seven adjuvants
NCT02018458	Safety study of chemotherapy combined with DCs vaccine to treat breast cancer
NCT00101101	Universal granulocyte macrophage colony-stimulating factor (GM-CSF)-producing and GM.CD40L for autologous tumor vaccine in mantle cell lymphoma
NCT00048893	Vaccine and chemotherapy for previously untreated metastatic breast cancer
NCT02270372	Study of ONT-10 and varlilumab to treat advanced ovarian or breast cancer
NCT00006184	Chemotherapy, stem cell transplantation and donor and patient vaccination for treatment of multiple myeloma
NCT00004197	Vaccine therapy plus sargramostim following chemotherapy in treating patients with previously untreated aggressive non-Hodgkin's lymphoma
NCT00324831	GM-CSF with or without vaccine therapy after combination chemotherapy and rituximab as first-line therapy in treating patients with stage II, stage III, or stage IV diffuse large B-cell lymphoma
NCT03206671	Treatment protocol of the NHL-BFM and the NOPHO study groups for mature aggressive B-cell lymphoma and leukemia in children and adolescents
NCT02107378	Efficacy of DCVAC/OvCa plus standard of care in relapsed platinum-resistant epithelial ovarian carcinoma

While water-soluble proteins would be included in the liposome core or bound to the polar surface of the vesicles, hydrophobic antigens would be inserted into the liposome membranes.

One of the main advantages of liposomes is that their physicochemical properties can be tuned to meet the requirements of the antigen. The characteristics of the head groups are crucial in determining the surface properties of the vesicles, while fluidity, permeability, and stability depend on the

length and degree of saturation of the acyl chains. All these characteristics will have a tremendous impact on the delivery of the antigen (e.g., liposomes containing phosphatidylserine enhance phagocytosis by macrophages [64,65]) and the type of immune response triggered by the vaccine (e.g., immunization with small liposomes induces a Th2 response, while large liposomes trigger a Th1 response [66]). There is a vast amount of literature on liposomal-based vaccines. The references used in this chapter are meant to introduce the reader to the field and not to serve as a comprehensive list. The physiochemical properties of liposomal vaccines were recently reviewed in Bernasconi et al. [67], while liposomal vaccine formulation was summarized by Charlton Hume and Lua [68].

4.3 Liposome Antigens and Adjuvants

There are many types of antigens that have been successfully incorporated into liposomes. Peptides and proteins are the most common antigens used for vaccination. Preparations of peptides and liposomes have shown their efficacy many times and for multiple applications, including protection against a large variety of pathogens [69,70] and cancer [71]. Furthermore, peptides delivered via liposome formulations have and are been extensively used in many immunology studies as stimulants of the humoral response (e.g., Taenechi and colleges recently demonstrated that liposome-coupled peptides induce long-lived memory CD8 T cells without CD4 T cells using liposome-based vaccines [72]).

Proteins (full-length or partial sequences) have also been successfully incorporated in liposomal vaccines. In fact, in the early works of liposomal vaccines, proteins were already used as immunogens [62,63]. Nonetheless, the use of proteins in liposome-based vaccines is still under investigation and new applications and formulations are currently being investigated (e.g., in a recent work, mimetics of the envelope protein of HIV were used to accomplish virus neutralization using a high-density liposomal formulation [73]).

Vaccination using nucleic acids relies on the cellular transcription/translation machinery to produce the antigen. The delivery of the antigen via DNA/RNA vaccination is a promising alternative to protein-based vaccines, especially to those that are difficult to produce in recombinant form. Additionally, current nucleic acid synthesis technologies allow tailored, rapid, and inexpensive production of new antigens. These characteristics would make the DNA/RNA vaccines ideal in changing scenarios such as the emergence of a new pandemic (e.g., the recent Ebola pandemic) or the constant

reemergence of a continuously evolving pathogen. However, the immunogenicity of naked nucleic acid vaccines is weak and, in most cases, insufficient for a successful vaccination due to degradation and/or inefficient targeting to immune cells of the DNA/RNA. Furthermore, delivery of genetic material into the cell has to overcome many obstacles (e.g., endosomal escape, lysosomal degradation, or nuclear uptake). Many of these hurdles could be overcome using an adequate vector making nucleic acid vaccines a viable alternative.

Liposomes were shown to be efficient on delivering nucleic acids in vitro and were used promptly for in vivo vaccination [74]. Nonetheless, the first formulations of liposomal vaccines, although capable of enhancing the efficiency of nucleic acid vaccines, did not completely overcome many of the previously mentioned obstacles associated with DNA/RNA vaccines. However, advances in liposome formulation are mitigating these limitations while reducing off-target effects. These new technologies are revolutionizing the DNA/RNA vaccine field and could become a viable option soon [75].

Liposome-based DNA/RNA vaccines are proven especially successful in the case of oral vaccines. Nucleic acid-based oral vaccines represent an ideal combination of antigen and delivery route. On top of the potential benefits of DNA/RNA vaccines, the oral administration might eliminate many of the prejudices associated with vaccines and could facilitate massive vaccination campaigns, even in third world countries. Consequently, nucleic acid antigens with liposomes as vehicles for oral administration are currently gathering a lot of attention not only for humans [76,77] but also for animal vaccines [78]. Once again, the enormous versatility in the physical parameters of the lipids constituting the liposomes can be utilized for constructing tailor-made vehicles for a wide range of applications [79].

Liposomes not only act as vaccine delivery systems but can also function as vaccine adjuvants. Liposomes, even those constituted mainly by phospholipids, have some adjuvant activity. There are two main reasons for this adjuvancy: 1. Liposomes enhance antigen uptake by some antigen-presenting cells, including CDs [80]. 2. Liposomes, like oil-in-water emulsions, have some *depot effect* [81]. The depot effect is the oldest adjuvant mechanism described, antigen entrapment at the site of injection slows down the release of the antigen which facilitates a continuous stimulation of the antigen-presenting cells that correlated with high antibody titers.

More advanced liposomes base their adjuvant properties on multiple mechanisms, besides the depot effect. Modification of the physicochemical characteristics of the liposomes can modify the type of immune response by

modulating their exposure and recruitment by immune cells [82,83]. E.g., Li and colleagues showed that small liposomes (~230 nm) induce a stronger response to the OVA peptide than larger vesicles (~708 nm) [84]. Later on, using leishmaniasis rgp63 as an antigen, it was shown that small liposomes stimulated a Th2 response, while large ones induce a Th1 response accompanied with higher interferon gamma levels [66]. Furthermore, the head group charge has been thoroughly investigated and it was shown that liposomes with different net charges had different adjuvant activities [85]. Nonetheless, the outcome of the response will be also influenced by the antigen and thus it is difficult to predict. Not only are the properties of the liposome important, but the location/interaction between the antigen and the liposome can also modulate the immune response. Barnier-Quer et al. showed that influenza hemagglutinin adsorbed on the surface of the liposome was more immunogenic than when encapsulated [86].

