Bifunctional lipids in tumor vaccines: an outstanding delivery carrier and promising immune stimulator

Zhiling Liu[1], Na Xu[1], Lin Zhao[1], Jia Yu[1], and Peng Zhang[1]

[1]Shenyang Pharmaceutical University

March 07, 2024

Abstract

Cancer is still a major threat for human life, and the cancer immunotherapy can be more optimized to prolong life. However, the effect of immunotherapy is not encouraging. In order to achieve outstanding immune effect, it is necessary to strengthen antigens uptake of antigen presenting cells. Adjuvants were added to vaccines to achieve this purpose, which could be divided into two types: as an immunostimulatory molecule, the innate immunities of the body were triggered; or as a delivery carrier, and antigens were cross-delivery through the "cytoplasmic pathway" and released at a specific location. This paper reviewed the relevant research status of tumor vaccine immune adjuvants in recent years. Among the review, the function, combination strategies and derivatives of lipid A were discussed in detail. In addition, some suggestions on the existing problems and research direction of lipids as tumor vaccine adjuvants were put forward.

Bifunctional lipids in tumor vaccines: an outstanding delivery carrier and promising immune stimulator

Zhiling Liu, Na Xu, Lin Zhao, Jia Yu*, Peng Zhang*

Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China.

* Corresponding Author Tel. and Fax: +86 24 2398 6256

E-mail address: zhangpengspu@163.com (Peng Zhang)

yujia_yc@163.com (Jia Yu)

Conflict of interest disclosure

We confirm that this work is new and original and not under consideration elsewhere. The submission of this manuscript has been approved by Shenyang Pharmaceutical University.

Bifunctional lipids in tumor vaccines: an outstanding delivery carrier and promising immune stimulator

Summary

Cancer is still a major threat for human life, and the cancer immunotherapy can be more optimized to prolong life. However, the effect of immunotherapy is not encouraging. In order to achieve outstanding immune effect, it is necessary to strengthen antigens uptake of antigen presenting cells. Adjuvants were added to vaccines to achieve this purpose, which could be divided into two types: as an immunostimulatory molecule, the innate immunities of the body were triggered; or as a delivery carrier, and antigens were cross-delivery through the "cytoplasmic pathway" and released at a specific location. This paper reviewed the relevant research status of tumor vaccine immune adjuvants in recent years. Among the review, the function,

combination strategies and derivatives of lipid A were discussed in detail. In addition, some suggestions on the existing problems and research direction of lipids as tumor vaccine adjuvants were put forward.

Keywords: tumor immunotherapy, adjuvant, TLR4, lipid-based, liposomes

Introduction

For decades, how to treat cancer effectively has been explored and studied, and the progresses and breakthroughs in this field have changed the prospect of cancer treatment. At present, there are many treatment ways to choose from, such as surgical resection, chemotherapy, radiotherapy, hormone therapy and immunotherapy [1]. However, the inevitably side effects of these methods and the risk of tumor recurrence contributed to cure cancer difficultly. Cancer vaccine is an innovative type of vaccine, which reduces the side effects of traditional cancer treatment and improves the treatment compliance of cancer patients by strengthening the body's natural defense against cancer. Although most cancer vaccines have not yet been mass-produced and widely used, there is no doubt that the treatment of cancer vaccine will be one of the most powerful cancer treatments in the future. The effective antigenic substances in cancer vaccines can be cancer cells, cell lysates, proteins, peptides, and nucleic acids that encode some cancer antigens [2]. In general, the immunogenicity of tumor antigens is poor, so it is not realistic to achieve the ideal tumor immune effect only by injecting tumor antigen.

Adding immune stimulants to the vaccine system as a way to stimulate the body's innate immunity has been proved by years of researches on the prevention of viruses and bacteria [3]. When injecting adjuvants before antigens injection or simultaneously injecting adjuvants to bind to antigens, nonspecific immune response is enhanced or the type of immune response is reversed. The adjuvants continue to evolve, from aluminum and Freund's adjuvants to lipopolysaccharide endotoxins, from gram-negative bacteria to various immunostimulatory complexes (ISCOM) [4]. The reasonable use of adjuvants can reduce antigenic applications, reduce the incidence of adverse reactions, thereby achieving better immune effects [5]. Successful vaccines should include three key components: an effective antigenic cargo, immunostimulatory agent, and a targeted delivery system that precise delivers antigens and immunostimulators to the appropriate site [6].

In the vaccine system, adjuvants can be divided into two roles, one is immune stimulant, the other is delivery carrier to exert immune effect. Because of its good safety, controllable size and easy functionalization, lipids can not only be used as immune stimulants (stimulate innate immunity), but also can be self-assembled into carriers for antigen delivery (delivery to some cells). It is considered be an excellent candidate for adjuvants, and the characteristics of lipid adjuvants provide a new prospect for the development of effective cancer vaccines. When lipid adjuvants are used in cancer vaccines, innate immune receptors are usually stimulated at once. After the innate immunity is activated, the affinity of T cells to tumor antigens are enhanced, thus the immune response to tumor is improved [7]. The duration of inflammatory response induced by lipid adjuvant such as MPLA is short and the effective activation time is no more than 24 hours [8]. When different types of antigens (tumor-associated antigens or tumor-specific antigens) are delivered together, the enhancement effect of immune adjuvants will be different. Combining various adjuvants or developing a new more effective adjuvant, which can improve the effectiveness of a single adjuvant in the vaccine while preventing from being suppressed by non-apoptotic tumor cells.

In this review, the research status and application prospects of lipid-based adjuvant in tumor vaccines were briefly discussed. The two applications of adjuvants are elaborated, and the most mature tumor immunostimulant: the function, chemical synthesis, combination strategy and derivatives of lipid A were discussed in detail. In addition, the problems and insights of immune adjuvants in the field of tumor vaccines would be proposed based on the current research.

As a delivery carrier to play the role of adjuvant

Liposome delivery carrier

Characteristics of liposome carriers. Liposomes are vesicles formed by self-assembly of phospholipid molecules, which can wrap water-soluble antigens in the center of the vesicles (proteins, peptides, nucleic

acids, etc.) (Fig. 1). While lipophilic substances can be embedded into the lipid bilayer by adsorption or stable chemical covalence. Liposomes are potential candidates for vaccine delivery because of their versatility, biocompatibility, and biodegradability [9]. As the length and saturation of the phospholipid molecule of liposomes is different, the membrane fluidity of the liposomes also changes [10]. The phase change temperature (Tc) of the phospholipid determines the morphology of phospholipids [11]. When the ambient temperature is lower than the liposome, the liposome is in a rigid state, and the cargo can be stored in the cavity. When the ambient temperature increases to liposome Tc, the liposome exhibits maximum permeability, and all goods can be released. The Tc of a single phospholipid molecule is determined, but the formulation of mixed different phospholipid molecules is usually used when preparing the liposome, and the Tc can be adjusted to the most suitable temperature. Further, the addition of cholesterol helps to maintain the conformational order of the lipid bilayer and increase the mechanical stiffness of the liposome, thus achieving the purpose of fluidity of the protective film [12]. Therefore, the addition of cholesterol to the construction of liposomes can significantly reduce the permeability of liposome membrane, prevent liposome leakage at higher ambient temperature, and enhance the ability of liposome vesicles to resist changes in external conditions [13]. Previous liposome studies had shown that polyethylene glycol (PEG) modification could effectively prolong the circulation time of drugs *in vivo* [14, 15]. Surface PEG modification has a spatially stable effect on the structure of liposomes, preventing the agglutination and fusion of liposomes and preventing the binding of plasma proteins to liposomes, thus producing a spatially stable effect on liposomes [16]. Surface charge is not only one of the key factors affecting liposomes stability, but also one of the key factors affecting liposomes immunostimulant. Depending on the surface charge, the liposomes can be divided into cationic liposomes, anionic liposomes, and neutral liposomes. The entrapment efficiency and stability of liposomes are closely related to the surface charge. First, the entrapment efficiency of liposomes largely depends on the electrostatic interaction between liposomes and goods, so the surface charge determines the interaction between liposomes, biological components, and immune cells, affecting the loading and release of liposome antigens. The change of physical size will cause different transport rates of liposomes to antigen-presenting cells (APC), resulting in different immune effects. It is believed that the smaller the particle size of liposomes, the higher the stability, and the liposomes with the particle size of about 100 nm have better enhanced permeability and retention effect (EPR) in the tumor site [17]. When the particle size is smaller than 150 nm, it mainly promotes the development of Th2 cells; when the particle size larger than 200 nm, the typical Th1 immune response is developed [18, 19].

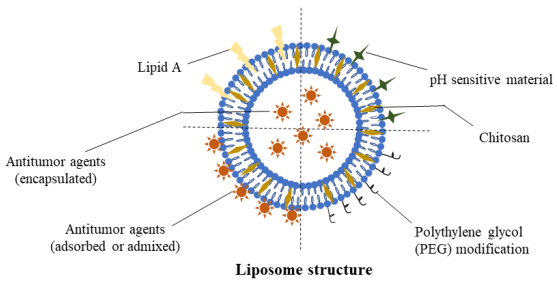


Fig. 1. General structure of liposome vaccines. The liposome vaccines consist of a two-layer structure with added cholesterol phospholipids, as well as pH-sensitive materials and (polyethylene glycol) PEG modifications for versatility. The ways in which liposomes carry antigens include encapsulation, absorption or

admixed. Lipid A is an adjuvant added in a liposome vaccine which is a lipid layer.

Preparation of liposome carriers. The properties of liposomes are closely related to the preparation liquid. In the past, liposome preparation techniques had similar general steps: drying from volatile solvents, dispersion, purification, and synthesis in aqueous medium, and finally liposomes were obtained [20].

