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EVALUATION OF NANO-SYSTEMS AS VACCINE PLATFORMS AGAINST INFECTIOUS DISEASES

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Table of contents

1 Abstract

Vaccines are one of the most important pages of human health by providing protection against various infections. Although the indisputable benefits of classic vaccines, they have some serious limitations such as low immunogenicity and short-term effectiveness, time-consuming developing and/or manufacturing processes, high cost, and limited variety of administration routes and storage. The above led to the development of nanovaccinology, a science area which concludes vaccine platforms of 1-1000 nm. These platforms function either as antigen delivery systems or as immune-stimulators and induce innate and adaptive immune responses. Nanovaccines can be classified by their physicochemical and morphological characteristics and, thus, platforms with unique properties are able to form. Liposomes, virus-like particles, nano-emulsions, polymeric or inorganic nanoparticles as well as viral vectors are the main categories that are currently on the market or in clinical and pre-clinical phases. Nano-formulations allow the manufacture of innovative vaccines, such as the third generation vaccines, but they shall be carefully studied to minimize probable dangers from their use in clinical practice. Medicine agencies are responsible for composing a legislative framework about nanovaccinology. In this way, both pharmaceutical companies and human populations will be benefited by eliminating the danger of upcoming pandemics or other infectious diseases.

Keywords: Nanovaccine, Vaccine, Antigen delivery system, Adjuvant, Immunology, Virus, Infection

2 Introduction

After the recent outbreak of COVID-19, caused by the virus SARS-CoV-2, a reminder of the severity of infectious diseases is obvious. Ebola (Kuroda et al., 2020), HIV/AIDS (Hemelaar et al., 2019), SARS (Ksiazek et al., 2003), and MERS (Assiri et al., 2013) are only some of the most known recent epidemic and pandemic examples. Changes in the lifestyle, such as the ease of traveling around the word and development of densely populated metropolitan cities, have led to an increase of the spreading of these diseases, making it clear that we cannot put boundaries in outbreaks (Bedford et al., 2019; Rauch et al., 2018). Direct solutions for the elimination of such situations are necessary. The rapid development of the technology has played a key role in the prophylaxis from infections. Notable, it is supported that preventing pathogens' transmission is not only feasible with vaccines, but also via digital contract tracing. According to Ferretti and colleagues, a mobile application that is compatible with national authorities could result in a much more beneficial outcome and decrease of the viral reproduction number (R) than quarantine or manual contact tracing for the case of SARS-CoV-2. Although promising, at the moment, ethical issues about the personal data and difficulties in a universal use of such an app delay its application in countries of Europe and USA (Ferretti et al., 2020). As a result, effective vaccines remain the number one choice for the transmission elimination of infectious diseases.

Vaccines are one of the most significant accomplishment in the history of medicine and human health. In 1796, Edward Jenner, an Englishman doctor, performed the first vaccination. He administrated pus from a lesion of a woman who was infected by cowpox to a boy and found out that the boy did not infected by smallpox. After further research, in the 1800s he published his paper: "Inquiry into the causes and effects of Viriolae vaccinae" (Stern & Markel, 2005). The word "vaccine" comes from the term Viriolae vaccinae, which has invented by Jenner to describe cowpox. It was many years later when Louis Pasteur, in 1881, proposed the utilize of the terms "vaccine" and "vaccination" to characterize all the immunizing procedures and not just those associated with smallpox (Baxby, 1999). Louis Pasteur was the first who achieved to attenuate microorganisms and inoculate people with them to provide protection against certain germs. Thus, in contrast with Jenner's technique to find a similar, milder disease to provide inoculation, Pasteur used the weakened germs that caused the infectious diseases. His work in chicken cholera, anthrax and rabies constitute the front lines of vaccinology (Berche, 2012).

Nowadays, we can classify the vaccines in three main generations. The first generation contains the vaccines of live-attenuated or inactivated pathogens. This approach was the continuation of Pasteur thoughts: cultures of pathogens undergo environmental (e.g. thermal) or chemical (e.g. changes in pH value) pressure, so that the pathogen loses its infectious ability but, on the same time, it remains immunogenic. Several efficacious vaccines of this type have been produced against smallpox (WHO, 2004), tuberculosis (Covián et al., 2019), poliomyelitis (Kew et al., 2005) and seasonal or pandemic flu (Herold & Sander, 2020). In fact, smallpox eradicated in the 1980s and poliomyelitis is nearly eradicated today (Minor, 2015).

Although first generation vaccines are responsible for the elimination of serious human diseases, they seem to be ineffective in some cases of lethal diseases caused by pathogens as HIV-1. Especially live-attenuated pathogens have the possibility to evoke the disease they are supposed to protect from, mostly in immunodeficient populations (Pliaka et al., 2012). Thus, scientists were motivated for the development of the next-generation vaccines: subunit vaccines. Subunit vaccines contain only specific immunogenic domains of the pathogen. Domains that are usually used for this purpose are the membrane or capsid proteins of germs and viruses (Bror Morein & Simons, 1985). Sometimes, these proteins have the ability to self-assemble into particulate systems, known as virus-like particles (Noad & Roy, 2003). Subunit vaccines seem to have a better safety profile than live-attenuated or inactivated vaccines, but, on the other hand, they are less immunogenic, resulting in weaker and sometimes insufficient response. Thus, the majority of these vaccines need an immunomodulator, an adjuvant, to induce the immune system (Cimica & Galarza, 2017). Some of the most widely used vaccines nowadays belong in this category, such as human papilloma virus and hepatitis B virus vaccines, as analyzed below.