The inclusion of additional adjuvant agents can further enhance and or modulate the vaccine immunoproperties. Some liposome formulations incorporate lipids or lipid-like adjuvants (such as TLR4 agonists discussed previously [87]) [88,89]. Beyond those, some of the most commonly used adjuvants in liposome-based vaccines are:

CpG. Short single-stranded DNA molecules containing a cytosine triphosphate deoxynucleotide and a guanine triphosphate deoxynucleotide. These DNA motifs are often found in bacterial DNA and are recognized as PAMP by TLR9. CpG is thought to activate DC maturation, increase antigen expression, and induce a Th1 immune response [90].

Poly I:C. Polyinosinic:polycytidylic acid is a synthetic mimetic of a double-stranded RNA capable of stimulating the immune system through the TLR and RLR families. Poly I:C strongly drives cell-mediated immunity and a potent type I interferon response [91].

CAF01. It consists of the synthetic glycolipid TDB incorporated into cationic DDA (dimethyldioctadecylammonium bromide) liposomes [92]. CAF01 leads to high levels of IFN-γ secretion and low levels of interleukin-5 (IL-5) secretion by CD4+ T-cells inducing a strong Th1 and Th17 immune response.

MDP. Muramyl dipeptide (MDP) is a synthetic immunoreactive peptide consisting of *N*-acetyl muramic acid attached to an L-alanine D-isoglutamine dipeptide. MDP, a constituent of both Gram-positive and Gram-negative bacteria, is recognized by the NOD-2 PRR as PAMP and activates the production of inflammatory cytokines. Fatty acyl analogs of MDP have also been investigated as adjuvants in liposomal preparations [93].

The breadth of the molecules incorporated into liposomes to enhance the vaccine efficacy is increasing rapidly and includes de-O-acylated lipooligosaccharide (a novel TLR4 agonist capable of enhancing both humoral and cellular immune responses [94]), CAF09 (a variation of the CAF01 formulation previously described [95]), and immune receptors such as the Fc receptor peptide (the receptor for the IgG Fc fragment) which enhances the uptake of the antigen by DCs [96].

4.4 VLPs-Based Vaccines

VLPs are noninfectious viral particles that resemble wild-type virions structurally but lack the viral genome—rendering them unable to replicate [97]. VLPs are typically generated by the viral-encoded capsid and/or envelope proteins that drive the budding of the virions. In the case of enveloped viruses the VLPs represent a lipid membrane derived from the host cell and thus similar in lipid composition. This membrane (now considered viral membrane) may also incorporate the virally encoded surface proteins. Given their intrinsic nature to self-assemble into particles and the relative ease of production, VLPs have greatly contributed over the last three decades to numerous fields of study such as vaccinology, virus–host interactions/virus replication cycle, structural biology, gene therapy, and bionanotechnology even in the case of highly pathogenic viruses (see below). Particularly, in vaccinology VLPs represent one of the most utilized tools due to their favorable immunological characteristics. Notably, candidate VLP-based vaccines against highly pathogenic viruses have been extensively pursued due for their ability to present the chief targets of the immune system in a noninfectious platform. Moreover, the ease by which VLP can be manufactured in large batches in several types of host expression systems (e.g., insects and bacteria) makes VLP-based vaccines a good alternative for a safe and fast method of developing prophylactics in urgent situations. Currently, several VLP-based vaccines are commercially available, including Cervarix or the third-generation hepatitis B virus Sci-B-Vac vaccine and many more are currently under investigation. The following text will mainly focus on VLPs derived from the family of viruses that infect mammals. However, it should be noted that VLPs derived from mammalian viruses can still be produced in heterologous host systems.

The first examples of recombinant VLPs were generated from the in vitro expression of the coat protein from the hepatitis B virus (HBV) core antigen (HBcAg) [98], the HBV surface antigen (HBsAg) [99], or the

tobacco mosaic virus [100]. Further analysis of HBcAg VLPs via electron microscopy subsequently showed that VLPs were identical to core shells harvested from HBV-infected liver cells demonstrating for the first time that recombinant VLPs resemble native viral particles isolated from infected cells [101]. The advent of a new generation of vaccines for HBV [102] and the human papilloma virus [103] based on VLPs has increased interest in generating novel strategies for VLP-based vaccines and nanoparticle delivery system. Thus, it is not surprising that most medical-based applications of VLPs are in the development of vaccines against infectious diseases.

4.4.1 Icosahedral and Spherical VLPs

A large number of VLPs are formed from highly ordered symmetric complexes derived from the structural capsid proteins of nonenveloped viruses. A list of icosahedral VLPs from different viruses is briefly described in Table 1. The 22 nm VLP generated from the HBV surface antigen is one of the best-characterized particles and has been the basis of a successful HBV vaccine since 1986 [104]. It structurally recapitulates the native subviral particles released in the bloodstream of HBV-infected individuals at up to 100,000-fold higher than infectious complete virions at 1014 particles/mL [104]. It has been speculated that the major role of subviral particles is to sequester-induced neutralizing antibodies against complete virions and acts as an immune evasion technique during viral infection. While the exact mechanism by which the HBsAg lipoprotein self-assembles is yet to be fully elucidated, there is evidence from VLP studies that intra- and intermolecular disulfide bonds are required for proper recapitulation of the antigenic and immunogenic properties of subviral particles [105]. It is interesting to note that the same subviral particle has been used to develop the first generation of HBV vaccine with great success. Spherical-shaped VLPs are also generated from several families of viruses (Table 6). VLPs generated from the Gag protein of retroviruses have also been pursued by many research groups. Expression of Gag is sufficient to form retrovirus VLPs, although there are multiple strategies in combining other viral proteins to generate VLPs—with the stipulation that viral replication and/or integration cannot be initiated. This may involve genetically removing viral enzymes such as the reverse transcriptase and integrase. Conversely, a retroviral VLP can be composed of multiple immunological targets that can stimulate both the humoral antibody and cellular responses without the deleterious effects of a live-attenuated vaccine. Indeed, VLPs expressing a native form of HIV-1 envelope protein can elicit strong humoral and cellular responses, which can lead to antibody-dependent cell-mediated immunity [113].