Thin film hydration is the earliest liposome preparation technology, which mainly includes dissolving phospholipids in organic solvents, removing phospholipids by evaporation, and finally pouring them into aqueous buffer solution, stirring to make the dried lipid membranes hydrate successfully [21]. However, this method will produce many heterogeneous paleosecular liposomes. The reverse phase evaporation method is to reisolate the phospholipid molecules in the organic phase, and then adding aqueous phase buffer to a mixed solution to decompress distillation [22]. The two-phase system was ultrasonic treated to transparent and uniform single-phase dispersion, and it was observed that the solution state did not change for at least 30 minutes, then the organic solvent was removed by rotary evaporation. The liposomes prepared by reversed-phase evaporation method have unique advantages in encapsulating water-soluble substances, but may have drug residue [23]. The solvent injection method does not need to be helpful in ultrasonic waves, simply dissolve lipids in the organic phase which is generally diethyl ether or ethanol, and then injects the lipid solution into the aqueous solution, the particle diameter is obtained by narrowing a smaller-distribution [24]. With using ethanol, it can remain stable at higher temperature, but it is difficult to remove all the solvent, and the possibility of biological deactivation is high. Compared with ethanol, ether injection can completely remove the solvent and form liposomes with high entrapment efficiency, but due to the low boiling point of ether, it will lead to uneven aggregation and dissolution at high temperature [25]. The bubble method is a method without the use of organic solvents, in which inert gas bubbles are introduced into the lipid mixture to prepare liposomes with mild preparation conditions and high entrapment efficiency [26]. The uniqueness of supercritical fluids is that there is no difference in liquid and gaseous. The researchers found that the properties of liposomes obtained by supercritical fluids were better than those prepared by conventional solvents [27]. Supercritical carbon dioxide has become a substitute for organic solvents because of its low critical temperature and pressure, similar solubility to non-polar solvents, low application cost, and non-toxicity [28].

Conventional preparation techniques generally lack the precise regulation of liposome particle size on macro levels, batch reproducibility and quality repetitive control. Microfluidics refers to the precise control of fluid behaviors in micro-channels or designing different microfluidic channels to realize the mixed reaction of different liquids. Delivery carriers with small particle size distribution, high repeatability between batches and significantly improved drug loading rate can be prepared by microfluidic technology [29]. Microfluidic techniques can overcome the disadvantages of conventional methods, a highly stabilizing liposomes that can accurately control physical parameters can be obtained by designing flow rate, flow rate ratio, mixing speed, and lipid concentration. In order to be able to scale production, the preparation steps should be as simple as possible. For human health, liposomes should avoid using hazardous chemicals or solvents when prepared. The residual solution is potentially toxic to human body and causes great environmental pollution, so it should be eliminated in the preparation process. Therefore, when preparing liposomes, we should consider comprehensively and choose the most suitable preparation method to achieve the most beneficial results.

Relationship between the properties and immunogenicity of liposome carrier. Since Allison first reported in 1974, liposomes mainly exert immunity through the "pool effect" and immune stimulation, and gradually become the research object of vaccines as adjuvants [30, 31]. A mass of liposomes containing antigens are ingested by macrophages through natural channels, they can be stored in macrophages for slow release, thus introducing antigens to APC, promoting downstream cells to secrete a series of cytokines, and enabling the body to maintain high-valence antibodies (Fig. 2). Although vaccine carriers based on other materials, such as metal nanoparticles, inorganic (such as silica) nanoparticles, and polymer nanoparticles [32, 33] have also shown advantages over traditional preparations in achieving delivery and adjuvant functions, most vaccine vectors are still in the preclinical development stage, and their feasibility needs to be further explored. Also, based on the studies of existing tumor-specific antigen delivery, we can tell liposome carriers show better

properties than non-liposomes [34].

Several key parameters mentioned above, such as surface charge, physical size, and degree of PEG modification, play a very important role in regulating the biological behavior of liposome vaccine vectors. The interaction of various factors finally plays a comprehensive role in the immunostimulatory activity of the whole carrier. In addition, the loaded way of antigen is also very important. Mixing the carrier with the antigen and using mild adsorption may be more beneficial to maintain the relative activity of the antigen, but the surface adsorption also means a higher antigen release rate, resulting in low efficiency of antigen uptake. Encapsulating antigens in liposome carriers can achieve sustained release of antigens or drugs, resulting in a more effective cross-presentation process and a better lysosome escape [35].

Cationic liposomes

The structure of cationic liposomes generally consists of three parts: one or more cationic heads, a linker bond, and a hydrophobic tail. The cationic head mostly contains amino groups, such as amino, methyl or hydroxyethyl substituted quaternary ammonium compound (Table 1). The multivalent polar head group or the polar head with multiple positive charges has higher transfection efficiency and allows the endosome to escape, and the tertiary amine group head has higher transfection activity and lower toxicity than the quaternary ammonium group head. Linkage bonds mainly include ester bond, amide bond and carbamate bond, which determine the chemical stability and biodegradability of cationic lipid molecules. Long-chain aliphatic hydrocarbons in cationic liposomes can significantly enhance the interaction with cell membrane and have high transfection efficiency. And the hydrophobic tails of common cationic liposomes are mainly composed of aliphatic hydrocarbon chains and cholesterol [36].

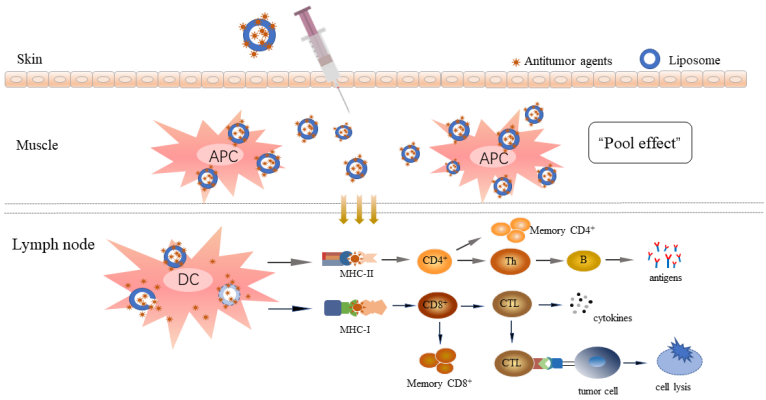


Fig. 2. The mechanism of immune function of vaccine liposomes. After being injected into the body, the liposome vaccine is ingested by macrophages and stored in it to produce "pool effect". Then the antigen is presented by APC cells (especially DC) and transferred to the lymph nodes. After extracting the antigen information, $CD4^+CD8^+$ was activated by MHC-I and MHC-II respectively to secrete specific antibodies and a series of cytokines. Tumor cells are cleaved and inactivated after binding to CTL.

Table 1. Normal liposome composition and classification

Category	Compound	Structure
Cationic liposomes	dimethyldioctadecyl ammonium (DDA)	

Category	Compound	Structure
	3β- (N- [N', N'-dimethyl aminoethane] -carbamoyl) cholesterol (DC-Chol)	
	N, N, N-trimethyl ammonium (DOTMA)	
	octadecenoyloxy (ethyl-2-heptadecenyl-3-hy-droxyethyl) imidazolinium (DOTIM)	
	1,2-dioleyl-sn-glycero-3-ethylphosphocoline (DOEPC)	
	1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP)	
Neutral Liposomes	phosphatidylcholine (PC)	
	phosphatidylethanolamine (PE)	
	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)	
	1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)	
Anionic liposomes	phosphatidylserine (PS)	
	phosphatidylglycerol (PG)	
	phosphatidylinositol (PI)	
	palmitic acid (PA)	
	1,2-dioleoyl-sn-glycero-3-phosphate (DOPA)	
	1,2-dipalmitoyl-sn-glycero-3-phosopho-(1'-rac-glycerol) (DPPG)	

The encapsulation rate of liposomes largely depends on the electrostatic attraction between liposomes and goods, so the surface charge determines the interaction between liposomes and biological components, immune cells, and other charged objects biological components, affecting the loading and release of liposome antigens. The surface charge of the cationic liposomes is positive, and an anionic matter, such as nucleic acids, polypeptides, or the like, is attracted by electrostatic action. Interestingly, the APC is usually negatively charged, easily adhered to the cationic liposome, thereby accelerating to phagocytosis. In addition, surface positive charge can extend the vaccine residence time in the injection site, increasing the antigen presence, extending the time of stimulating cellular immunity in the body [37]. Studies have shown that cationic liposomes can stimulate the expression of dendritic cell (DC) maturity markers CD80 and CD86, and induce the expression of $CD4^+$ T cells and the immune response of $CD8^+$ T cells [38]. Hence, cationic liposomes have intense immunogenicity.

As early as 2011, scientists summarized the applications of cationic liposomes in the vaccine [39]. Cationic lipids (Table 1) had adjuvant activity, including dimethyldioctadecyl ammonium (DDA) and 3β-(N-[N',N'-dimethylaminoethane]- carbamoyl) cholesterol (DC-Chol), a cholesterol derivative containing tertiary amine group, which induced Th1/Th2 immune response and antibody response, showing good mucosal immunity. N,N,N-trimethylammonium (DOTMA) and octadecenoyloxy (ethyl-2-heptadecenyl-3-hy-droxyethyl) imida-

zolinium (DOTIM) are excellent non-viral gene transfection materials, especially for nucleic acid substances, often in combination with plasmids [39]. Moreover, 1,2-dioleyl-sn-glycero-3-ethylphosphocoline (DOEPC) and 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP) are the most used cationic lipids in the construction of tumor vaccine delivery vectors, because they have higher membrane fusion rates and more multidirectional immunostimulatory effects than other cationic liposomes [40]. Eleni et al. analyzed the potency of SLP (synthetic long peptides)-loaded formulation (DOTAP: DOPC, molar ratio 1:1) [41]. The results showed that the SLP model delivery system could achieve complete cure of two invasive tumors (TC-1 and B16 melanoma), demonstrating that the cationic liposome was a good delivery platform for polypeptide-based tumor vaccine. Among them, E7 liposomes with poly (ibuprofen C) as adjuvant can cure 75-100% of the large tumors in immunized mice. The delivery of cGAMP (a STING ligands) by liposome composed of soybean L-α-phosphatidylcholine (Soy-PC) and DOTAP (100:1, weight ratio) activated STING more effectively than soluble cGAMP [42]. In tumor microenvironment, the liposome induced mice and human macrophages (M) reprogramed from M2-like phenotype to M1-like phenotype, and enhanced the expression and costimulatory effect of MHC to increase the apoptosis of tumor cells [42]. Besides, cationic liposomes can also overcome the shortcomings of polymer material PLGA's adsorption efficiency for nucleic acid molecules. The polymer nanoparticles encapsulated by DC-CHOL form hybrid nanoparticles with a lipid shell, which can absorb more nucleic acid adjuvants or nucleic acid antigens and enhance cell adhesion and absorption [43].

However, the advantages of cationic liposomes in attracting anionic substances may be paradoxical. While stably loading nucleic acid or polypeptide molecules and giving priority to targeting APC, cationic liposomes inevitably attracted other anionic components in the blood, resulting in accumulation and embolism, which is very fatal to patients. Consequently, the establishment of cationic liposomes should fully consider this problem, optimize the formulation of liposomes, so that the amount of surface charge in a safe and effective range.