The third generation vaccines do not contain proteins of the pathogens but part of their genetic material. Such vaccines have recently been approved for human use but many assume that they will bring a revolution in the field of vaccinology. The genetic material is encapsulated into nanoparticles and transferred inside the target host cells. When released, the genetic material, DNA or RNA, is expressed by the host expression system, as in the case of an infection. Hence an accurate architecture of the protein is produced. The cost of production for nucleic acid vaccines is one of their main advantages as the manufacturing process is simpler and has higher repeatability in comparison with previous generation vaccines (K. Lee et al., 2018; Tejeda-Mansir et al., 2019). On the other hand, when DNA is the cargo, it should pass into the nucleus to produce the intermediate molecule -mRNA- that

will be then, translated into the immunogenic protein. mRNA vaccines need to pass only the plasma membrane which is easier than passing the nuclei membrane. The mRNA molecules have a dual role in the induction of the immune system. First, they are the templates for the formation of the desired antigenic protein and second, the can act as adjuvants that are recognized by endosomal and cytosolic innate immune receptors, such as Toll-like receptors 3,7 and 8 (Rauch et al., 2018). Despite their important advantages, nucleic acid vaccines have not been evaluated for a long period and some consideration about their mechanism still exists. For example, the exogenous plasmid DNA that is inserted into the nucleus might remain there for a longer time than it is supposed to, leading to worries for genomic integration and mutagenesis (Rauch et al., 2018). Since EMA differentiate gene therapy medicinal products and vaccines against infectious diseases, no guidance is available for nucleic acid vaccines. However, the agency has recognized the need for a central, common source since 2007 (EMA, 2012). In December 2019, WHO consulted for the evaluation of DNA vaccines, after the pressure for the finding of a vaccine against SARS-CoV-2. The consultation concluded that the changes that proposed would be discussed at the next meeting of WHO ECBS (Oct. 2020) and suggested that guidelines for RNA vaccines shall also be prepared (Sheets et al., 2020). These guidelines will open the road for the authorization of third generation vaccines.

At this point, it is important to note that many of the innovative technologies, which mentioned above, follow the physicochemical rules of nanotechnology. Pharmaceutical nanotechnology and nanomedicine are the areas that evaluate all the types of nanomaterials for application in health sciences. In 2011 the European Commission recommended that a material shall be characterized as nanomaterial if 50% or more of the constituent particles have one or more external dimensions in the size range 1-100 nm. A material can also be considered as nanomaterial if its volume – specific surface area is larger than 60 m^2/cm^3 (EC, 2011). However, EMA has not yet taken an official thesis about the boundaries of nanomedicine and it is proposed that nanotechnology is the science that uses structures less than 1,000 nm across (https://www.ema.europa.eu/en/glossary/nanotechnology). In the last decades, nanomedicine has swift progress and some impressive results are presented in its short-term history. Indicative, the authorization of the first nanoparticulated drug, containing doxorubicin, in 1995, altered the way we can handle molecules with low hydrophilicity or small therapeutic window. In vaccinology, nanoparticles, have a dual role as they can play a key role in both antigen delivery and adjuvanticity. Nanoparticles' unique properties such as the high surface-to-volume ratio (**Figure 1)** and small size (10^{-9} m), at which quantum

effects are significant, are responsible for their promising results (https://www.nano.gov/nanotech-101/special).

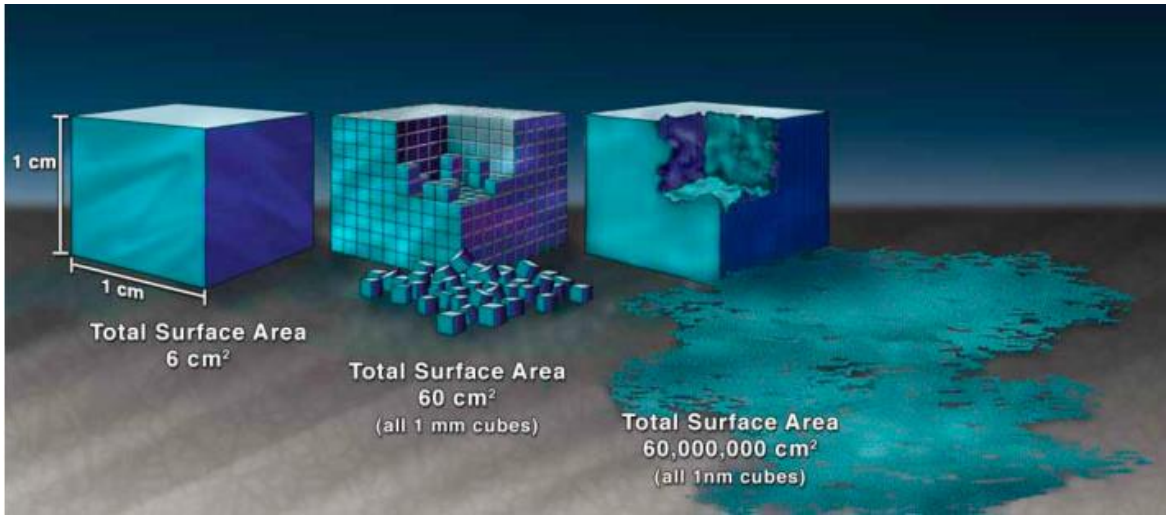


Figure 1: Nanoparticles unique properties. Adapted from https://www.nano.gov/nanotech-101/special

Furthermore, the classic technique of developing vaccines, requires valuable time. Hence, it is necessary to develop vaccine platforms for the delivery of the antigen or as adjuvants that could act as a trump card in a group of cases. In this way, the time for the construction of a novel platform for every pathogen is minimized, resulting in a decrease in the cost, time and effort needed. Moreover, novel biomaterials with unique physicochemical properties, can self-assemble into smart formulations that can change their behavior, and thus the visibility and immunogenicity of the antigen in vaccinology, by responsiveness in the environmental stimuli. New administrating approaches are also feasibly with novel nanoparticles such as intradermal or intranasal vaccines. However, as novel formulations, at least in the beginning, they should be strictly designed and evaluated to prevent the appearance of undesirable pharmacological effects. As toxicological issues of nano-structures have not been solved till now and each formulation constitutes a unique case, trials should begin from point 0 each time.