Table 6 Spherical VLPs

Virus Family	Genome	Example VLPs	References
Arenaviridae	Negative-sense RNA	Lassa virus	[106]
Bunyaviridae	Negative-sense RNA	Hantaan virus	[107]
Coronaviridae	Positive-sense RNA	Severe acute respiratory syndrome corona virus	[108]
Flaviviridae	Positive-sense RNA	West Nile virus	[109]
Retroviridae	Positive-sense RNA	Human immunodeficiency virus 2	[110]
Orthomyxoviridae	Negative-sense RNA	Influenza A virus	[111]
Paramyxoviridae	Negative-sense RNA	Nipah virus	[112]

Table 7 Filamentous VLPs

Virus Family	Genome	Example VLPs	References
Filoviridae	Negative-sense RNA	Ebola virus	[114]
Orthomyxoviridae	Negative-sense RNA	Influenza A virus	[115]

4.4.2 Filamentous VLPs

A third group of viruses can be used to generate a helical or filamentous type of VLP which is summarized in Table 7. It is notable that influenza viruses of the *Orthomyxoviridae* family of viruses can produce both spherical and helical VLP depending on the experimental condition. However, it must also be emphasized that most clinical isolates of influenza viruses produce pleomorphic viral particles, which includes long filamentous virions [116]. Nonetheless, production of influenza virus-like particles requires the hemagglutinin, neuraminidase, and matrix protein one for efficient egress. Transient expression of the two surface glycoproteins, hemagglutinin and neuraminidase, is sufficient in the budding and production of VLPs [117]. However, the inclusion of the matrix protein with both the hemagglutinin and neuraminidase generated filamentous VLPs more similar in nature of clinically isolated viral particles [115]. Numerous approaches have generated a plethora of influenza virus-like particles against a wide range of influenza viral subtypes—specifically zoonotic viruses that have caused yearly or sporadic outbreaks. However, lots of work remain to be done to address breadth, efficacy, and longevity of the response elicited [118]. Of note, the prototypical virusus that produce filamentous mature VLPs are members of *Filoviridae*.

In the following section, the role of VLP in filovirus research, specifically, Ebola virus (EBOV), will be discussed.

VLP vaccines can also benefit from the incorporation of an adjuvant. Like liposomal-based vaccines, VLP immunogenicity has been boosted using a wide range of adjuvants including PRR [119], Lipid A analogs [120], AS01 [121], AS04 [122], poly I:C [123], CpG [111], and Iscomatrix a complex adjuvant composed by saponin, the glycoside extracted from *Quillaja saponaria* (QS21), cholesterol, and phospholipids [124]. For a complete review on the adjuvant formulations for VLP-based vaccines, visit Ref. [125].

4.5 Utilizing VLPs in the Context of a Highly Pathogenic Virus Research

EBOV is the prototypical viral species of the *Filoviridae* family of pathogens that has caused deadly and sporadic outbreaks of hemorrhagic fever [126]. The disease known as Ebola virus disease is generally characterized by malaise, headache, fever, hypotension, myalgia, edema, vascular permeability, blood coagulation dysregulation, and hemorrhages [127–129]. While the administration of antibody-based therapy has shown some promise in the most recent outbreak of EBOV in West Africa [130], there is currently no FDA-approved vaccine for EBOV. Toward better understanding the clinical hallmarks of EBOV infection, much work is still needed to elucidate the molecular determinants of EBOV pathogenesis and define the optimal immune responses for protection. One major obstacle to EBOV research is the requirement for performing experiments under the highest biosafety level conditions, BSL-4, which can significantly curtail and slow down scientific progress. The experimental use of EBOV VLPs, however, has greatly contributed to elucidating viral replication and identifying molecular determinants of pathogenesis.

EBOV is a negative-sense nonsegmented RNA virus that encodes for seven structural proteins and one nonstructural protein. The viral RNA genome is encapsidated by the nucleoprotein (NP), which is further associated with the viral L polymerase alongside its noncatalytic cofactor VP35 and transcription activator VP30—forming a complex called the ribonucleoprotein (RNP). A mature and infectious EBOV particle is composed of the viral RNP surrounded by a host-derived plasma membrane or envelope studded with virally encoded glycoprotein (GP). The two matrix proteins, VP40 and VP24, bridge the gap between the RNP and associate with the internal region of the viral envelope. The findings that transient expression of EBOV

VP40 was solely sufficient for virus budding through a conserved proline-rich motif (PY) at the N-terminus and a C-terminus region that generated filovirus-like particles were a significant contribution to the field [131–133]. A subsequent study demonstrated that coexpression of GP with VP40 produced filamentous virion-like particles that expressed GP on the surface [134]. These early observations of generating noninfectious EBOV VLPs have greatly contributed in describing steps in the viral replication cycle that bypass work performed in BSL-4 conditions.

4.5.1 Entry

In one example, work performed by Martinez and colleagues revealed important host- and tissue-specific restrictions to EBOV infection and disease utilizing a VLP entry assay [135]. While wild-type EBOV infection of nonhuman primates is highly lethal, experimental infection of mice and guinea pigs is not lethal and animals do not succumb to disease [136,137]. Toward elucidating the determinants of EBOV host- and tissue-specific tropism, the researchers focused on the GP, which mediates viral attachment and entry into permissive cells [138]. To study the role of GP in tropism, 293T cells were transiently transfected with a plasmid encoding EBOV VP40 fused to a β-lactamase and another plasmid encoding EBOV GP—resulting in EBOV VLPs expressing the β-lactamase reporter gene. Successful entry of VLP was detected by measuring enzyme activity after addition of a substrate. It was previously shown that a mutation at site 88 on the GP from a phenylalanine to an alanine (F88A) decreases the affinity of GP to the human Niemann-Pick C1 (NPC1) protein, an essential filovirus receptor and that higher expression of NPC1 can rescue VLPs with the F88A mutation [139]. A closer look at the differential expression of NPC1 in murine cells reveals a pattern: cells with higher NPC1 expression are more permissive in internalizing VLPs expressing a mutant version of the F88A than cells that do not. This finding provided evidence that a region within the GP can influence affinity to a host cell receptor and affect the host cell range of EBOV.

4.5.2 Budding

The ability to produce EBOV VLPs has also provided a method by which to investigate virus–host interactions that govern the budding phase of filoviruses. A similar method as described earlier can be utilized to generate VLPs expressing a reporter gene. While it is generally understood that the self-assembly and oligomerization of viral proteins, such as EBOV VP40,

are the major driving force of the budding of mature virions during later stages of viral replication, there are virus–host interactions that play crucial roles in facilitating efficient viral egress. In a recent study [140], investigators showed that the interaction of host suppressor of cytokine signaling 3 (SOCS3) with VP40 enhances ubiquitinylation and egress of VP40. Indeed, overexpression of SOCS3 correlated with the increased budding of EBOV VP40 VLPs [140]. In a previous study, overexpression of a host E3 ubiquitin ligase, Nedd4, through its interaction with the PPxY domain of VP40 enhanced EBOV VP40 VLP budding [141].