Anionic liposomes

The negatively charged lipids mainly include phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), palmitic acid (PA), and so on (Table 1). The liposomes formed or modified by these molecules are called anionic liposomes. Since the APC usually carries a negative charge, an electrostatic repulsion reaction occurs with anionic liposomal. From intuition, the anionic liposome is difficult to promote cellular uptake as an antigen delivery vector. However, in fact, the PS is one of the lipids of the cell membrane. When the anionic liposome of the PS component entered body, a great many specific PS receptors are activated to direct the APC to take the antigen to take place. In a study comparing the activity of lipid adjuvants with different charges, anionic liposomes made from DOPA mixed with OVA antigens could significantly delay the growth of B16-OVA tumors in mice. In the stage of cell culture, the effect of DOPA anionic liposomes on stimulating DC to extract OVA antigen was not as good as that of cationic liposomes, but the intensity of anti-OVA IgG response induced by cationic liposomes was the same, and the mixture of OVA-DOPA liposomes could also induce OVA specific CTL response. Moreover, enzyme linked immunosorbent assay (ELLISA) results showed that DOPA-OVA liposome could significantly up-regulate the expression of MHC II on DC cells and enhance the expression of cytokines IL-6 and CCL-17, which confirmed that both cationic liposomes and anionic liposomes could show strong adjuvant activity [44].

When combined with ID93/GLA (mycobacterial antigen and lipid adjuvant),*in vivo* experiments showed that anionic liposome (CHO/DPPC/DPPG) produced Th2 cells at the highest rate, which could further promote the immune response of mice [45]. It has been seen that the adjuvant activity of anionic liposomes may activate DC maturation by up-regulating the expression of related genes. It is noteworthy that anionic liposomes are a favorable drug delivery system for mucosal vaccines. The mucus gel layer is negatively charged and is repelled by anionic liposomes. Using an anionic liposome, the progression of particles and the speed of epithelial penetration are accelerated, which is promoted to absorb by M cells through endocytosis [46]. So anionic liposomes are more suitable for oral vaccines, nasal vaccines, and other mucosal vaccines.

Neutral Liposomes

The materials of the neutral liposomes (Table 1) including natural product phosphatidylcholine (PC), phosphatidyl ethanolamine (PE) and synthetic product 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). The non-charged lipids can also be referred to as helper lipids, which can stabilize the bilayer membrane and reduce the toxicity of the charged lipid component, wherein DOPE and cholesterol are most used. The role of cholesterol has been described above, the addition of DOPE can interfere with the lipid membrane, make the inclusion body unstable, promote the release of nucleic acid substances, and play an auxiliary role in cell osmosis.

Neutral liposomes can be transformed into lipids with different charges by modifying lipid molecules with different charges. Furthermore, in targeted tumor delivery, neutral liposomes play an advantage in EPR effect, and play a priority role in the treatment of solid tumors [47]. In immunization, using neutral liposome can induce a Th1 type immune response than cationic liposomes in the mice model [48]. In delivery, a neutral liposome loaded with focal adhesion kinase short interference RNA (FAKsiRNA) to treat ovarian cancer. The Western blotting and immunohistochemical analysis showed that a single dose of FAKsiRNA-liposome could effectively reduce the expression of FAK *in vivo* for up to 4 days, and the average tumor weight decreased significantly (44%-72%) [49]. In another cervical cancer treatment study, the stability, cytotoxicity, and cell uptake of targeted delivery of arsenic trioxide (ATO) to cervical cancer cells by liposomes of different charge types were investigated. The authors concluded that neutral liposomes prepared from PC and cholesterol selectively produced a high mortality rate in HeLa cells and minimal toxicity in control cells. Although ATO encapsulated by liposomes is less easily absorbed by cells than free ATO, delivered ATO can reduce the expression of oncogenes and show reduced toxicity [50].

Multifunctional liposomes

An effective antigen carrier requires a variety of functions to enable it to release and deliver antigens to APC at a defined location. Multifunctional liposomes were constructed by doping lipid molecules with different response signals in the assembled liposome structure or modifying liposomes with different functional structures. By adding pH-sensitive lipids DOPE (1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine) to the formula, Banu et al. constructed a pH-sensitive liposome co-encapsulating CpG ODN and cGAMP (TLR9 and STING ligands) to release contents in lysosomal acidic environment [51]. And the synergistic innate immune response was induced by increasing the level of type I and type II interferon. Most importantly, the vaccine formula made the established melanoma regression rate up to 70%. Yuta et al. used liposome modified with 3-methylglutarylated hyperbranched poly (glycidol) (MGlu-HPG)-a pH sensitive polymer [52]. At a weakly acidic pH, after the carboxyl matrix is protonated, the nature of the lipid membrane changed from hydrophilic to hydrophobic, so the stability of the lipid membrane was destroyed and the inclusions were released. Furthermore, their study showed that the introduction of more hydrophobic spacing next to the carboxyl group could obtain 10-fold higher dendritic cell uptake than the short-chain carboxyl modified liposome, which greatly improved the uptake of tumor antigens [53].

The purpose of temperature response can be achieved by adding lipids with different Tc or embedding temperature-sensitive materials into liposomes. The results show that the thermosensitive liposomes composed of DPPC enhance the release of goods near the Tc (Fig. 3A). But thermosensitive liposomes are mainly used in tumor thermotherapy [54].

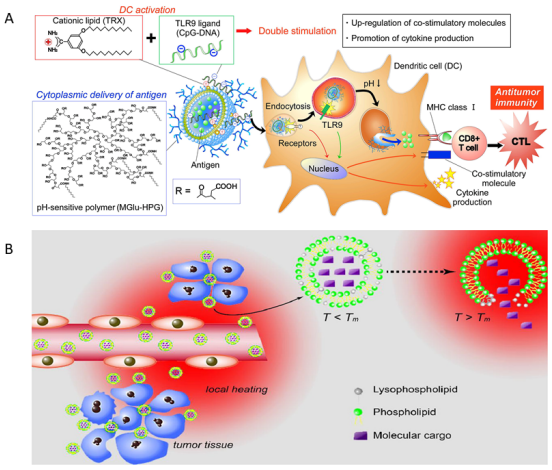


Fig. 3. A, a pH sensitive polymer (MGlu-HPG) modified cationic liposomes (TRX) for efficient antigen delivery and activation of dendritic cells [52]. B, the drug is released when exceeded the liposome phase transition temperature [54].

To grasp the particularity of tumor environment or immune cells, it is helpful to establish an efficient immune induction system or drug delivery system by modifying different targeted ligands on liposomes. Sakurai et al. established a new drug delivery system for malignant pleural mesothelioma (MPM) cells with high expression and high affinity with HA receptor CD44 for the treatment of MPM [55]. The drug delivery system connects cationic liposomes and HA derivatives through the reductive amination of carboxyl and amino groups, which improves the targeting effect of drug therapy. DC-SIGN is known to be pathogen-associated molecular patterns (PAMPs) receptor and the C-type lectin receptor that can recognize and combine mannose structure and fucose structure. Martine et al. generated a liposome containing the glycan Lewis $(Le)^X$ to target DC with tumor antigen [56]. The glycol-liposome increased the targeting of the drug to DC-SIGN, enhanced the uptake of liposome antigen conjugate by DC, and ensured the presentation of tumor antigen in MHC molecule.

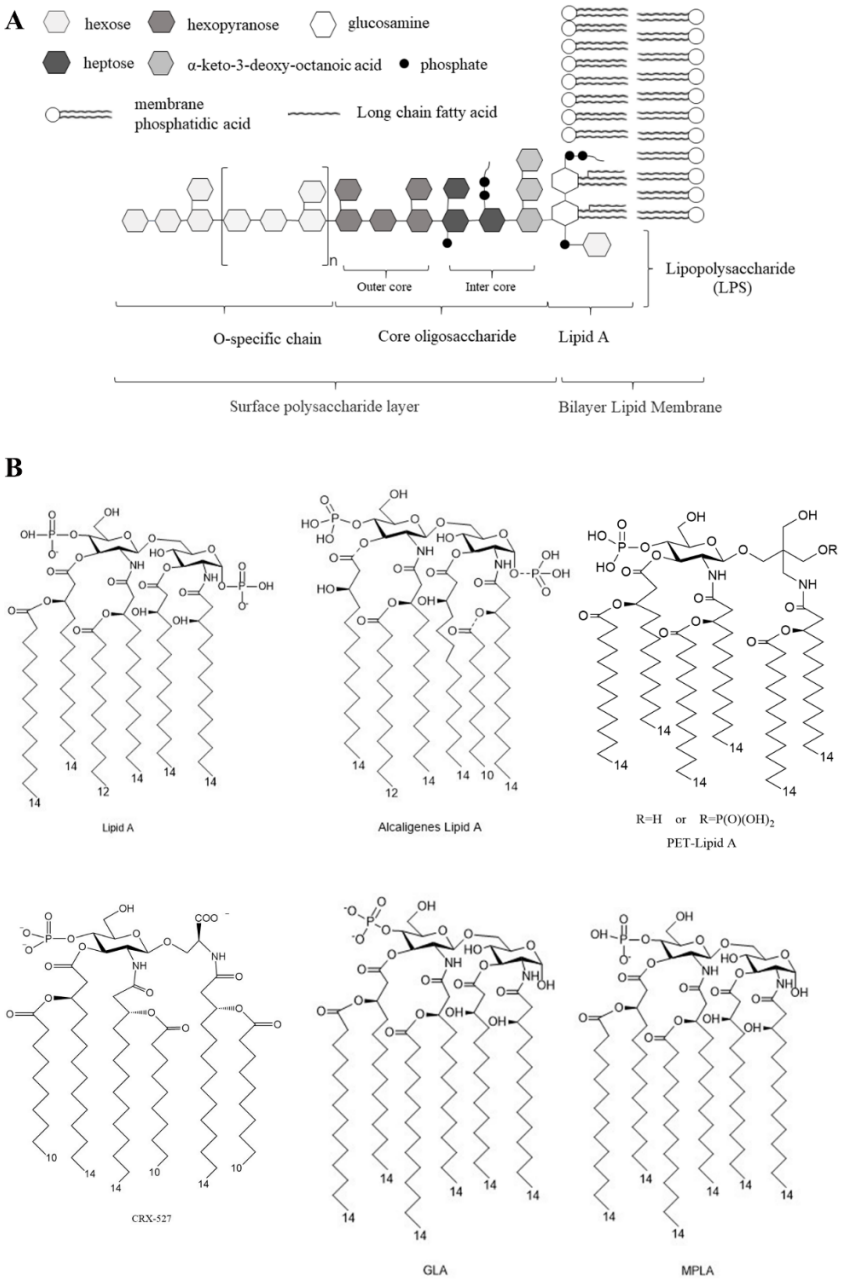


Fig. 4. A, Lipopolysaccharide (LPS) component. B, lipid A derivatives structure, including lipid A [57], alcaigenes lipid A [58], PET-lipid A [59], CRX-527 [60], GLA [61], MPLA [62].