The aim of the present study is to classify the nano-formulations that have been investigated in the area of vaccinology and present nano-vaccines that are in the market currently. For the purposes of this review, we consider as nano-vaccines the formulations that contain particles 1-1,000 nm. The classification is based on the material composition. Morphological and physicochemical characteristics of each group are analyzed and the most recent research

and active clinical trials are presented. A spherical cover of the issue as well as the advantages and difficulties of each formulation are mentioned. Thus, at the end of the review, the reader will be able to understand the area of nano-vaccinology and the novelties of its use for the development of potent and safe vaccines.

3 Theory: Overview of the immune system

Although notable progress in the understanding of the immune system is observed, there are still some unexplored paths. Generally, the mechanism of action of human immunity consists of two main types of response: the innate and the adaptive response. Both are equally important in the protection against infections and cellular malfunctions, producing an enhanced humoral and cellular cascade.

3.1 Innate Immunity:

When a pathogen or antigen is presented into a tissue or in body fluids the innate response is immediately activated. Complement, granulocytes, macrophages, and dendritic cells promotes the release of a plethora of active molecules, as chemokines, cytokines, interferons, and complement components. Firstly, complement activation leads to the opsonization or lysis of the antigen membrane via three routes: the classical, the alternative and the mannan binding lectin pathway. As complement contains about 20 serum active molecules – glycoproteins – the existence of a pathogen in the blood is recognized and entitled within a few minutes (Zipfel & Skerka, 2009). Furthermore, neutrophils are granulocytes that normally flow in the blood and can recognize signals from infected cells and macrophages. After the recognition, the neutrophils gather in the site of the infection and phagocytose the antigens, while simultaneously producing chemokines and other chemoattractants for a further activation of the immune response. Eosinophils and basophils are other granulocytes of the bloodstream but they do exist in much lower concentration in contrast with neutrophils. These two types of leucocytes have an important role in hypersensitivity responses, such as allergies or autoimmunity via the production of IgE antibodies but they also are valuable for anti-parasite immunity. This action is due to the activation of a T-helper type 2 (T_H2) immune response via the production of the cytokines interleukin 4 (IL-4), IL-5 and IL-13 (Falcone et al., 2001; Jiao et al., 2016; Voehringer, 2009).

However, except blood protective molecules and cells, tissues also have their own immune cells. Immature dendritic cells and macrophages are located in peripheric tissues and in the presence of a pathogen they have the ability of phagocytosis. The main difference between these two cell types of the innate system is that dendritic cells do not only phagocytose the pathogens but as well, are capable of antigen presentation in contrast with macrophages. The presentation occurs via the binding of the antigen with major histocompatibility complex class I or II (MHC I or II). MHC class II is associated with the presentation of extracellular

peptides derived from allergens, bacteria, protozoa, or dead host cells, while MHC class I is used for the presentation of intracellular proteins such as viral proteins expressed by infected cells. MHC I can be expressed by almost all nucleated cells while, MHC II is a privilege presentation complex of only professional antigen-presenting cells (Mellman, 2013), as presented in **Figure 2**.

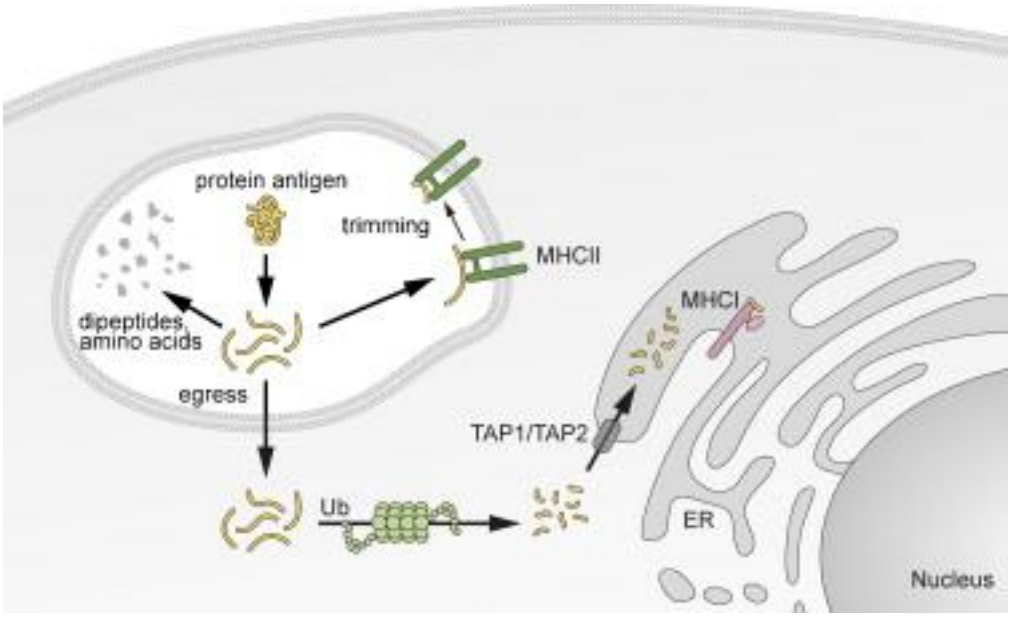


Figure 2: MHC presentation pathways of captured antigens. Lysosomes contain an acidic and proteolytic environment, which favors antigen processing and loading onto the MHCII molecules. The newly formed MHCII/peptide complexes are then transported to the cell surface. The harsh lysosomal environment however does not appear to favor MHCI cross-presentation. To be presented on MHCI molecules, most captured antigens must be transferred into the cytosol to access the conventional MHCI pathway. The molecular mechanism underlying this transfer and the compartment where it takes place are still unknown. After transfer to the cytosol, antigens are processed by the proteasome, and the resulting peptides are then transported by TAP for loading on MHCI in the ER, and possibly in the endocytic compartments. Adapted from (Delamarre & Mellman, 2011).