REFERENCES

[1] E.M. Crimmins, C.E. Finch, Infection, inflammation, height, and longevity, Proc. Natl. Acad. Sci. U.S.A. 103 (2) (2006 Jan 10) 498–503.
[2] WHO. Estimated economic impact of vaccinations in 73 low- and middle-income countries, 2001–2020 [Internet]. WHO. [cited 2017 October 3]. Available from: http://www.who.int/bulletin/volumes/95/9/16-178475/en/.
[3] R. Rappuoli, C.W. Mandl, S. Black, E. De Gregorio, Vaccines for the twenty-first century society, Nat. Rev. Immunol. 11 (12) (2011) 865–872.
[4] MODULE 2—Components of a vaccine—WHO Vaccine Safety Basics [Internet]. [cited 2017 November 2]. Available from: http://vaccine-safety-training.org/vaccine-components.html.
[5] E. Bell, Cytokines: linking innate and adaptive immunity, Nat. Rev. Immunol. 2 (6) (2002) 381.
[6] R. Toporovski, M.P. Morrow, D.B. Weiner, Interferons as potential adjuvants in prophylactic vaccines, Expert Opin. Biol. Ther. 10 (10) (2010) 1489–1500.
[7] P.H. Goff, T. Hayashi, L. Martínez-Gil, M. Corr, B. Crain, S. Yao, et al., Synthetic TLR4 and TLR7 ligands as influenza virus vaccine adjuvants induce rapid, sustained and broadly protective responses, J. Virol. 89 (6) (2015) 3221–3235.
[8] L. Martínez-Gil, P.H. Goff, R. Hai, A. García-Sastre, M.L. Shaw, P. Palese, A Sendai virus-derived RNA agonist of RIG-I as a virus vaccine adjuvant, J. Virol. 87 (3) (2013) 1290–1300.
[9] L. Martínez-Gil, J. Ayllon, M.B. Ortigoza, A. García-Sastre, M.L. Shaw, P. Palese, Identification of small molecules with type I interferon inducing properties by high-throughput screening, PLoS One 7 (11) (2012), e49049.
[10] The UniProt Consortium, UniProt: the universal protein knowledgebase, Nucleic Acids Res. 45 (D1) (2017) D158–69.
[11] C.R.H. Raetz, C. Whitfield, Lipopolysaccharide endotoxins, Annu. Rev. Biochem. 71 (2002) 635–700.
[12] Y. Rosenfeld, Y. Shai, Lipopolysaccharide (endotoxin)-host defense antibacterial peptides interactions: role in bacterial resistance and prevention of sepsis, Biochim. Biophys. Acta 1758 (9) (2006) 1513–1522.
[13] E.F. Kenny, L.A.J. O'Neill, Signalling adaptors used by toll-like receptors: an update, Cytokine 43 (3) (2008) 342–349.
[14] B. Kobe, A.V. Kajava, The leucine-rich repeat as a protein recognition motif, Curr. Opin. Struct. Biol. 11 (6) (2001) 725–732.
[15] B.S. Park, D.H. Song, H.M. Kim, B.-S. Choi, H. Lee, J.-O. Lee, The structural basis of lipopolysaccharide recognition by the TLR4–MD-2 complex, Nature 458 (7242) (2009) 1191–1195.

[16] T. Ve, P.R. Vajjhala, A. Hedger, T. Croll, F. DiMaio, S. Horsefield, et al., Structural basis of TIR-domain-assembly formation in MAL- and MyD88-dependent TLR4 signaling, Nat. Struct. Mol. Biol. 24 (9) (2017) 743–751.
[17] K.S. Mineev, S.A. Goncharuk, M.V. Goncharuk, P.E. Volynsky, E.V. Novikova, A.S. Aresinev, Spatial structure of TLR4 transmembrane domain in bicelles provides the insight into the receptor activation mechanism, Sci. Rep. 7 (1) (2017) 6864.
[18] K. Miyake, Roles for accessory molecules in microbial recognition by Toll-like receptors, J. Endotoxin Res. 12 (4) (2006) 195–204.
[19] R. Shimazu, S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, et al., MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4, J. Exp. Med. 189 (11) (1999) 1777–1782.
[20] H.M. Kim, B.S. Park, J.-I. Kim, S.E. Kim, J. Lee, O. SC, et al., Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist eritoran, Cell 130 (5) (2007) 906–917.
[21] U. Ohto, K. Fukase, K. Miyake, T. Shimizu, Structural basis of species-specific endotoxin sensing by innate immune receptor TLR4/MD-2, Proc. Natl. Acad. Sci. U.S.A. 109 (19) (2012) 7421–7426.
[22] E. Guven-Maiorov, O. Keskin, A. Gursoy, C. VanWaes, Z. Chen, C.-J. Tsai, et al., The architecture of the TIR domain signalosome in the Toll-like receptor-4 signaling pathway, Sci. Rep. 5 (2015). Available from:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4544004/.
[23] L.A.J. O'Neill, A.G. Bowie, The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling, Nat. Rev. Immunol. 7 (5) (2007) 353–364.
[24] E. Valkov, A. Stamp, F. Dimaio, D. Baker, B. Verstak, P. Roversi, et al., Crystal structure of Toll-like receptor adaptor MAL/TIRAP reveals the molecular basis for signal transduction and disease protection, Proc. Natl. Acad. Sci. U.S.A. 108 (36) (2011) 14879–14884.
[25] M. Landström, The TAK1-TRAF6 signalling pathway, Int. J. Biochem. Cell Biol. 42 (5) (2010) 585–589.
[26] S. Akira, K. Takeda, Toll-like receptor signalling, Nat. Rev. Immunol. 4 (7) (2004) 499–511.
[27] Y. Enokizono, H. Kumeta, K. Funami, M. Horiuchi, J. Sarmiento, K. Yamashita, et al., Structures and interface mapping of the TIR domain-containing adaptor molecules involved in interferon signaling, Proc. Natl. Acad. Sci. U.S.A. 110 (49) (2013) 19908–19913.
[28] S. Liu, X. Cai, J. Wu, Q. Cong, X. Chen, T. Li, et al., Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation, Science 347 (6227) (2015). aaa2630.
[29] K. Honda, A. Takaoka, T. Taniguchi, Type I interferon gene induction by the interferon regulatory factor family of transcription factors, Immunity 25 (3) (2006) 349–360.
[30] A. Zariri, P. van der Ley, Biosynthetically engineered lipopolysaccharide as vaccine adjuvant, Expert Rev. Vaccines 14 (6) (2015) 861–876.
[31] J. Geurtsen, L. Steeghs, H.-J. Hamstra, J. Ten Hove, A. de Haan, B. Kuipers, et al., Expression of the lipopolysaccharide-modifying enzymes PagP and PagL modulates the endotoxic activity of Bordetella pertussis, Infect. Immun. 74 (10) (2006) 5574–5585.
[32] N. Qureshi, P. Mascagni, E. Ribi, K. Takayama, Monophosphoryl lipid A obtained from lipopolysaccharides of Salmonella minnesota R595. Purification of the dimethyl derivative by high performance liquid chromatography and complete structural determination, J. Biol. Chem. 260 (9) (1985) 5271–5278.