As an immune stimulator to play the role of adjuvants

Lipid A and its derivatives: the most mature tumor immunostimulant

Mechanism of lipid A. Lipid A is the hydrophobic region of lipopolysaccharide (LPS) (Fig. 4A), a glycolipid composed of the membrane of gram-negative bacteria, recognized by Toll-like receptors 4 (TLR4) that highly observed in immune cells, such as monocytes, macrophages, dendritic cells, lymphocytes, and NK

cells. Consequently, among all the TLR4 ligands, lipid A is the most famous and studied one, which is the lipophilic anchoring and main bioactive motif of LPS.

To be specific, the myeloid differentiation factor 88 (My88-dependent) signaling pathway and the My88-independent signaling pathway were involved in TLR4 activation by lipid A (Fig. 5). TLR4 can mediate pathogen-associated molecular patterns (PAMPs), when TLR4 is activated by lipid A and binds to myeloid differentiation factor 2 (MD2), the signal is transduced to the toll or interleukin-1 receptor (TIR) domain. Then interacting with toll or interleukin-1 receptor-domain-containing adapter-inducing interferon-β (TRIF) and MyD88 to enable to activate downstream signals [63]. Subsequently, various types of inflammatory cytokine gene expressions is released as well as the maturation of the immune cells, and signal transducer and activator of transcription play important roles in mediating process [64]. The ultimate result is the innate and adaptive immune responses [57].

Previous studies have shown that the continuous increase of inflammatory factors may lead to the generation and persistence of chronic inflammation microenvironment, and occasionally accelerate tumor growth and tumor cell proliferation through autocrine pathways. Although the potential of relying on humoral immunity to kill tumors is low, effective cellular immunity can be activated by CTL, especially $CD8^+$ T cells, which can be activated in cancer immunotherapy. And a massive of investigations demonstrated that a mouse tumor model was treated with lipid A observed tumor regression [57]. In cancer immunotherapy, adjuvants have been successfully incorporated into vaccine delivery with antigen to improve the bioactivity [5, 6]. Additionally, it is suggestable that TLR4 agonist like MPLA can also as a safe and effective radio protector for clinical application [65].

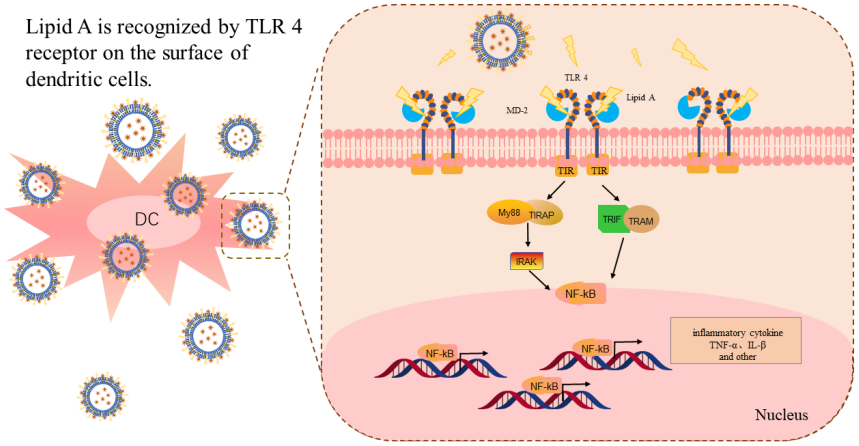


Fig. 5. Mechanism of Lipid A. When TLR4 is activated by lipid A on the liposome vaccine,and binds to myeloid differentiation factor 2 (MD2) to form A complex, the signal is transduced to the Toll/IL-1R1 homologous region (TIR) domain, followed by two My88: myeloid differentiation factor 88 (My 88)-dependent and My88-independent or TIR-domain-containing adaptor (TRIF)-dependent pathways. My 88-dependent pathway: TLR binds to the TIR domain containing adaptor protein (TIRAP) of MyD88 and activates MyD88, forming an active TLR4/MyD88 complex that activates IL-1R-associated kinase (IRAK), which eventually causes nuclear factor-κB (NF-κB) into the nucleus and triggers subsequent reactions. My88-independent pathway or TRIF-dependent pathway: complex's signal is transmitted to TIRF, the interferon transcription is regulated by activating TRIF to further activate the NF-κB. Impelling the body to produce inflammation-related factors.

Optimization of lipid A. It is difficult to obtain structurally homogeneous lipid A in varied forms from biological sources. Moreover, lipid A from natural sources may be contaminated by other components of

the bacterial cell wall. Chemical synthesis as an effective tool can perfectly solve this problem. Previous review, from the perspective of chemical synthesis of each part in detail elaborated the lipid A chemical synthesis process and related derivatives, such as containing saturated phospholipids and double phosphate group of natural lipids, containing unsaturated double phosphate groups of lipid A, containing carboxylic acid derivatives, single phosphorus acyl lipid A and the phosphorylation of lipid A [63].

As the premise, various positions in the disaccharide backbone of lipid A with the same or different acyl lipid chain, thus lipid A and its analogues synthesis can be divided into two basic strategies. One is to build an asymmetric distribution of lipid chains, that is to construct general disaccharides skeleton and install the same length or different length of lipid acyl chain in a particular location. Another strategy is to synthesize a monosaccharide with acyl lipid chain and then stitch them into a disaccharide structure through glycosylation reaction. However, the latter is often hard to implement due to the steric hindrance. Shimoyama et al. synthesized *Alcaligenes* lipid A (3+3) (Fig. 4B) by the introduction of benzyl-protected β-hydroxy fatty acid into the disaccharide with an appropriate acylation reagent and repeated similar operations [58].

Jiang et al. provided a synthesis method of pentaerythritol lipid A (PET-LA) (Fig. 4B) which contains an amine-substituted pentaerythritol instead of a glucosamine unit found in natural lipid A [59].

After a period of research, Kawther et al. proved that PET-LA had powerful adjuvant properties in immunogenic mouse tumor models by loading CD40 and CD86 into polylactic acid-glycolic acid (PLGA) particles to stimulates the upregulation of CD40 and CD86 [66]. Compared to the glucosamine structure, PET can keep the anomeric phosphate group well, and because of its simple structure, the synthesis process is simplified and the molecular stability is also improved. Niels et al. studied the immune activity of lipid A analogue CRX-527 conjugated with long peptides in different ways to determine whether it had the potential to be vaccinated. CRX-527 (Fig. 4B), one of aminoalkyl glucosamine 4-phosphates (AGPs), which contains monosaccharide with a long-chain acyl group [60]. *In vitro* , ester-linked compounds performed better in inducing DC maturation, while ammonia-linked compounds showed greater activity in presentation. However, according to the data studied *in vivo* , it seemed that a conjugate of mounting the free carboxyl ligand at the N-terminus of the model peptide antigen can recruit enough protective immune response to effectively kill cancer cells and exert a powerful immune function for the development of anti-cancer.

Glucopyranosyl lipid adjuvant (GLA) is a synthetic non-toxic derivative of lipid A (Fig. 4B). When it is used as adjuvant and co-delivery with tumor antigen, it plays the role of TLR4 agonist. GLA targeting human DC and peripheral blood mononuclear cells (PBMC) has 10-100-fold more activity and safety than semi-synthetic MPLA. The intensity of immune-induced response is also different due to the change of the type of preparation. GLA prepared as stable oil-in-water emulsion (GLA-SE) formulations can induce stronger Th1 reaction than that prepare as an aqueous(GLA-AF) [61]. In a trial of an advanced solid tumor vaccine expressing NY-ESO-1, after stimulating the systemic innate immune response, locally intra-tumoral injection of GLA-SE can recruit NY-ESO-1-specific $CD8^+$ T cells into the tumor [67]. In previous dose-increasing trials, GLA-SE at doses of 2-10 mg showed potential clinical benefits and reliable safety in the experimental patient population. Six of the eleven patients showed increased expression of $CD4^+$ T cells after vaccination and four patients showed increased expression of $CD8^+$T cells, but none of them showed a correlation between GLA-SE dose and induction of immune [68]. And when the vaccine is injected away from the tumor, Deepak et al. proved that the addition of GLA led to alters the kinetics of changes in T cell trafficking, and it can induce a massive proliferation of vaccine primed antigen-specific T cells in the spleen [8]. The most fundamental reason is that GLA precociously matures APC at the injection site, reduces the phagocytic function of APC and reduces the delivery of antigens to draining lymph nodes. But the use of GLA adjuvant can lead to a great number of antigen-specific T cells proliferation induced by vaccine in immune organs, driving more T cells into tumor tissue and infiltrating tumor T cells, resulting in stronger cytotoxicity.

Derivatives of lipid A. All lipid A contain a highly similar core structure of β-1,6-linked disaccharide of D-glucosamine. Specially, subtle changes in chemical structure can result in dramatically different immune activities. An understanding of structure–activity relationships help to provide a better choice when devel-

oping a cancer vaccine. In previous studies, many factors such as the number and length of the acyl chain, the structure and substituent pattern of the disaccharide scaffold, the length and phosphorylation state of the acyl chain at positions 1 and 40 of the disaccharide backbone had a strong effect on the activation of the immune response [69].

Gram-negative bacteria have evolved the diversified of their lipid A structures. A typical lipid containing six acyl chains exhibits severe immunological and endotoxic activities, such as asymmetrically distributed (4+2) from *E. coli*[70], or symmetrically (3+3) from *Neisseria meningitidis*[71] or *Alcaligenes* [58, 72]. However, removal of the phosphate group at the O-1-position to generate monophosphoryl lipid A (MPLA) derived from *Salmonella Minnesota* (Fig. 4B), which greatly eliminates the endotoxin properties without affecting its immune-stimulating activity, has been approved by the FDA for using as an adjuvant vaccine [62]. Most studies have tried to prove that MPLA is a favorable candidate for the formulation of different cancer vaccines with better immune potency.