Phagocytic cells interact with the pathogens via recognition of the pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) by their pathogen recognition receptors (PRRs). There are four types of PRRs: i) toll-like receptors (TLRs), ii) NOD-like receptors (NLRs), iii) RIG-like receptors (RLRs) and iv) C-type lectin receptors (CLRs). All play an important role in the induction of the immune system. Eleven

TLR members have been identified in the human organism. TLR1, TLR2, TLR4, TLR5 and TLR6 are located in the cell membrane and identify bacterial cell components (e.g. bacterial lipoproteins) or host heat shock proteins. One the other hand, TLR3, TLR8 and TLR9 recognize nucleic acids that exist in viruses and bacteria (Frazão et al., 2013). Interestingly, TLR9 agonists, which are CpG motifs have been successfully evaluated as vaccine adjuvants, as analyzed in Chapter 5.

Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are similar to the TLRs, but differ in their position. In contrast with TLRs, which are transmembrane receptors, NLRs are cytoplasmic receptors. NLRs consist of three domains: The C-terminal leucine rich repeats, which is responsible for the recognition of PAMPs and DAMPs, the intermediate domain and the N-terminal effector domain that interacts with other host proteins to activate the response. NLRs can recognize a variety of antigens such as peptidoglycan, flagellin, viral RNA, alum, silica, etc., as well as signals from host cells. The above result that NLRs have an important role in pathogens' recognition (Y. K. Kim et al., 2016).

C-type lectin receptors (CLRs) are carbohydrate recognition receptors (mainly galactose and mannose) that exist both soluble and in transmembrane formation. Nowadays, 17 groups of CLRs have been reported to be connected with innate immunity. The main role of this type of PRRs is the protection against fungal. Dectin1, Dectin2, macrophage mannose receptor (MMR) and dendritic cell-specific intracellular adhesion molecule 3-grabing non-integrin (DC-SIGN) are some of the most well-characterized receptors of CLRs (Nikolakopoulou et al., 2020).

Lastly, retinoic acid-inducive gene-like receptors (RIG-like receptors or RLRs) are cytosolic proteins connected with innate signaling from the biosynthetic pathway. In this way, the cells can separate pathogenic microbes from the non-pathogenic normal flora. RLRs identify viral RNA by recognition of certain patterns found in pathogens. For instance, 5' triphosphate RNA, long double-stranded RNA or poly-uridine domains act as signals for the activation of RLRs immunological pathway leading to induction of inflammatory cytokines and type I interferons (Brubaker et al., 2015; H. Kumar et al., 2011).

The variety of PRRs permits the pathogen-recognition and activation of innate immunity in several stages and against different types of pathogens as presented in **Table 1**.

Table 1: Pathogen-recognition receptors (PRRs) of the innate immune system their location and targets.

Types	Receptor location	Target groups
TLR	Cell membrane Endosomal compartments	Bacterial proteins Host heat shock proteins Bacterial & viral nucleic acid
NLR	Cytoplasm (endosomal membrane-associated)	Bacterial components Viral RNA Inorganic molecules (e.g. Al & Si) DAMPS
CLR	Cell membrane	Fungal molecules
RLR	Cytoplasm	Viral RNA

Abbreviations: TLR, Toll-like receptor; NLR, NOD-like receptor; CLR, C-type lectin receptor; RLR, RIG-like receptor.

To conclude, one of the most interesting categories of innate cells is the one containing natural killer cells (NK). NK are lymphocytes that can detect infected host cells, allogenic cells, or cancer cells and cytolyze them. Thus, concerning infectious diseases, NK seem to be key molecules for the protection against viral infections. NK can sense inflammatory signals such as cytokines, antibodies, viral signals, or host cell stress-signals (Hammer et al., 2018). The interesting part is that although NK belong to the innate immune system, they have the ability to produce memory. Antigen-specific or non-antigen- specific NK memory cells are induced after activation from viruses or haptens. Hence, certain markers that are present in the surface of NK memory cells provide long-lasting protection against stimuli (Cerwenka & Lanier, 2016).

3.2 Adaptive Immunity:

It would be true if someone claimed that adaptive response is the elegant function of our immune system, as it is responsible for the specialized combat of any pathogen infection or host cells malignancies. There are two main arms of adaptive immune cells, T-lymphocytes and B-lymphocytes. As in innate immune response, the adaptive system also produces both

humoral and cellular responses. Generally, T cells are responsible for the cellular induction while B cells produce high quantities of specialized antibodies that neutralize and signal the antigens. After the activation of the innate response, the antigen-presenting cells, mostly the dendritic cells, migrate from the peripheral tissues to the lymph nodes. In lymph nodes they present, via the help of MHC class I or II, antigens from the pathogen they have phagocytosed.

T-cells mature in the thymus and are classified in $CD4^+$ and $CD8^+$ T-cells. $CD8^+$ T-cells, also called cytotoxic T-cells, recognize antigens presented in MHC I and help in the defense against intracellular pathogenic proteins, such as viral peptides. The interaction is based on the recognition of the MHC I by the CD8 domain of the T-cell receptor (TCR). After the recognition, the cytotoxic T-cell will evoke lysis of the infected cell membrane, concluding in the cell's death. On the other hand, $CD4^+$ T-cells or T-helper cells (T_H) are activated by antigens in MHC II and produce cytokines to interact with other cells. Naïve $CD4^+$ T-cells differentiate in five subcategories: T_H1, T_H2, T_H17, T_{FH}, and T_{REG}. The differentiation is associated with the presence of certain cytokines, as it is mentioned in **Figure 3**.