[33] D.C. Beachler, A.R. Kreimer, M. Schiffman, R. Herrero, S. Wacholder, A.C. Rodriguez, et al., Multisite HPV16/18 vaccine efficacy against cervical, anal, and oral HPV infection, J. Natl. Cancer Inst. 108 (1) (2016).
[34] M. Lehtinen, J. Paavonen, C.M. Wheeler, U. Jaisamrarn, S.M. Garland, X. Castellsagué, et al., Overall efficacy of HPV-16/18 AS04-adjuvanted vaccine against grade 3 or greater cervical intraepithelial neoplasia: 4-year end-of-study analysis of the randomised, double-blind PATRICIA trial, Lancet Oncol. 13 (1) (2012) 89–99.
[35] C.J.P.A. Hoebe, A.P.A. Vermeiren, N.H.T.M. Dukers-Muijrers, Revaccination with Fendrix® or HBVaxPro® results in better response rates than does revaccination with three doses of Engerix-B® in previous non-responders, Vaccine 30 (48) (2012) 6734–6737.
[36] E. Pupo, H.-J. Hamstra, H. Meiring, P. van der Ley, Lipopolysaccharide engineering in Neisseria meningitidis: structural analysis of different pentaacyl lipid A mutants and comparison of their modified agonist properties, J. Biol. Chem. 289 (12) (2014) 8668–8680.
[37] Y. Zhang, J. Gaekwad, M.A. Wolfert, G.-J. Boons, Synthetic tetra-acylated derivatives of lipid A from Porphyromonas gingivalis are antagonists of human TLR4, Org. Biomol. Chem. 6 (18) (2008) 3371–3381.
[38] C.R. Casella, T.C. Mitchell, Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant, Cell. Mol. Life Sci. 65 (20) (2008) 3231–3240.
[39] A. Poltorak, X. He, I. Smirnova, M.Y. Liu, C. Van Huffel, X. Du, et al., Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene, Science 282 (5396) (1998) 2085–2088.
[40] V. Mata-Haro, C. Cekic, M. Martin, P.M. Chilton, C.R. Casella, T.C. Mitchell, The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4, Science 316 (5831) (2007) 1628–1632.
[41] T. Kawai, S. Akira, The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors, Nat. Immunol. 11 (5) (2010) 373–384.
[42] T. Kawai, O. Adachi, T. Ogawa, K. Takeda, S. Akira, Unresponsiveness of MyD88-deficient mice to endotoxin, Immunity 11 (1) (1999) 115–122.
[43] C.H. Clegg, R. Roque, N. Van Hoeven, L. Perrone, S.L. Baldwin, J.A. Rininger, et al., Adjuvant solution for pandemic influenza vaccine production, Proc. Natl. Acad. Sci. U.S.A. 109 (43) (2012) 17585–17590.
[44] D.A. Johnson, C.G. Sowell, C.L. Johnson, M.T. Livesay, D.S. Keegan, M.J. Rhodes, et al., Synthesis and biological evaluation of a new class of vaccine adjuvants: aminoalkyl glucosaminide 4-phosphates (AGPs), Bioorg. Med. Chem. Lett. 9 (15) (1999) 2273–2278.
[45] G.L. Morefield, L.D. Hawkins, S.T. Ishizaka, T.L. Kissner, R.G. Ulrich, Synthetic Toll-like receptor 4 agonist enhances vaccine efficacy in an experimental model of toxic shock syndrome, Clin. Vaccine Immunol. 14 (11) (2007) 1499–1504.
[46] W.S. Bowen, L.A. Minns, D.A. Johnson, T.C. Mitchell, M.M. Hutton, J.T. Evans, Selective TRIF-dependent signaling by a synthetic Toll-like receptor 4 agonist, Sci. Signal. 5 (211) (2012) ra13.
[47] S.B. Sirima, C. Durier, L. Kara, S. Houard, A. Gansane, P. Loulergue, et al., Safety and immunogenicity of a recombinant Plasmodium falciparum AMA1-DiCo malaria vaccine adjuvanted with GLA-SE or Alhydrogel® in European and African adults: a phase 1a/1b, randomized, double-blind multi-centre trial, Vaccine 35 (45) (2017) 6218–6227.
[48] M. Chan, T. Hayashi, R.D. Mathewson, A. Nour, Y. Hayashi, S. Yao, et al., Identification of substituted pyrimido[5,4-b]indoles as selective Toll-like receptor 4 ligands, J. Med. Chem. 56 (11) (2013) 4206–4223.

[49] A. Nour, T. Hayashi, M. Chan, S. Yao, R.I. Tawatao, B. Crain, et al., Discovery of substituted 4-aminoquinazolines as selective Toll-like receptor 4 ligands, Bioorg. Med. Chem. Lett. 24 (21) (2014) 4931–4938.
[50] J.D. Marshall, D.S. Heeke, E. Rao, S.K. Maynard, D. Hornigold, C. McCrae, et al., A novel class of small molecule agonists with preference for human over mouse TLR4 activation, PLoS One 11 (10) (2016), e0164632.
[51] M. Rehli, Of mice and men: species variations of Toll-like receptor expression, Trends Immunol. 23 (8) (2002) 375–378.
[52] C.R.H. Raetz, C.M. Reynolds, M.S. Trent, R.E. Bishop, Lipid A modification systems in gram-negative bacteria, Annu. Rev. Biochem. 76 (2007) 295–329.
[53] A.M. Hajjar, R.K. Ernst, E.S. Fortuno, A.S. Brasfield, C.S. Yam, L.A. Newlon, et al., Humanized TLR4/MD-2 mice reveal LPS recognition differentially impacts susceptibility to Yersinia pestis and Salmonella enterica, PLoS Pathog. 8 (10) (2012), e1002963.
[54] J.-M. Billod, A. Lacetera, J. Guzmán-Caldentey, S. Martín-Santamaría, Computational approaches to Toll-like receptor 4 modulation, Molecules 21 (8) (2016).
[55] O.C. Farokhzad, R. Langer, Impact of nanotechnology on drug delivery, ACS Nano 3 (1) (2009) 16–20.
[56] J.-S. Lee, J.J. Green, K.T. Love, J. Sunshine, R. Langer, D.G. Anderson, Gold, poly (beta-amino ester) nanoparticles for small interfering RNA delivery, Nano Lett. 9 (6) (2009) 2402–2406.
[57] N. Nasongkla, E. Bey, J. Ren, H. Ai, C. Khemtong, J.S. Guthi, et al., Multifunctional polymeric micelles as cancer-targeted, MRI-ultrasensitive drug delivery systems, Nano Lett. 6 (11) (2006) 2427–2430.
[58] C. Zhang, Z. Zhao, J.-W. Zha, G.-X. Wang, B. Zhu, Single-walled carbon nanotubes as delivery vehicles enhance the immunoprotective effect of a DNA vaccine against spring viremia of carp virus in common carp, Fish Shellfish Immunol. 71 (2017) 191–201.
[59] I. Neamtu, A.G. Rusu, A. Diaconu, L.E. Nita, A.P. Chiriac, Basic concepts and recent advances in nanogels as carriers for medical applications, Drug Deliv. 24 (1) (2017) 539–557.
[60] N. Grimaldi, F. Andrade, N. Segovia, L. Ferrer-Tasies, S. Sala, J. Veciana, et al., Lipid-based nanovesicles for nanomedicine, Chem. Soc. Rev. 45 (23) (2016) 6520–6545.
[61] A.D. Bangham, M.M. Standish, J.C. Watkins, Diffusion of univalent ions across the lamellae of swollen phospholipids, J. Mol. Biol. 13 (1) (1965) 238–252.
[62] G. Gregoriadis, E.J. Wills, C.P. Swain, A.S. Tavill, Drug-carrier potential of liposomes in cancer chemotherapy, Lancet 1 (7870) (1974) 1313–1316.
[63] A.G. Allison, G. Gregoriadis, Liposomes as immunological adjuvants, Nature 252 (5480) (1974) 252.
[64] V.A. Fadok, D.R. Voelker, P.A. Campbell, J.J. Cohen, D.L. Bratton, P.M. Henson, Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages, J. Immunol. 148 (7) (1992) 2207–2216.
[65] N.C. Phillips, J. Rioux, M.S. Tsao, Activation of murine Kupffer cell tumoricidal activity by liposomes containing lipophilic muramyl dipeptide, Hepatology 8 (5) (1988) 1046–1050.
[66] A. Badiee, A. Khamesipour, A. Samiei, D. Soroush, V.H. Shargh, M.T. Kheiri, et al., The role of liposome size on the type of immune response induced in BALB/c mice against leishmaniasis: rgp63 as a model antigen, Exp. Parasitol. 132 (4) (2012) 403–409.
[67] V. Bernasconi, K. Norling, M. Bally, F. Höök, N.Y. Lycke, Mucosal vaccine development based on liposome technology, J. Immunol. Res. 2016 (5482087) (2016).