Combination strategy of lipid A. The construction of a multi-functional liposome vaccine delivery platform with a variety of immune stimulants is the focus of the current preclinical research, researchers are committed to adding vaccine adjuvants and antigen delivery to achieve preferable anti-tumor effects. In order to improve the immunogenicity of the vaccine and the survival rate of cancer patients, the combination strategy of lipid A is a good choice. The combined use of adjuvants achieved dual effects, and the combination of cancer drugs expanded the therapeutic effect of drugs.

Currently, mature adjuvant combination formulations have been tested in preclinical or clinical trials of cancer vaccines. For example, in AS01, MPLA is combined with QS-21, a triterpenoid glycoside saponin extracted from the bark of Quillaja Saponaria Molina, which is a powerful adjuvant [73]. AS02 precipitates MPLA and QS-21 in a stable oil-in-water emulsion [74]. The TLR9 agonist CpG ODN was added to the existing formula, and then the whole was wrapped in lipids to form an AS15 combination [75]. Based on these approved formulations, scientists have developed several new adjuvant coordination strategies. Zhu et al. synergistically activated the immune response with TLR7/8 agonist imiquimod and TLR4 agonist MPLA. From immune study *in vivo* , the addition of combined adjuvant effectively improved the proliferation and activation of immune cells, especially antigen-specific T lymphocytes. Surprisingly, the mice received the tumor challenge again after vaccination, of which 2/8 mice maintain a tumor-free state to 92 days [76]. The vaccination greatly prolonged the median survival time of the mice. Both complex vaccine adjuvants ((HA/MPLA/QS21), HMQ and (HA/MPLA/R837), HMR) were prepared by Shin, which exerted important immunostimulatory effects *in vitro* and essential antitumor therapy *in vivo* [77]. With the help of HA, HMQ or HMR were easily prepared into aqueous solution and freeze-dried in the form of powder, showing high stability, even when stored at room temperature. TLR4 agonist MPLA and 9 agonist oligonucleotide CpG combined with*in vitro* stimulating DC secretion IL-12, TNF-α, and proliferation of the same foreign $CD8^+$ T cells have unique intensive effects, producing adding adjuvant effect [78]. The combination of TLR agonists and non-TLR agonists also presented an effective power [62]. The strong anti-tumor cytotoxic response dependent on natural killer cells can be induced by α-galactose ceramide (α-GalCer) and when cooperated with MPLA and DNA antigens, multiple immune targets are activated at the same time, resulting in more lasting immunotherapy [79]. The cell killing activity of mice generated by the combined strategy is more than twice that of the single adjuvant. Not only that, the use of combined adjuvants can also reduce the amount of antigen in the vaccine to avoid possible problems and obstacles in the use of high doses of antigen.

The efficacy can also be amplified in combination with commonly used cancer drugs. Doxorubicin (DOX), as a broad-spectrum anti-tumor chemotherapy drug commonly used in clinical practice, promoted immunogenic cell death by increasing the expression of tumor antigen *in vivo* , thus triggering immune response. However, its anti-tumor effect is limited, and it is also necessary to maintain high doses of drug concentrations in the tumor site. This requires increased DOX administration, which inevitably causes drug toxicity and drug resistance problems. The addition of MPLA assists in the immune response triggered by DOX. The joint processing of MPLA-DOX significantly increases the number of invasive $CD8^+$ or $CD4^+$ T cells, which basically inhibits tumor growth [80]. Immune checkpoint inhibitors can inhibit the related receptors PD-L1

or PD-1, which are a successful treatment for patients with advanced melanoma. When combined with MPLA, MPLA-induced interferon γ (IFN-γ) production by T cells can further promote PD-L1 expression, promote the proliferation and CTL of the infiltrated regulatory T cell, thereby finally eradicating the primary tumor and inhibiting tumor transfer and recurrence [81, 82]. Therefore, the combined strategy can make the tumor treatment effects better, which provides a desired strategy for research work.

Mycophenolic acid: an immunostimulant for bladder cancer

The standard treatment to prevent the recurrence and progression of bladder cancer is a topical application of high-dose Mycobacterium bovis bacillus Calmette-Gue ´rin (BCG) [83]. Mycolic acids (MA) (Fig. 6), which is major and essential lipid components of the genus Mycobacterium, has been shown to play a key role in the host immune reaction. Yoshino et al. first reported the anti-tumor effects of purified MAs in animal models. It exerted a distinctive tumor cell growth retardation *in vivo* through T-cell-dependent antitumor immunity. Simply, MA triggered the collection of various cytokines and MHC class II on the cell surface. Then the immune cells, including $CD4^+$ and $CD8^+$ lymphocytes, NK cells, granulocytes, macrophages, and dendritic cells, were recruited to surround the tumor region and exhibit an immune reaction to the cancer cells. In another report, Kubota et al. had demonstrated that MA induced sufficient humoral response comparable to that of conventional CFA when loaded with antigen (Fig. 7) [84]. Ultimately, after receiving MA, the body tends to develop a Th1-biased mild inflammatory response at the injection site, while CFA induced Th17-biased inflammatory responses are severe and destructive. Therefore, MA is a promising adjuvant candidate material for clinical value of tumor treatment.

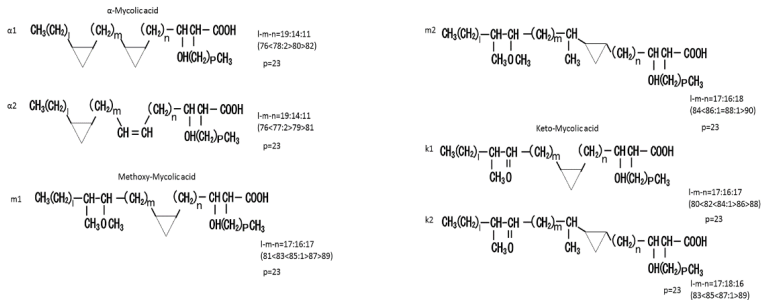


Fig. 6. Structure of mycophenolic acid (MA) subclass derived from M. bovis BCG [83].

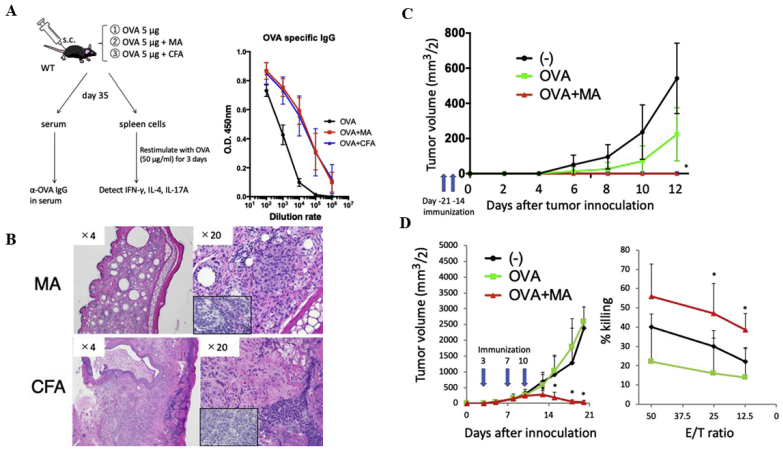
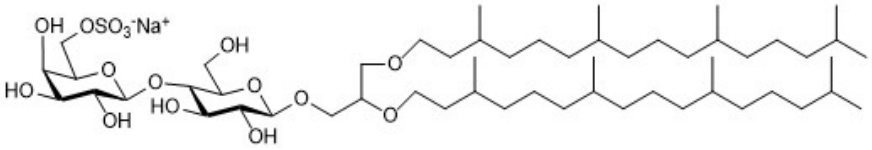


Fig. 7. A, antibody response induced of MA. Left, Schematic diagram of vaccination program and examination strategy in mice. Right, Serum OVA IgG content was detected 35 days after immunization. B, After the injection of the MA or CFA, histological examination of tissue samples on day 8. C, Immunotherapy was performed on days -21 and -14, tumor cells were inoculated on day 0 and tumor size changes were recorded. D, tumor cells were inoculated on day 0 and vaccinated on day 3, 7 and 10. Tumor size changes were recorded. E/T ratio: effector to target ratio (ratio of number of spleen cells from mice to that of tumor cell [84].

SLA: a new tumor immune stimulant

SLA (6'-sulfate-β-D-Galp-(1,4)-β-D-Glcp-(1,1)-archaeol) is a new formulation of archaeosome, which was first proposed by McCluskie. Its composition is archaeosome core lipid modified by adding sulfated saccharide (Fig. 8) [85]. SLA has the characteristics of stabilizing archaea in extreme environments. Early studies on archaeal membranes explained the source of archaea. Archaea lack peptidoglycan and isoprenoid parts are ether connected to the l-glycerol backbone. In bacteria, esters are linked to fatty acid-derived hydrocarbon chains. Like liposomes, archaesome can constitute effective carrier vesicles for entrapped antigens, and the inherent stability and unique structure enable it more potential to be used as drug delivery carrier.



SLA：6'-sulfate-β-D-Galp-(1,4)-β-D-Glcp-(1,1)-archaeol

Fig. 8. Structure of SLA [85].

Researchers have reviewed that archaeosome possesses strong immune adjuvant activity for induction of cell-mediated immunity [86]. However, traditional archaeosome formulations are complex. SLA is a simpler formulation that possesses immunostimulatory properties and elicit an CD 8^+ T cell response [87]. Moreover, it also induces strong adjuvant effect on the production of multiple cytokines/chemokines including IL-6, G-CSF, KC & MI P-2, and CTL activity[88].

To confirm the potential immunostimulatory of SLA, Stark et al. have evaluated it as an adjuvant with different antigens, including ovalbumin (OVA) and hepatitis B surface antigen (HBsAg) [89]. The result revealed that SLA has strong adjuvant activity, and it attractively superior to various other established adjuvants including TLR3/4/9 agonists, oil-in-water and water-in-oil emulsions and aluminum hydroxide (Fig. 9).