Briefly, T_H1 induce the activation of innate cellular immunity (macrophages), while T_H2 is associated with the production of antibodies via the activation of B-lymphocytes. T_H17, apart from inducing innate immunity, also seem to play a key role in autoimmune diseases (Leung et al., 2010). Follicular T-helper cells (T_{FH}) are located in the germinal centers inside the tonsils and physiologically, they are responsible for the differentiation and the proliferation of B-cell clones (Crotty, 2011). Thus, T_{FH} are necessary for the induction of the adaptive immune response. However, the scientific community has not yet fully understood the function of T_{FH} cells in immunodeficient organisms. It is probable that T_{FH} cells have a beneficial role in immunodeficient malignancies such as HIV-1 by modulating the maturation of B-cells in plasma cells (Tangye et al., 2013). Nevertheless, these cells, as the other types of $CD4^+$ T-cells, are substrates for the virus proliferation. Interestingly, some results show that T_{FH} can carry more copies of the viral nucleic acid than other T-cells (Perreau et al., 2012). Hence, whether T_{FH} have a beneficial role in infectious immunodeficient diseases still constitutes a question (Xu et al., 2019). In conclusion, T_{REG} are the immunosuppressive cells of the immune system. They can be further sub-classified, and depending on their special active biomolecules e.g. FOXP3, down-regulate the proliferation of certain types of T-cells. Their functionality is significant for the prevention of cancer or autoimmune diseases development. Moreover, they can protect organs (e.g. liver) or immune cells from immune mediated injury by suppressing the immune response.

The equilibrium between the necessity to encounter the "enemy" and preserve a functional and effective immunity is therefore based on the work of T_{REG} cells (Karkhah et al., 2018).

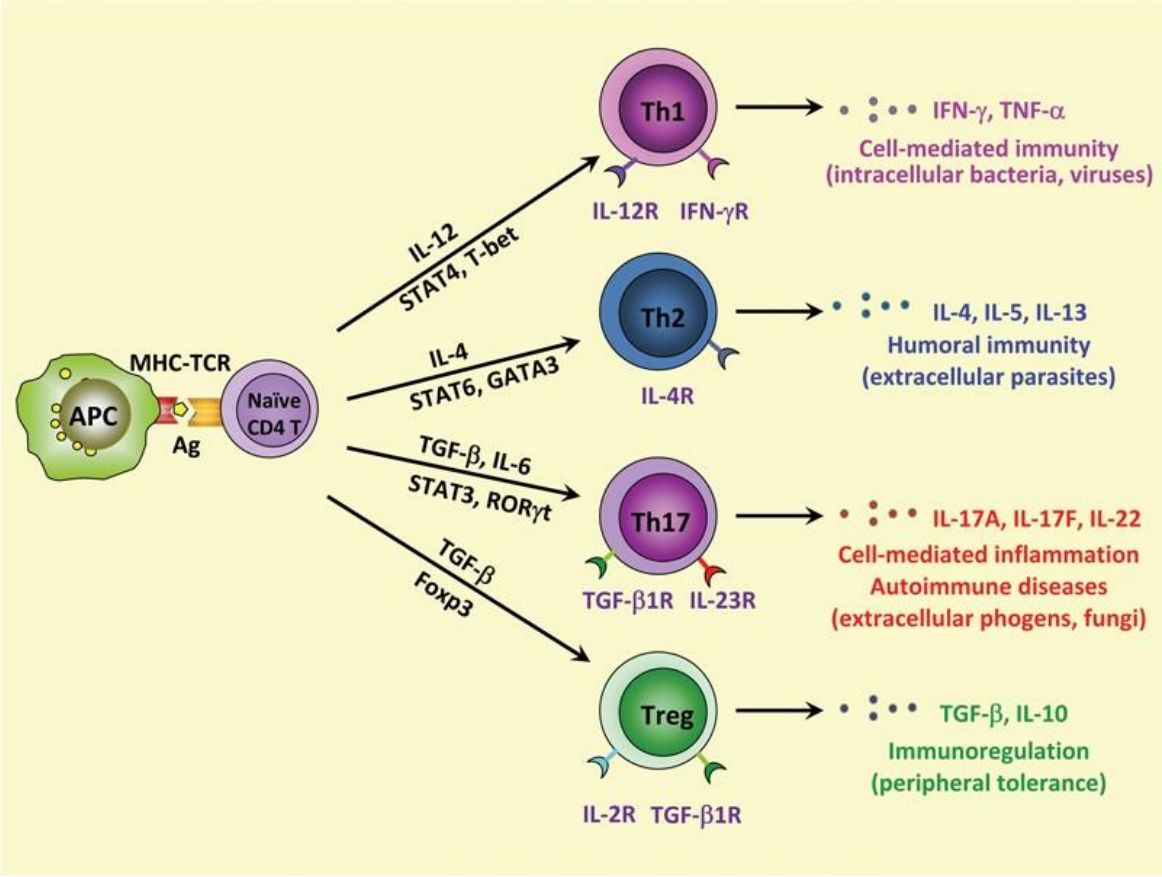


Figure 3: Model for Th cell differentiation from naive CD4+ T cells. In the presence of IL-12, differentiation of naive CD4+ T cells into Th1 cells requires activation of the master regulator transcription factor T-bet through STAT1 and STAT4. Th1 cells produce IFN-γ and are involved in cell-mediated immunity against intracellular bacteria and viruses. IL-4 promotes the activation of STAT6 and GATA3, which are responsible for Th2 cell differentiation. Th2 cells are important in humoral immunity against parasites, an action that is mediated through their production of IL-4, IL-5 and IL-13. The combination of TGF-β and proinflammatory cytokines, such as IL-6 and IL-23, drives the differentiation of naive CD4+T cells into IL-17-producing Th cells (Th17) through the regulation of STAT3 and RORγt. Th17 cells play a critical role in host protection against extracellular pathogens and in inflammatory autoimmune diseases. In addition, TGF-β can induce differentiation of naive CD4+ T cells into Foxp3+ Treg cells, which produce TGF-β and IL-10 and act as modulators of immune responses. APC, antigen-presenting cell; Foxp3+, forkhead box p3+; IFN, interferon; MHC–TCR, major histocompatibility complex–T-cell receptor; ROR,

retinoid-related orphan receptor; TGF, transforming growth factor; Th, T helper; Treg, regulatory T. Adapted from (Leung et al., 2010)**.**