[68] H.K. Charlton Hume, L.H.L. Lua, Platform technologies for modern vaccine manufacturing, Vaccine 35 (35,Pt. A) (2017) 4480–4485.
[69] W. Zeng, Y.-L. Chen, Editorial: advances in therapeutic glycopeptides, Protein Pept. Lett. 21 (10) (2014) 975.
[70] B. Apellániz, J.L. Nieva, The use of liposomes to shape epitope structure and modulate immunogenic responses of peptide vaccines against HIV MPER, Adv. Protein Chem. Struct. Biol. 99 (2015) 15–54.
[71] A. Razazan, J. Behravan, A. Arab, N. Barati, L. Arabi, Z. Gholizadeh, et al., Conjugated nanoliposome with the HER2/neu-derived peptide GP2 as an effective vaccine against breast cancer in mice xenograft model, PLoS One 12 (10) (2017), e0185099.
[72] M. Taneichi, Y. Tanaka, T. Kakiuchi, T. Uchida, Liposome-coupled peptides induce long-lived memory CD8 T cells without CD4 T cells, PLoS One 5 (11) (2010), e15091.
[73] P. Martinez-Murillo, K. Tran, J. Guenaga, G. Lindgren, M. Àdori, Y. Feng, et al., Particulate array of well-ordered HIV clade C Env trimers elicits neutralizing antibodies that display a unique V2 cap approach, Immunity 46 (5) (2017) 804–817.e7.
[74] R.A. Schwendener, Liposomes as vaccine delivery systems: a review of the recent advances, Ther. Adv. Vaccines 2 (6) (2014) 159–182.
[75] C. Zylberberg, K. Gaskill, S. Pasley, S. Matosevic, Engineering liposomal nanoparticles for targeted gene therapy, Gene Ther. 24 (8) (2017) 441–452.
[76] D. Wang, J. Xu, Y. Feng, Y. Liu, S.S.S. Mchenga, F. Shan, et al., Liposomal oral DNA vaccine (Mycobacterium DNA) elicits immune response, Vaccine 28 (18) (2010) 3134–3142.
[77] S. Jain, P. Singh, V. Mishra, S.P. Vyas, Mannosylated niosomes as adjuvant-carrier system for oral genetic immunization against hepatitis B, Immunol. Lett. 101 (1) (2005) 41–49.
[78] M. Reyes, C. Ramírez, I. Ñancucheo, R. Villegas, G. Schaffeld, L. Kriman, et al., A novel "in-feed" delivery platform applied for oral DNA vaccination against IPNV enables high protection in Atlantic salmon (Salmon salar), Vaccine 35 (4) (2017) 626–632.
[79] A.S. Ulrich, Biophysical aspects of using liposomes as delivery vehicles, Biosci. Rep. 22 (2) (2002) 129–150.
[80] M.J. Copland, M.A. Baird, T. Rades, J.L. McKenzie, B. Becker, F. Reck, et al., Liposomal delivery of antigen to human dendritic cells, Vaccine 21 (9) (2003) 883–890.
[81] G.W. Siskind, B. Benacerraf, Cell selection by antigen in the immune response, Adv. Immunol. 10 (1969) 1–50.
[82] Y. Perrie, F. Crofts, A. Devitt, H.R. Griffiths, E. Kastner, V. Nadella, Designing liposomal adjuvants for the next generation of vaccines, Adv. Drug Deliv. Rev. 99 (Pt. A) (2016) 85–96.
[83] S. Tandrup Schmidt, C. Foged, K.S. Korsholm, T. Rades, D. Christensen, Liposome-based adjuvants for subunit vaccines: formulation strategies for subunit antigens and immunostimulators, Pharmaceutics 8 (1) (2016).
[84] X. Li, B.R. Sloat, N. Yanasarn, Z. Cui, Relationship between the size of nanoparticles and their adjuvant activity: data from a study with an improved experimental design, Eur. J. Pharm. Biopharm. 78 (1) (2011) 107–116.
[85] N. Yanasarn, B.R. Sloat, Z. Cui, Negatively charged liposomes show potent adjuvant activity when simply admixed with protein antigens, Mol. Pharm. 8 (4) (2011) 1174–1185.
[86] C. Barnier-Quer, A. Elsharkawy, S. Romeijn, A. Kros, W. Jiskoot, Adjuvant effect of cationic liposomes for subunit influenza vaccine: influence of antigen loading method, cholesterol and immune modulators, Pharmaceutics 5 (3) (2013) 392–410.