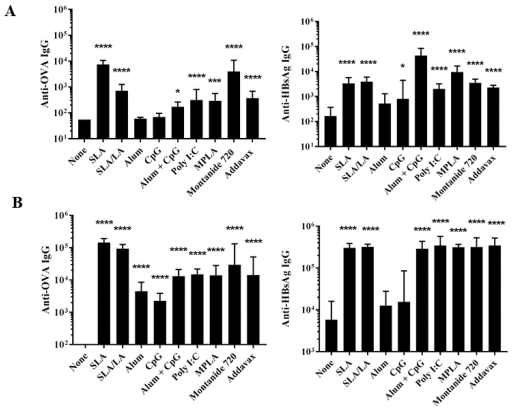


Fig. 9. Mice were immunized with OVA/HBsAg with or without adjuvants. Mice inoculated with OVA/HBsAg. A, serum analyzed for anti-OVA IgG on the 20th day. B, serum analyzed for anti-OVA IgG on the 28th day [89].

Recently, researchers have proved SLA exhibit high thermal stability as vaccine adjuvant: after six months of storage at 4 °C and 37 °C, the adjuvant properties of blank SLA remain unchanged, and the immunogenicity of the vaccine formulations does not change for one month after binding to the antigen [90]. It means that the use of SLA could solve the problem of cold chain transport of vaccines, thereby making the vaccine more effective and stabler.

In order to improve the body's response to antigens, SLA was used as an immune adjuvant in cancer vaccines resulting in enhancing protection from solid and metastatic tumors [86]. Ovalbumin (OVA) is entrapped within SLA given in murine, a large amount of IFN-γ production and strong $CD8^+$ T cell response are detected, and immune cells are recruited at the injection site and the antigens are uptake to the local draining lymph nodes in against B16 melanoma tumor challenge. When entrapped the cancer self-antigen peptide, it induced similar great response.

In addition to delivery antigen alone, it also can combine with other therapeutics to improve tumor curative effect. Stark et al. tested the combination of archaeosome from *Methanobrevibacter smithii*(MS) with checkpoint inhibitor immunotherapies, including αCTLA-4/αPD-1/αPD-L1, and the encouraging result is that 70% of C57BL/6 mice survived beyond 100 days from the B16-OVA tumor [91]. After two years, archaeosome combine with checkpoint inhibitor was detected again [92]. The difference is that the vaccine formulation is a simple blend of SLA and OVA(SLA-OVA). The research shows that tumor grows slower with SLA–OVA, a mass of $CD45^+$ T cell and $CD8^+$ T cell gather around the dying or dead tumor cells and exist in the survived tumor tissues widely. Furthermore, immunohistochemical tests demonstrated that most of tumor mass was consisted of dead cells.

Whether adjuvants and antigens are co-encapsulated in liposomes (SLA-Enc) or simply mixed with antigen-containing liposomes (SLA-Adm) affect the intensity and quality of T cell-induced reactions are related to the type of adjuvants. In terms of simply admixed SLA with antigen, the way of entrapped cargo may induce a high antigen loss during preparation, while a study has revealed that this new formulation can obtain similar induction of robust adjuvant activity as an encapsulated formulation deliver encapsulated antigen with SLA and without antigen loss during production. The new formulation may lead to excellent reproducibility, while compared to the encapsulation, the mixed formula shows negative energy to induce antigen $CD8^+$ T cell to activate dendritic cells *in vitro* , and the mixed formula retains ovarian injection for 24 h, but the encapsulated formulation maintains it for 48 h *in vitro* (Fig. 10) [92]. SLA has become a new

attractive role as effective adjuvant, the advantages of two formulation are of great significance in a cancer vaccine, so proposing a new formulation with no antigen loss, and better immune stimulation, is a key that researchers need to tackle in the future.

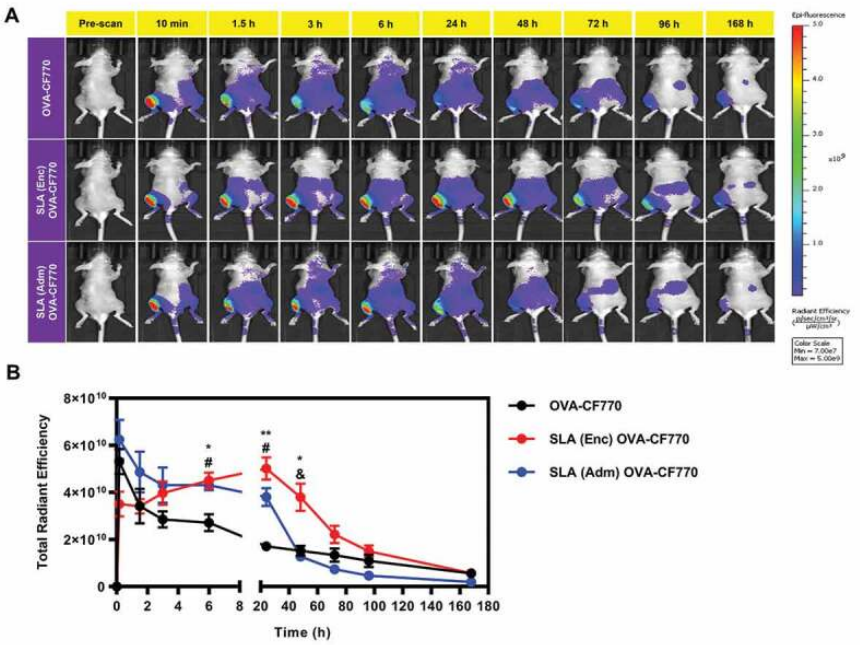


Fig. 10. A, bioimaging of *in vivo* biological distribution of OVA-CF770 at shown time after immunization with SLA (Enc) and (Adm). B, Linear view of total fluorescence signal *in vivo* [92].

Conclusion and prospect

Lipid adjuvants have made remarkable achievements in the treatment of cancer, and their good immunogenicity, safety and controllability have been proved. In this review, we summarized two roles in adjuvants of lipids: delivery carrier and immune stimulants. According to the new understanding of current immune cell biology, we can create new lipids with immunogenicity, or carry out structural modification based on the preexisting lipids to obtain more optimized products, to further reduce the clinical dosage of drugs and improve the compliance of cancer patients.

Furthermore, due to the complexity of the human environment, especially the variability of the tumor environment, it is not only need to consider the internal association between the carriers and antigens, but also the influence of the biological characteristics *in vivo*[93]. A new platform must be developed to respond to the structure and biological characteristics of the target cells to overcome the complex mechanism of immunosuppression in tumor microenvironments. Based on the key parameters such as surface charge, membrane fluidity, and*in vivo* transfection rate, new lipid materials or functionalized liposomes will be added to establish a new liposome delivery platform. Using this platform to deliver antigens and immune stimulants and promote the cross presentation of antigens is a development direction of cancer vaccines in the future.

Whereas, the safety and stability of new materials are the keys to the successful establishment of the delivery platform [7]. Liposome cancer vaccine has no long-term safety record and needs to be further verified. The cancer vaccine containing lipid adjuvant is amazing in clinical trials, the existing experimental data were only for individual types of tumors, and there is still a lack of extensive and comprehensive clinical trial data, which indicates that it is still a long way to go from laboratory to clinical application. At the same

time, the induction data of this new adjuvant *in vivo* and*in vitro* were not consistent, so an efficient and simple method should be established to visualize the immune response, that is, to monitor the changes of the number, function, and immune behavior of immune cells *in vivo* [94]. Only by solving all the above problems, the application of lipid adjuvants in cancer vaccines can support the future demand.

Conflict of interest disclosure

The authors declare that no competing interest, financial or otherwise, exists in relation to this study.

Data Availability statement

Not applicable.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Acknowledgements

We thank all the researchers who contributed to the lipid adjuvants cited and uncited in this review.

Author contributions .

Liu ZL, Xu N, Zhao L, Yu J and Zhang P were responsible for writing of the manuscript. All authors contributed to substantial discussion of content, reviewing, and revising the manuscript before submission.

Reference

1. Gonzalez-Fierro A, Duenas-Gonzalez A. Drug repurposing for cancer therapy, easier said than done. Semin Cancer Biol. 2021;**68** :123-31.

2. Shemesh CS, Hsu JC, Hosseini I, Shen BQ, Rotte A, Twomey P, et al. Personalized Cancer Vaccines: Clinical Landscape, Challenges, and Opportunities. Mol Ther. 2021; **29** :555-70.

3. Facciola A, Visalli G, Lagana P, La Fauci V, Squeri R, Pellicano GF, et al. The new era of vaccines: the "nanovaccinology". Eur Rev Med Pharmacol Sci. 2019; **23** :7163-82.

4. Del Giudice G, Rappuoli R, Didierlaurent AM. Correlates of adjuvanticity: A review on adjuvants in licensed vaccines. Semin Immunol. 2018; **39** :14-21.

5. Temizoz B, Kuroda E, Ishii KJ. Vaccine adjuvants as potential cancer immunotherapeutics. Int Immunol. 2016; **28** :329-38.

6. Alving CR, Beck Z, Matyas GR, Rao M. Liposomal adjuvants for human vaccines. Expert Opin Drug Deliv. 2016; **13** :807-16.

7. Le QV, Yang G, Wu Y, Jang HW, Shokouhimehr M, Oh YK. Nanomaterials for modulating innate immune cells in cancer immunotherapy. Asian J Pharm Sci. 2019; **14** :16-29.

8. Kadayakkara DK, Korrer MJ, Bulte JW, Levitsky HI. Paradoxical decrease in the capture and lymph node delivery of cancer vaccine antigen induced by a TLR4 agonist as visualized by dual-mode imaging. Cancer Res. 2015; **75** :51-61.

9. Yuba E. Liposome-based immunity-inducing systems for cancer immunotherapy. Mol Immunol. 2018; **98** :8-12.

10. Bompard J, Rosso A, Brizuela L, Mebarek S, Blum LJ, Trunfio-Sfarghiu AM, et al. Membrane Fluidity as a New Means to Selectively Target Cancer Cells with Fusogenic Lipid Carriers. Langmuir. 2020;**36** :5134-44.

11. Takechi-Haraya Y, Sakai-Kato K, Abe Y, Kawanishi T, Okuda H, Goda Y. Atomic Force Microscopic Analysis of the Effect of Lipid Composition on Liposome Membrane Rigidity. Langmuir. 2016; **32** :6074-82.

12. Raffy S, Teissie J. Control of lipid membrane stability by cholesterol content. Biophys J. 1999; **76** :2072-80.

13. Zhang JA, Pawelchak J. Effect of pH, ionic strength and oxygen burden on the chemical stability of EPC/cholesterol liposomes under accelerated conditions. Part 1: Lipid hydrolysis. Eur J Pharm Biopharm. 2000; **50** :357-64.