The other main class of adaptive immunity cells are B cells. As it is mentioned above B-cells are responsible for the production of specialized antibodies - immunoglobulin A (IgA), IgE, IgM, IgG, and IgD – that can inactivate the pathogens and simultaneously render them more recognizable from other immune cells. B-cells mature in the bone marrow and afterward locate in the lobules of the lymph nodes. There, they form certain structures, named B cell follicles. When activated by APCs, B cells start to proliferate and form the germinal centers (Schudel et al., 2019). In germinal centers, B cells undergo a series of mutations in the domain of IgG genes. Only B cells with high affinity to the present antigen develop and mature to plasma cells, which then secrete antibodies of high quality and quantity (Kräutler et al., 2017). For the process of B cell proliferation and maturation, the role of T_{FH}, as well as follicular DCs, is extremely important, as it is presented in **Figure 4**. Both classes of adaptive lymphocytes produce immunity memory as they are able to provide long-lasting protection against the same or even very similar antigens (an action called cross-protection) (Netea et al., 2019). Indeed, memory B cells seem to have a much broader repertoire leading to more efficient and faster antigen neutralization in the case of infection by a closely related antigenic epitope as the first one. T cell-dependent and T cell-independent memory B cells are the two major types of long-lasting B-cells (Kurosaki et al., 2015). In contrast with memory B-cells, which induce prophylaxis through humoral memory, T-cells can also induce memory. Central memory T cells (T_{CM}) trafficking through lymphoid tissues, whereas effector memory T cells (T_{EM}) can accumulate other tissues. Thus, T_{CM} and T_{EM} can provide the functionalities of mature T cells in the case of reinfection (Jameson & Masopust, 2018).

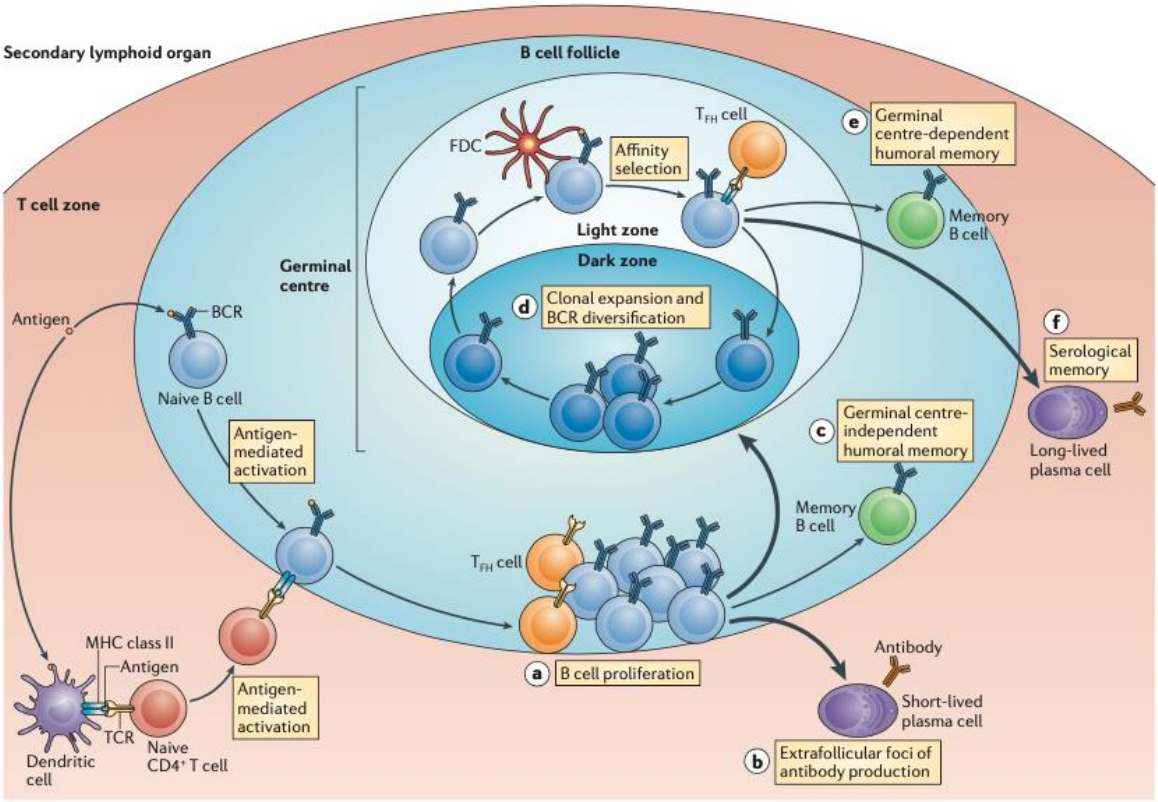


Figure 4: T cell-dependent memory B cell generation. Antigen-activated B cells and T cells migrate towards the borders of the B cell follicles and the T cell zones of secondary lymphoid organs, respectively, which leads to them establishing stable B cell–T cell interactions and enables B cells to receive helper signals from cognate CD4+ T cells. Activated B cells and T cells then migrate to the outer follicles, where B cells undergo proliferation (part a). Some of the proliferating B cells differentiate into short-lived plasma cells (part b), which give rise to the extrafollicular foci, and some develop into memory B cells (part c; germinal centre-independent memory B cells). Alternatively, the activated B cells can return to the follicle and can undergo rapid proliferation to form the germinal centre (part d). In the dark zone of the germinal centre, the clonal expansion of antigen-specific B cells is accompanied by B cell receptor (BCR) diversification through somatic hypermutation. The B cells that exit the cell cycle relocate to the light zone, where affinity selection takes place through interaction with immune complex-coated follicular dendritic cells (FDCs) and antigen-specific T follicular helper cells (TFH cells). The affinity-matured germinal centre B cells can re-enter the germinal centre cycle. Alternatively, these germinal centre B cells exit the germinal centre, either as memory B cells (part e; germinal centre-dependent memory B cells) or as long-lived plasma cells (part f) that contribute to serological memory. The strength of signals that B cells receive is likely to determine their fate; stronger signals (indicated by bold arrows) favour development into plasma cells or germinal centre B cells, whereas weaker