[87] J.P. Adler-Moore, W. Ernst, H. Kim, N. Ward, S.M. Chiang, T. Do, et al., Monomeric M2e antigen in VesiVax(®) liposomes stimulates protection against type a strains of influenza comparable to liposomes with multimeric forms of M2e, J. Liposome Res. 27 (3) (2017) 210–220.
[88] G.B. Patel, H. Zhou, A. Ponce, W. Chen, Mucosal and systemic immune responses by intranasal immunization using archaeal lipid-adjuvanted vaccines, Vaccine 25 (51) (2007) 8622–8636.
[89] U. Glück, J.O. Gebbers, R. Glück, Phase 1 evaluation of intranasal virosomal influenza vaccine with and without Escherichia coli heat-labile toxin in adult volunteers, J. Virol. 73 (9) (1999) 7780–7786.
[90] H. Shirota, D.M. Klinman, Recent progress concerning CpG DNA and its use as a vaccine adjuvant, Expert Rev. Vaccines 13 (2) (2014) 299–312.
[91] A.M. Hafner, B. Corthésy, H.P. Merkle, Particulate formulations for the delivery of poly(I:C) as vaccine adjuvant, Adv. Drug Deliv. Rev. 65 (10) (2013) 1386–1399.
[92] D. Christensen, E.M. Agger, L.V. Andreasen, D. Kirby, P. Andersen, Y. Perrie, Liposome-based cationic adjuvant formulations (CAF): past, present, and future, J. Liposome Res. 19 (1) (2009) 2–11.
[93] R. Effenberg, P. Turánek Knötigová, D. Zyka, H. Čelechovská, J. Mašek, E. Bartheldyová, et al., Nonpyrogenic molecular adjuvants based on norAbu-muramyldipeptide and norAbu-glucosaminyl muramyldipeptide: synthesis, molecular mechanisms of action, and biological activities in vitro and in vivo, J. Med. Chem. 60 (18) (2017) 7745–7763.
[94] A. Ko, S.R. Wui, J.I. Ryu, Y.J. Lee, D.T.T. Hien, I. Rhee, et al., Potentiation of Th1-type immune responses to m. tuberculosis antigens in mice by cationic liposomes combined with de-O-acylated lipooligosaccharide, J. Microbiol. Biotechnol. 27 (7) (2017) 136–144.
[95] D.A. Espinosa, D. Christensen, C. Muñoz, S. Singh, E. Locke, P. Andersen, et al., Robust antibody and CD8(+) T-cell responses induced by P. falciparum CSP adsorbed to cationic liposomal adjuvant CAF09 confer sterilizing immunity against experimental rodent malaria infection, NPJ Vaccines 2 (2017) 10.
[96] L.J. Cruz, F. Rueda, B. Cordobilla, L. Simón, L. Hosta, F. Albericio, et al., Targeting nanosystems to human DCs via Fc receptor as an effective strategy to deliver antigen for immunotherapy, Mol. Pharm. 8 (1) (2011) 104–116.
[97] L.K. Pattenden, A.P.J. Middelberg, M. Niebert, D.I. Lipin, Towards the preparative and large-scale precision manufacture of virus-like particles, Trends Biotechnol. 23 (10) (2005) 523–529.
[98] C.J. Burrell, P. Mackay, P.J. Greenaway, P.H. Hofschneider, K. Murray, Expression in Escherichia coli of hepatitis B virus DNA sequences cloned in plasmid pBR322, Nature 279 (5708) (1979) 43–47.
[99] P. Valenzuela, A. Medina, W.J. Rutter, G. Ammerer, B.D. Hall, Synthesis and assembly of hepatitis B virus surface antigen particles in yeast, Nature 298 (5872) (1982) 347–350.
[100] J.R. Haynes, J. Cunningham, A. Seefried, M. von Lennick, R.T. Garvin, S.-H. Shen, Development of a genetically-engineered, candidate polio vaccine employing the self-assembling properties of the tobacco mosaic virus coat protein. Nat. Biotechnol. 4 (7) (1986) 637–641, https://doi.org/10.1038/nbt0786-637.
[101] B.J. Cohen, J.E. Richmond, Electron microscopy of hepatitis B core antigen synthesized in E. coli, Nature 296 (5858) (1982) 677–679.
[102] I. Sominskaya, D. Skrastina, A. Dislers, D. Vasiljev, M. Mihailova, V. Ose, et al., Construction and immunological evaluation of multivalent hepatitis B virus (HBV) core virus-like particles carrying HBV and HCV epitopes, Clin. Vaccine Immunol. 17 (6) (2010) 1027–1033.
[103] I.H. Frazer, Prevention of cervical cancer through papillomavirus vaccination, Nat. Rev. Immunol. 4 (1) (2004) 46–54.

[104] G.N.A. Vyas, Brief overview of the new vaccines against hepatitis B virus infection: immunogenic gene products and peptide analogues of antigenic epitopes, Dev. Biol. Stand. 63 (1986) 141–146.
[105] S. Mishiro, M. Imai, K. Takahashi, A. Machida, T. Gotanda, Y. Miyakawa, et al., A 49,000-dalton polypeptide bearing all antigenic determinants and full immunogenicity of 22-nm hepatitis B surface antigen particles, J. Immunol. 124 (4) (1980) 1589–1593.
[106] L.M. Branco, J.N. Grove, F.J. Geske, M.L. Boisen, I.J. Muncy, S.A. Magliato, et al., Lassa virus-like particles displaying all major immunological determinants as a vaccine candidate for lassa hemorrhagic fever, Virol. J. 7 (2010) 279.
[107] Q. Ying, T. Ma, L. Cheng, X. Zhang, A.D. Truax, R. Ma, et al., Construction and immunological characterization of CD40L or GM-CSF incorporated Hantaan virus like particle, Oncotarget 7 (39) (2016) 63488–63503.
[108] K.G. Lokugamage, N. Yoshikawa-Iwata, N. Ito, D.M. Watts, P.R. Wyde, N. Wang, et al., Chimeric coronavirus-like particles carrying severe acute respiratory syndrome coronavirus (SCoV) S protein protect mice against challenge with SCoV, Vaccine 26 (6) (2008) 797–808.
[109] G. Spohn, G.T. Jennings, B.E. Martina, I. Keller, M. Beck, P. Pumpens, et al., A VLP-based vaccine targeting domain III of the West Nile virus E protein protects from lethal infection in mice, Virol. J. 7 (2010) 146.
[110] C.Y. Kang, L. Luo, M.A. Wainberg, Y. Li, Development of HIV/AIDS vaccine using chimeric Gag-Env virus-like particles, Biol. Chem. 380 (3) (1999) 353–364.
[111] J.M. Galarza, T. Latham, A. Cupo, Virus-like particle vaccine conferred complete protection against a lethal influenza virus challenge, Viral Immunol. 18 (2) (2005) 365–372.
[112] P. Walpita, J. Barr, M. Sherman, C.F. Basler, L. Wang, Vaccine potential of Nipah virus-like particles, PLoS One 6 (4) (2011), e18437.
[113] K.R. Young, S.P. McBurney, L.U. Karkhanis, T.M. Ross, Virus-like particles: designing an effective AIDS vaccine, Methods 40 (1) (2006) 98–117.
[114] D.L. Swenson, K.L. Warfield, D.L. Negley, A. Schmaljohn, M.J. Aman, S. Bavari, Virus-like particles exhibit potential as a pan-filovirus vaccine for both Ebola and Marburg viral infections, Vaccine 23 (23) (2005) 3033–3042.
[115] P. Chlanda, O. Schraidt, S. Kummer, J. Riches, H. Oberwinkler, S. Prinz, et al., Structural analysis of the roles of influenza A virus membrane-associated proteins in assembly and morphology, J. Virol. 89 (17) (2015) 8957–8966.
[116] B. Dadonaite, S. Vijayakrishnan, E. Fodor, D. Bhella, E.C. Hutchinson, Filamentous influenza viruses, J. Gen. Virol. 97 (8) (2016) 1755–1764.
[117] B.J. Chen, G.P. Leser, E. Morita, R.A. Lamb, Influenza virus hemagglutinin and neuraminidase, but not the matrix protein, are required for assembly and budding of plasmid-derived virus-like particles, J. Virol. 81 (13) (2007) 7111–7123.
[118] F.-S. Quan, Y.-T. Lee, K.-H. Kim, M.-C. Kim, S.-M. Kang, Progress in developing virus-like particle influenza vaccines, Expert Rev. Vaccines 15 (10) (2016) 1281–1293.
[119] E. Poteet, P. Lewis, C. Chen, S.O. Ho, T. Do, S. Chiang, et al., Toll-like receptor 3 adjuvant in combination with virus-like particles elicit a humoral response against HIV, Vaccine 34 (48) (2016) 5886–5894.
[120] D.I. Bernstein, R.L. Atmar, G.M. Lyon, J.J. Treanor, W.H. Chen, X. Jiang, et al., Norovirus vaccine against experimental human GII.4 virus illness: a challenge study in healthy adults, J. Infect. Dis. 211 (6) (2015) 870–878.
[121] P. Van Damme, G. Leroux-Roels, P. Simon, J.-M. Foidart, G. Donders, K. Hoppenbrouwers, et al., Effects of varying antigens and adjuvant systems on the immunogenicity and safety of investigational tetravalent human oncogenic papillomavirus vaccines: results from two randomized trials, Vaccine 32 (29) (2014) 3694–3705.