14. Schottler S, Becker G, Winzen S, Steinbach T, Mohr K, Landfester K, et al. Protein adsorption is required for stealth effect of poly(ethylene glycol)- and poly(phosphoester)-coated nanocarriers. Nat Nanotechnol. 2016; **11** :372-7.

15. Kaur R, Bramwell VW, Kirby DJ, Perrie Y. Pegylation of DDA:TDB liposomal adjuvants reduces the vaccine depot effect and alters the Th1/Th2 immune responses. J Control Release. 2012; **158** :72-7.

16. Schmidt ST, Olsen CL, Franzyk H, Worzner K, Korsholm KS, Rades T, et al. Comparison of two different PEGylation strategies for the liposomal adjuvant CAF09: Towards induction of CTL responses upon subcutaneous vaccine administration. Eur J Pharm Biopharm. 2019; **140** :29-39.

17. Mercer LK, Lunt M, Low AL, Dixon WG, Watson KD, Symmons DP, et al. Risk of solid cancer in patients exposed to anti-tumour necrosis factor therapy: results from the British Society for Rheumatology Biologics Register for Rheumatoid Arthritis. Ann Rheum Dis. 2015;**74** :1087-93.

18. Manolova V, Flace A, Bauer M, Schwarz K, Saudan P, Bachmann MF. Nanoparticles target distinct dendritic cell populations according to their size. Eur J Immunol. 2008; **38** :1404-13.

19. Mann JF, Shakir E, Carter KC, Mullen AB, Alexander J, Ferro VA. Lipid vesicle size of an oral influenza vaccine delivery vehicle influences the Th1/Th2 bias in the immune response and protection against infection. Vaccine. 2009; **27** :3643-9.

20. Filipczak N, Pan J, Yalamarty SSK, Torchilin VP. Recent advancements in liposome technology. Adv Drug Deliv Rev. 2020; **156** :4-22.

21. Vicario-de-la-Torre M, Benitez-del-Castillo JM, Vico E, Guzman M, de-Las-Heras B, Herrero-Vanrell R, et al. Design and characterization of an ocular topical liposomal preparation to replenish the lipids of the tear film. Invest Ophthalmol Vis Sci. 2014; **55** :7839-47.

22. Aburai K, Yagi N, Yokoyama Y, Okuno H, Sakai K, Sakai H, et al. Preparation of liposomes modified with lipopeptides using a supercritical carbon dioxide reverse-phase evaporation method. J Oleo Sci. 2011; **60** :209-15.

23. Szoka F, Jr., Papahadjopoulos D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc Natl Acad Sci U S A. 1978;**75** :4194-8.

24. Stano P, Bufali S, Pisano C, Bucci F, Barbarino M, Santaniello M, et al. Novel camptothecin analogue (gimatecan)-containing liposomes prepared by the ethanol injection method. J Liposome Res. 2004;**14** :87-109.

25. Deamer DW. Preparation and properties of ether-injection liposomes. Ann N Y Acad Sci. 1978; **308** :250-8.

26. Talsma H, van Steenbergen MJ, Borchert JC, Crommelin DJ. A novel technique for the one-step preparation of liposomes and nonionic surfactant vesicles without the use of organic solvents. Liposome formation in a continuous gas stream: the 'bubble' method. J Pharm Sci. 1994; **83** :276-80.

27. Meure LA, Foster NR, Dehghani F. Conventional and dense gas techniques for the production of liposomes: a review. AAPS PharmSciTech. 2008; **9** :798-809.

28. Huang Z, Li X, Zhang T, Song Y, She Z, Li J, et al. Progress involving new techniques for liposome preparation. Asian Journal of Pharmaceutical Sciences. 2014; **9** :176-82.

29. van Swaay D, deMello A. Microfluidic methods for forming liposomes. Lab Chip. 2013; **13** :752-67.

30. Allison AG, Gregoriadis G. Liposomes as immunological adjuvants. Nature. 1974; **252** :252.

31. Wang N, Chen M, Wang T. Liposomes used as a vaccine adjuvant-delivery system: From basics to clinical immunization. J Control Release. 2019; **303** :130-50.

32. Zamani P, Momtazi-Borojeni AA, Nik ME, Oskuee RK, Sahebkar A. Nanoliposomes as the adjuvant delivery systems in cancer immunotherapy. J Cell Physiol. 2018; **233** :5189-99.

33. Liu J, Miao L, Sui J, Hao Y, Huang G. Nanoparticle cancer vaccines: Design considerations and recent advances. Asian J Pharm Sci. 2020;**15** :576-90.

34. Varypataki EM, Silva AL, Barnier-Quer C, Collin N, Ossendorp F, Jiskoot W. Synthetic long peptide-based vaccine formulations for induction of cell mediated immunity: A comparative study of cationic liposomes and PLGA nanoparticles. J Control Release. 2016;**226** :98-106.

35. Liu L, Ma P, Wang H, Zhang C, Sun H, Wang C, et al. Immune responses to vaccines delivered by encapsulation into and/or adsorption onto cationic lipid-PLGA hybrid nanoparticles. J Control Release. 2016;**225** :230-9.

36. Kolasinac R, Kleusch C, Braun T, Merkel R, Csiszar A. Deciphering the Functional Composition of Fusogenic Liposomes. Int J Mol Sci. 2018;**19** :346.

37. Gregoriadis G. The immunological adjuvant and vaccine carrier properties of liposomes. J Drug Target. 1994; **2** :351-6.

38. Ma Y, Zhuang Y, Xie X, Wang C, Wang F, Zhou D, et al. The role of surface charge density in cationic liposome-promoted dendritic cell maturation and vaccine-induced immune responses. Nanoscale. 2011;**3** :2307-14.

39. Christensen D, Korsholm KS, Andersen P, Agger EM. Cationic liposomes as vaccine adjuvants. Expert Rev Vaccines. 2011; **10** :513-21.

40. Riehl M, Harms M, Hanefeld A, Baleeiro RB, Walden P, Mader K. Combining R-DOTAP and a particulate antigen delivery platform to trigger dendritic cell activation: Formulation development and in-vitro interaction studies. Int J Pharm. 2017; **532** :37-46.

41. Varypataki EM, Benne N, Bouwstra J, Jiskoot W, Ossendorp F. Efficient Eradication of Established Tumors in Mice with Cationic Liposome-Based Synthetic Long-Peptide Vaccines. Cancer Immunol Res. 2017; **5** :222-33.

42. Cheng N, Watkins-Schulz R, Junkins RD, David CN, Johnson BM, Montgomery SA, et al. A nanoparticle-incorporated STING activator enhances antitumor immunity in PD-L1-insensitive models of triple-negative breast cancer. JCI Insight. 2018; **3** : e120638.

43. Liu C, Chu X, Yan M, Qi J, Liu H, Gao F, et al. Encapsulation of Poly I:C and the natural phosphodiester CpG ODN enhanced the efficacy of a hyaluronic acid-modified cationic lipid-PLGA hybrid nanoparticle vaccine in TC-1-grafted tumors. Int J Pharm. 2018; **553** :327-37.

44. Yanasarn N, Sloat BR, Cui Z. Negatively charged liposomes show potent adjuvant activity when simply admixed with protein antigens. Mol Pharm. 2011; **8** :1174-85.

45. Orr MT, Fox CB, Baldwin SL, Sivananthan SJ, Lucas E, Lin S, et al. Adjuvant formulation structure and composition are critical for the development of an effective vaccine against tuberculosis. J Control Release. 2013; **172** :190-200.

46. Shakweh M, Besnard M, Nicolas V, Fattal E. Poly (lactide-co-glycolide) particles of different physicochemical properties and their uptake by peyer's patches in mice. Eur J Pharm Biopharm. 2005;**61** :1-13.

47. Zhao W, Zhuang S, Qi XR. Comparative study of the in vitro and in vivo characteristics of cationic and neutral liposomes. Int J Nanomedicine. 2011; **6** :3087-98.

48. Badiee A, Jaafari MR, Khamesipour A, Samiei A, Soroush D, Kheiri MT, et al. The role of liposome charge on immune response generated in BALB/c mice immunized with recombinant major surface glycoprotein of Leishmania (rgp63). Exp Parasitol. 2009; **121** :362-9.

49. Halder J, Kamat AA, Landen CN, Jr., Han LY, Lutgendorf SK, Lin YG, et al. Focal adhesion kinase targeting using in vivo short interfering RNA delivery in neutral liposomes for ovarian carcinoma therapy. Clin Cancer Res. 2006; **12** :4916-24.

50. Akhtar A, Wang SX, Ghali L, Bell C, Wen X. Effective Delivery of Arsenic Trioxide to HPV-Positive Cervical Cancer Cells Using Optimised Liposomes: A Size and Charge Study. Int J Mol Sci. 2018;**19** :1081.

51. Kocabas BB, Almacioglu K, Bulut EA, Gucluler G, Tincer G, Bayik D, et al. Dual-adjuvant effect of pH-sensitive liposomes loaded with STING and TLR9 agonists regress tumor development by enhancing Th1 immune response. J Control Release. 2020; **328** :587-95.

52. Yoshizaki Y, Yuba E, Sakaguchi N, Koiwai K, Harada A, Kono K. pH-sensitive polymer-modified liposome-based immunity-inducing system: Effects of inclusion of cationic lipid and CpG-DNA. Biomaterials. 2017;**141** :272-83.

53. Yuba E, Uesugi S, Miyazaki M, Kado Y, Harada A, Kono K. Development of pH-sensitive Dextran Derivatives with Strong Adjuvant Function and Their Application to Antigen Delivery. Membranes (Basel). 2017;**7** .

54. Bi H, Xue J, Jiang H, Gao S, Yang D, Fang Y, et al. Current developments in drug delivery with thermosensitive liposomes. Asian J Pharm Sci. 2019; **14** :365-79.

55. Sakurai Y, Kato A, Hida Y, Hamada J, Maishi N, Hida K, et al. Synergistic Enhancement of Cellular Uptake With CD44-Expressing Malignant Pleural Mesothelioma by Combining Cationic Liposome and Hyaluronic Acid-Lipid Conjugate. J Pharm Sci. 2019;**108** :3218-24.

56. Boks MA, Ambrosini M, Bruijns SC, Kalay H, van Bloois L, Storm G, et al. MPLA incorporation into DC-targeting glycoliposomes favours anti-tumour T cell responses. J Control Release. 2015;**216** :37-46.