signals (indicated by narrow arrows) determine memory B cell differentiation. TCR, T cell receptor. Adapted from (Kurosaki et al., 2015)

4 Methods

Several approaches were followed to ensure a high-quality literature review dissertation of novelties in nano-vaccinology against infectious diseases. Three main databases - Scopus, PubMed, and Google Scholar - utilized for a first comprehensive and a second in-depth research of the topic. Nanovaccine (or nano-vaccine), infection, VLP, liposomes, LNP, inorganic nanoparticles, adjuvant, and antigen delivery system are the basic keywords of the present search. Indicative, the search "nanovaccine AND infection" results in 76 articles in Scopus and 116 in PubMed, most of whom can be found in both databases. As the theme of the present review is the prevention of infections, articles that discuss the anti-cancer vaccines or the therapeutic vaccines were excluded. Importantly to note, cancer cases that relate to pathogens, such as the prevention of cervical cancer, mainly caused by human papilloma viruses, are not excluded and they are further analyzed. Articles of interest, scientific reports or review papers, were isolated and extensively studied. Moreover, the bibliography section of each paper was checked for further information. Concerning vaccines' clinical trials, we used the platform ClinicalTrials.gov to verify the most recent information on the phase of vaccines that have not been approved in the time of writing this dissertation (April-August 2020). European Medicine Agency (EMA) and Food and Drug Administration (FDA) websites were used on information of already licensed vaccines. Specific findings of dangerous infectious diseases as well as perspectives of the worldwide vaccines and vaccination programs, derived by respectful websites such as the World Health Organization website or other formal sites (e.g. *https://pave.niaid.nih.gov*, in the case of HPV). All the above sources were studied and are included in the review to verify an in-depth and complete presentation of nano-platforms' role in prophylaxis against pathogens.

5 Nanosystems classification as vaccine platforms

5.1 Virus-Like Particles (VLPs)

Virus-like particles (VLPs) are the most common vaccine platforms in nanoscale for both prophylactic and therapeutic purposes (Smith et al., 2013). The first nanovaccine, which was authorized in 1986 for hepatitis B prophylaxis, is classified in VLP-based vaccines (Zhao, Li, et al., 2013). From that moment a new approach in vaccine development started and until today, authorized VLP vaccines against three viruses (HBV, HEV, and HPV) are globally used and many others are in clinical trials (Qian et al., 2020). According to ClinicalTrials.gov, currently (June 2020), 22 clinical trials are active, 20 of whom involve prophylactic vaccines against infectious diseases. In fact, some of them concern vaccines for viruses with no other commercialized, approved vaccine such as Chikungunya virus (phase 2 - NCT03483961, sponsored by Emergent BioSolutions), Encephalitis virus (phase 1 - NCT03776994, sponsored by SRI International and U.S. Army Medical Research Institute of Infectious Diseases) and Norovirus (phase 2 - NCT03039790, sponsored by Takeda).

Most VLPs have a size of 20-100 nm and they are consisting of pathogens' surface proteins, without the presence of genetic material (Smith et al., 2013). This is the main difference between VLPs and viruses, which leads to the advantage that there is no danger of pathogen's proliferation. In other words, VLPs combine the good immunogenicity of the viruses, based on highly organized supramolecular structures, without their pathogenicity, leading to safe and effective vaccines. In fact, they constitute the evolution of live attenuated vaccines (LAV) and classical subunit vaccines as they present many similarities with viruses and provide higher titers of antibodies than monomeric proteins (Mohsen et al., 2017).

The proteins used, should have the ability to self-assemble into functional and immunogenic nano-structures, mainly with the use of an expression system, prokaryotic or eukaryotic cell line (Qian et al., 2020). Yeast (Engerix-B®: S. cerevisiae) (EMA, 2000) bacteria (Hecolin®: E. coli) (Li et al., 2015), insect cells (Cervarix®: Trichoplusia ni) (EMA, 2019a), mammalian cells (Sci-B-Vac®: CHO) (Shouval et al., 2015) and most recently plant cells (rabies vaccine: Spinacia oleracea) (Balke & Zeltins, 2020; Yusibov et al., 2002) have been utilized for this purpose. The above is achieved via the right conformational orientation of the antigenic epitopes in the VLP surface and thus, the production of high titers of specialized neutralizing antibodies (nAbs). The structure is stabilized with many intra- or inter-molecular covalent or hydrophobic interactions. Amino acids such as cysteine and lysine have an important role

in this activity due to their physicochemical properties (Berthier et al., 2020; Z. Li et al., 2016). Moreover, equally important is the switching of hydrophobic and hydrophilic domains for the creation of stable formulations (Berthier et al., 2020). The VLP technology is mostly used for protection against viruses, as the mechanism of the viral protein capsid formation follows the same rules: a vector takes advantage of the host translational and post-translational properties to replicate and form stable virions. As in the case of virus capsids, the antigenic proteins, which are used for the production of VLPs, self-assemble into highly symmetrical and strict architectures, usually icosahedral and octahedral, with statistically preferable repeatability (Gilbert et al., 2005; Lu et al., 2020). Even in cases of complex hybrid systems that consist of more than one protein the final formations do not present high lot-to-lot deviation (T. Zhang et al., 2020), maybe because the information of the functional conformation is encrypted in the monomers' structure. On the other hand, significantly important variations may be observed if the expression system change. For example, prokaryotic cell lines do not have the ability of post-translational processing that may conclude in a difference of glycosylation and quadruple structure of the VLPs (Mohsen et al., 2017). Such differences might lead to alteration of the immunogenic response of the vaccine receiver.