[122] J. Beran, Safety and immunogenicity of a new hepatitis B vaccine for the protection of patients with renal insufficiency including pre-haemodialysis and haemodialysis patients, Expert Opin. Biol. Ther. 8 (2) (2008) 235–247.
[123] K. Schneider-Ohrum, B.M. Giles, H.K. Weirback, B.L. Williams, D.R. DeAlmeida, T.M. Ross, Adjuvants that stimulate TLR3 or NLPR3 pathways enhance the efficiency of influenza virus-like particle vaccines in aged mice, Vaccine 29 (48) (2011) 9081–9092.
[124] K.Y. Chung, E.M. Coyle, D. Jani, L.R. King, R. Bhardwaj, L. Fries, et al., ISCOMATRIX™ adjuvant promotes epitope spreading and antibody affinity maturation of influenza A H7N9 virus like particle vaccine that correlate with virus neutralization in humans, Vaccine 33 (32) (2015) 3953–3962.
[125] V. Cimica, J.M. Galarza, Adjuvant formulations for virus-like particle (VLP) based vaccines, Clin. Immunol. 183 (2017) 99–108.
[126] J.H. Kuhn, S. Becker, H. Ebihara, T.W. Geisbert, K.M. Johnson, Y. Kawaoka, et al., Proposal for a revised taxonomy of the family Filoviridae: classification, names of taxa and viruses, and virus abbreviations, Arch. Virol. 155 (12) (2010) 2083–2103.
[127] C.F. Basler, Molecular pathogenesis of viral hemorrhagic fever, Semin. Immunopathol. 39 (5) (2017) 551–561.
[128] S. Paessler, D.H. Walker, Pathogenesis of the viral hemorrhagic fevers, Annu. Rev. Pathol. 8 (2013) 411–440.
[129] H.-J. Schnittler, H. Feldmann, Viral hemorrhagic fever—a vascular disease? Thromb. Haemost. 89 (6) (2003) 967–972.
[130] E.J. Mendoza, T. Racine, G.P. Kobinger, The ongoing evolution of antibody-based treatments for ebola virus infection, Immunotherapy 9 (5) (2017) 435–450.
[131] R.N. Harty, M.E. Brown, G. Wang, J. Huibregtse, F.P. Hayes, A PPxY motif within the VP40 protein of Ebola virus interacts physically and functionally with a ubiquitin ligase: implications for filovirus budding, Proc. Natl. Acad. Sci. U.S.A. 97 (25) (2000) 13871–13876.
[132] J. Timmins, S. Scianimanico, G. Schoehn, W. Weissenhorn, Vesicular release of ebola virus matrix protein VP40, Virology 283 (1) (2001) 1–6.
[133] L.D. Jasenosky, G. Neumann, I. Lukashevich, Y. Kawaoka, Ebola virus VP40-induced particle formation and association with the lipid bilayer, J. Virol. 75 (11) (2001) 5205–5214.
[134] T. Noda, H. Sagara, E. Suzuki, A. Takada, H. Kida, Y. Kawaoka, Ebola virus VP40 drives the formation of virus-like filamentous particles along with GP, J. Virol. 76 (10) (2002) 4855–4865.
[135] O. Martinez, E. Ndungo, L. Tantral, E.H. Miller, L.W. Leung, K. Chandran, et al., A mutation in the Ebola virus envelope glycoprotein restricts viral entry in a host species- and cell-type-specific manner, J. Virol. 87 (6) (2013) 3324–3334.
[136] M. Bray, K. Davis, T. Geisbert, C. Schmaljohn, J. Huggins, A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever, J. Infect. Dis. 179 (Suppl. 1) (1999) S248–258.
[137] B.M. Connolly, K.E. Steele, K.J. Davis, T.W. Geisbert, W.M. Kell, N.K. Jaax, et al., Pathogenesis of experimental Ebola virus infection in guinea pigs, J. Infect. Dis. 179 (Suppl. 1) (1999) S203–217.
[138] S. Bär, A. Takada, Y. Kawaoka, M. Alizon, Detection of cell-cell fusion mediated by ebola virus glycoproteins, J. Virol. 80 (6) (2006) 2815–2822.
[139] E.H. Miller, G. Obernosterer, M. Raaben, A.S. Herbert, M.S. Deffieu, A. Krishnan, et al., Ebola virus entry requires the host-programmed recognition of an intracellular receptor, EMBO J. 31 (8) (2012) 1947–1960.
[140] A. Okumura, A.L. Rasmussen, P. Halfmann, F. Feldmann, A. Yoshimura, H. Feldmann, et al., Suppressor of cytokine signaling 3 is an inducible host factor that regulates virus egress during ebola virus infection, J. Virol. 89 (20) (2015) 10399–10406.
[141] J. Yasuda, M. Nakao, Y. Kawaoka, H. Shida, Nedd4 regulates egress of ebola virus-like particles from host cells, J. Virol. 77 (18) (2003) 9987–9992.

FURTHER READING

[142] A. Zeltins, Construction and characterization of virus-like particles: a review, Mol. Biotechnol. 53 (1) (2013) 92–107.

[143] M.O. Mohsen, L. Zha, G. Cabral-Miranda, M.F. Bachmann, Major findings and recent advances in virus-like particle (VLP)-based vaccines, Semin. Immunol. 34 (2017) 123–132.