57. Shetab Boushehri MA, Lamprecht A. TLR4-Based Immunotherapeutics in Cancer: A Review of the Achievements and Shortcomings. Mol Pharm. 2018;**15** :4777-800.

58. Shimoyama A, Di Lorenzo F, Yamaura H, Mizote K, Palmigiano A, Pither MD, et al. Lipopolysaccharide from Gut-Associated Lymphoid Tissue-Resident Alcaligenes faecalis: Complete Structure Determination and Chemical Synthesis of its Lipid As. Angew Chem Int Ed Engl. 2021.

59. Jiang ZH, Budzynski WA, Skeels LN, Krantz MJ, Koganty RR. Novel lipid A mimetics derived from pentaerythritol: synthesis and their potent agonistic activity. Tetrahedron. 2002; **58** :8833-42.

60. Reintjens NRM, Tondini E, de Jong AR, Meeuwenoord NJ, Chiodo F, Peterse E, et al. Self-Adjuvanting Cancer Vaccines from Conjugation-Ready Lipid A Analogues and Synthetic Long Peptides. J Med Chem. 2020; **63** :11691-706.

61. Coler RN, Bertholet S, Moutaftsi M, Guderian JA, Windish HP, Baldwin SL, et al. Development and characterization of synthetic glucopyranosyl lipid adjuvant system as a vaccine adjuvant. PLoS One. 2011;**6** :e16333.

62. Vacchelli E, Galluzzi L, Eggermont A, Fridman WH, Galon J, Sautes-Fridman C, et al. Trial watch: FDA-approved Toll-like receptor agonists for cancer therapy. Oncoimmunology. 2012; **1** :894-907.

63. Gao J, Guo Z. Progress in the synthesis and biological evaluation of lipid A and its derivatives. Med Res Rev. 2018; **38** :556-601.

64. Varikuti S, Oghumu S, Natarajan G, Kimble J, Sperling RH, Moretti E, et al. STAT4 is required for the generation of Th1 and Th2, but not Th17 immune responses during monophosphoryl lipid A adjuvant activity. Int Immunol. 2018; **30** :385.

65. Guo J, Chen Y, Lei X, Xu Y, Liu Z, Cai J, et al. Monophosphoryl lipid a attenuates radiation injury through TLR4 activation. Oncotarget. 2017; **8** :86031-42.

66. Ahmed KK, Geary SM, Salem AK. Development and Evaluation of Biodegradable Particles Coloaded With Antigen and the Toll-Like Receptor Agonist, Pentaerythritol Lipid A, as a Cancer Vaccine. J Pharm Sci. 2016; **105** :1173-9.

67. Somaiah N, Chawla SP, Block MS, Morris JC, Do K, Kim JW, et al. A Phase 1b Study Evaluating the Safety, Tolerability, and Immunogenicity of CMB305, a Lentiviral-Based Prime-Boost Vaccine Regimen, in Patients with Locally Advanced, Relapsed, or Metastatic Cancer Expressing NY-ESO-1. Oncoimmunology. 2020; **9** :1847846.

68. Mahipal A, Ejadi S, Gnjatic S, Kim-Schulze S, Lu H, Ter Meulen JH, et al. First-in-human phase 1 dose-escalating trial of G305 in patients with advanced solid tumors expressing NY-ESO-1. Cancer Immunol Immunother. 2019; **68** :1211-22.

69. Xiao X, Sankaranarayanan K, Khosla C. Biosynthesis and structure-activity relationships of the lipid a family of glycolipids. Curr Opin Chem Biol. 2017; **40** :127-37.

70. Jeong Y, Kim GB, Ji Y, Kwak GJ, Nam GH, Hong Y, et al. Dendritic cell activation by an E. coli-derived monophosphoryl lipid A enhances the efficacy of PD-1 blockade. Cancer Lett. 2020; **472** :19-28.

71. Zhou Z, Mondal M, Liao G, Guo Z. Synthesis and evaluation of monophosphoryl lipid A derivatives as fully synthetic self-adjuvanting glycoconjugate cancer vaccine carriers. Org Biomol Chem. 2014;**12** :3238-45.

72. Yoshii K, Hosomi K, Shimoyama A, Wang Y, Yamaura H, Nagatake T, et al. Chemically Synthesized Alcaligenes Lipid A Shows a Potent and Safe Nasal Vaccine Adjuvant Activity for the Induction of Streptococcus pneumoniae-Specific IgA and Th17 Mediated Protective Immunity. Microorganisms. 2020; **8** :1102.

73. Beck Z, Matyas GR, Jalah R, Rao M, Polonis VR, Alving CR. Differential immune responses to HIV-1 envelope protein induced by liposomal adjuvant formulations containing monophosphoryl lipid A with or without QS21. Vaccine. 2015; **33** :5578-87.

74. Vantomme V, Dantinne C, Amrani N, Permanne P, Gheysen D, Bruck C, et al. Immunologic analysis of a phase I/II study of vaccination with MAGE-3 protein combined with the AS02B adjuvant in patients with MAGE-3-positive tumors. J Immunother. 2004; **27** :124-35.

75. Slingluff CL, Jr., Petroni GR, Olson WC, Smolkin ME, Chianese-Bullock KA, Mauldin IS, et al. A randomized pilot trial testing the safety and immunologic effects of a MAGE-A3 protein plus AS15 immunostimulant administered into muscle or into dermal/subcutaneous sites. Cancer Immunol Immunother. 2016; **65** :25-36.

76. Zhu D, Hu C, Fan F, Qin Y, Huang C, Zhang Z, et al. Co-delivery of antigen and dual agonists by programmed mannose-targeted cationic lipid-hybrid polymersomes for enhanced vaccination. Biomaterials. 2019;**206** :25-40.

77. Shin WJ, Noh HJ, Noh YW, Kim S, Um SH, Lim YT. Hyaluronic acid-supported combination of water insoluble immunostimulatory compounds for anti-cancer immunotherapy. Carbohydr Polym. 2017;**155** :1-10.

78. Ko EJ, Lee YT, Lee Y, Kim KH, Kang SM. Distinct Effects of Monophosphoryl Lipid A, Oligodeoxynucleotide CpG, and Combination Adjuvants on Modulating Innate and Adaptive Immune Responses to Influenza Vaccination. Immune Netw. 2017; **17** :326-42.

79. Gableh F, Saeidi M, Hemati S, Hamdi K, Soleimanjahi H, Gorji A, et al. Combination of the toll like receptor agonist and alpha-Galactosylceramide as an efficient adjuvant for cancer vaccine. J Biomed Sci. 2016; **23** :16.

80. Mei L, Liu Y, Rao J, Tang X, Li M, Zhang Z, et al. Enhanced Tumor Retention Effect by Click Chemistry for Improved Cancer Immunochemotherapy. ACS Appl Mater Interfaces. 2018;**10** :17582-93.

81. Mahoney KM, Rennert PD, Freeman GJ. Combination cancer immunotherapy and new immunomodulatory targets. Nat Rev Drug Discov. 2015;**14** :561-84.

82. Babjuk M, Bohle A, Burger M, Capoun O, Cohen D, Comperat EM, et al. EAU Guidelines on Non-Muscle-invasive Urothelial Carcinoma of the Bladder: Update 2016. Eur Urol. 2017; **71** :447-61.

83. Yoshino T, Miyazaki J, Kojima T, Kandori S, Shiga M, Kawahara T, et al. Cationized liposomal ketomycolic acids isolated from Mycobacterium bovis bacillus Calmette-Guerin induce antitumor immunity in a syngeneic murine bladder cancer model. PLoS One. 2019; **14** :e0209196.

84. Kubota M, Iizasa E, Chuuma Y, Kiyohara H, Hara H, Yoshida H. Adjuvant activity of Mycobacteria-derived mycolic acids. Heliyon. 2020;**6** :e04064.

85. McCluskie MJ, Deschatelets L, Krishnan L. Sulfated archaeal glycolipid archaeosomes as a safe and effective vaccine adjuvant for induction of cell-mediated immunity. Human Vaccines Immunotherapeutics 2017; **13** :2772-9.

86. Haq K, Jia Y, Krishnan L. Archaeal lipid vaccine adjuvants for induction of cell-mediated immunity. Expert Review of Vaccines. 2016;**15** :1557-66.

87. Akache B, Stark FC, Jia Y, Deschatelets L, Dudani R, Harrison BA, et al. Sulfated archaeol glycolipids: Comparison with other immunological adjuvants in mice. PLoS One. 2018; **13** :e0208067.

88. Akache B, Stark FC, Iqbal U, Chen W, Jia Y, Krishnan L, et al. Safety and biodistribution of sulfated archaeal glycolipid archaeosomes as vaccine adjuvants. Human Vaccines Immunotherapeutics. 2018;**14** :1746-59.

89. Jia Y, Chandan V, Akache B, Qian H, Jakubek ZJ, Vinogradov E, et al. Assessment of stability of sulphated lactosyl archaeol archaeosomes for use as a vaccine adjuvant. Journal of Liposome Research. 2020:1-9.

90. Stark FC, Weeratna RD, Deschatelets L, Gurnani K, Dudani R, McCluskie MJ, et al. An Archaeosome-Adjuvanted Vaccine and Checkpoint Inhibitor Therapy Combination Significantly Enhances Protection from Murine Melanoma. Vaccines (Basel). 2017; **5:** 38.

91. Stark FC, Agbayani G, Sandhu JK, Akache B, McPherson C, Deschatelets L, et al. Simplified admix archaeal glycolipid adjuvanted vaccine and checkpoint inhibitor therapy combination enhances protection from murine melanoma. Biomedicines. 2019; **7** .

92. Agbayani G, Jia Y, Akache B, Chandan V, Iqbal U, Stark FC, et al. Mechanistic insight into the induction of cellular immune responses by encapsulated and admixed archaeosome-based vaccine formulations. Human Vaccines Immunotherapeutics 2020; **16** :2183-95.

93. Li R, Peng F, Cai J, Yang D, Zhang P. Redox dual-stimuli responsive drug delivery systems for improving tumor-targeting ability and reducing adverse side effects. Asian J Pharm Sci. 2020; **15** :311-25.

94. Sen D, Deerinck TJ, Ellisman MH, Parker I, Cahalan MD. Quantum dots for tracking dendritic cells and priming an immune response in vitro and in vivo. PLoS One. 2008; **3** :e3290.