Concerning the immunogenicity, VLPs have proved to activate both the innate and the adaptive response. Complement activation via the classical pathway occurs after the vaccination, concluding to the opsonization of VLPs (Gomes et al., 2017). In this way, the repetitive epitopes of VLPs, by mimicking the immunogenicity of viruses and the behavior of pathogen-associated molecular patterns (PAMPs), and with the help of natural IgM, get recognized by the Toll-like receptors and become more easily visible from the components of the cellular immune system, especially dendritic cells (DCs) (Tagliamonte et al., 2017). DCs belong to antigen presenting cells (APC) and after the uptake of the antigen, they mature and present the antigenic epitopes, such as peptides, to the specialized cells of adaptive immune systems in the lymph nodes. The presentation happens by the loading of the epitopes in major histocompatibility complexes class II (MHC II) and results in the stimulation of $CD4^+$ T-helper cells - T_H1 and T_H2. Furthermore, because of the virus-like behavior of VLPs, DCs cross-present the epitopes in MHC I, in contrast with other platforms, concluding in the activation of $CD8^+$ T-cells and a more intense immune response (Bachmann & Jennings, 2010). Additionally, it is interesting that small particulate antigens (<200 nm), such as VLPs, have the ability to enter the lymphatic system without the need of APCs (Manolova et al., 2008). This is extremely important as the cell-free antigenic VLPs can directly interact

with the follicular B cells in the secondary lymphoid organs (Bachmann & Jennings, 2010). The cross-linking interaction is much stronger than the DC one and result in a more effective activation of immune response with a much lower quantity of antigens (Hong et al., 2018). Recently, Hong and his team, found that, indeed, antigen-specific B cells are the main initiators for activation of $CD4^+$ T cells in the case of VLPs, and not the DCs, as previously was thought (Hong et al., 2018). That point, further highlights the importance of direct initiation of B cells by the highly rigid and repetitive VLP platforms, without them been phagocytosed by APCs.

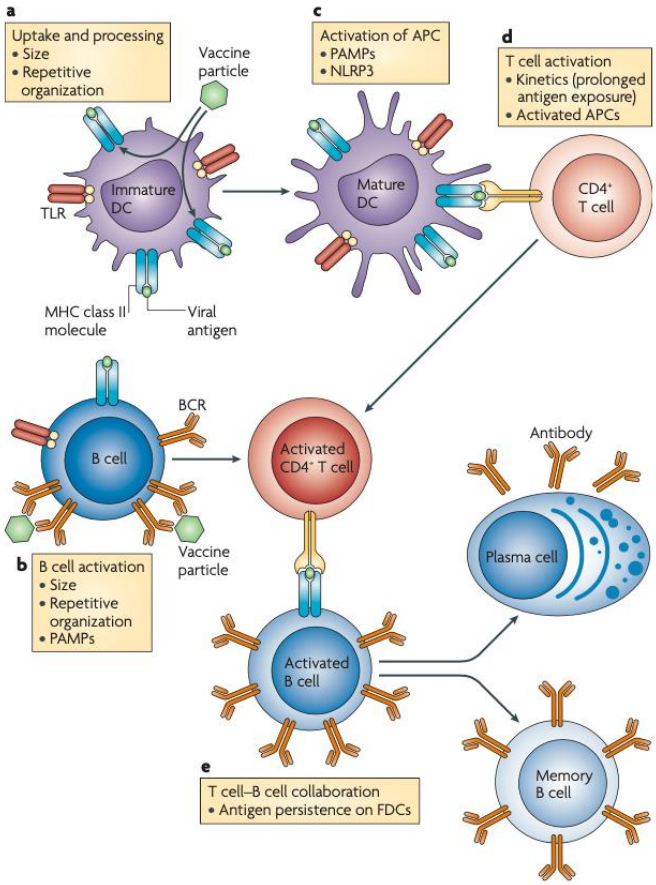


Figure 5: Key steps during the generation of protective immune responses.

a | Antigen processing is facilitated if antigens are particulate and have a repetitive surface organization, which increases phagocytosis and the ability to activate complement and recruit other molecules of the innate humoral immune system.

b | B cell activation is also facilitated by antigens that have a repetitive surface organization (through cross-linking of the B cell receptor (BCR) and activation of complement), that are 20–200 nm in size (which allows them direct access to the lymphatic system) and that contain pathogen-associated molecular patterns (PAMPs).

c | Activation of antigen-presenting cells (APCs) is facilitated by the recognition of PAMPs by Toll-like receptors (TLRs) or other pattern-recognition receptors (such as NOD-like receptor family pyrin domain-containing protein 3 (NLRP3)), or by other mechanisms.

d | T cell activation is facilitated by the prolonged presence of antigen through depot-forming adjuvants or perhaps vaccination regimens.

e | T cell–B cell collaboration is essential for the generation of antibody-producing plasma cells and memory B cells but not much is known about the factors that influence this interaction. It is likely that factors that increase persistence of antigen on follicular dendritic cells (FDCs) would be beneficial. Adopted from (Bachmann & Jennings, 2010).

Although we are familiar with the VLP technology and billions of people have been vaccinated by these supramolecular formulations, they remain in the center of the modern vaccine's technology with innovative inventions. Hence, numerous interesting revolutions are rapidly presented by the experts in that area of immunology.

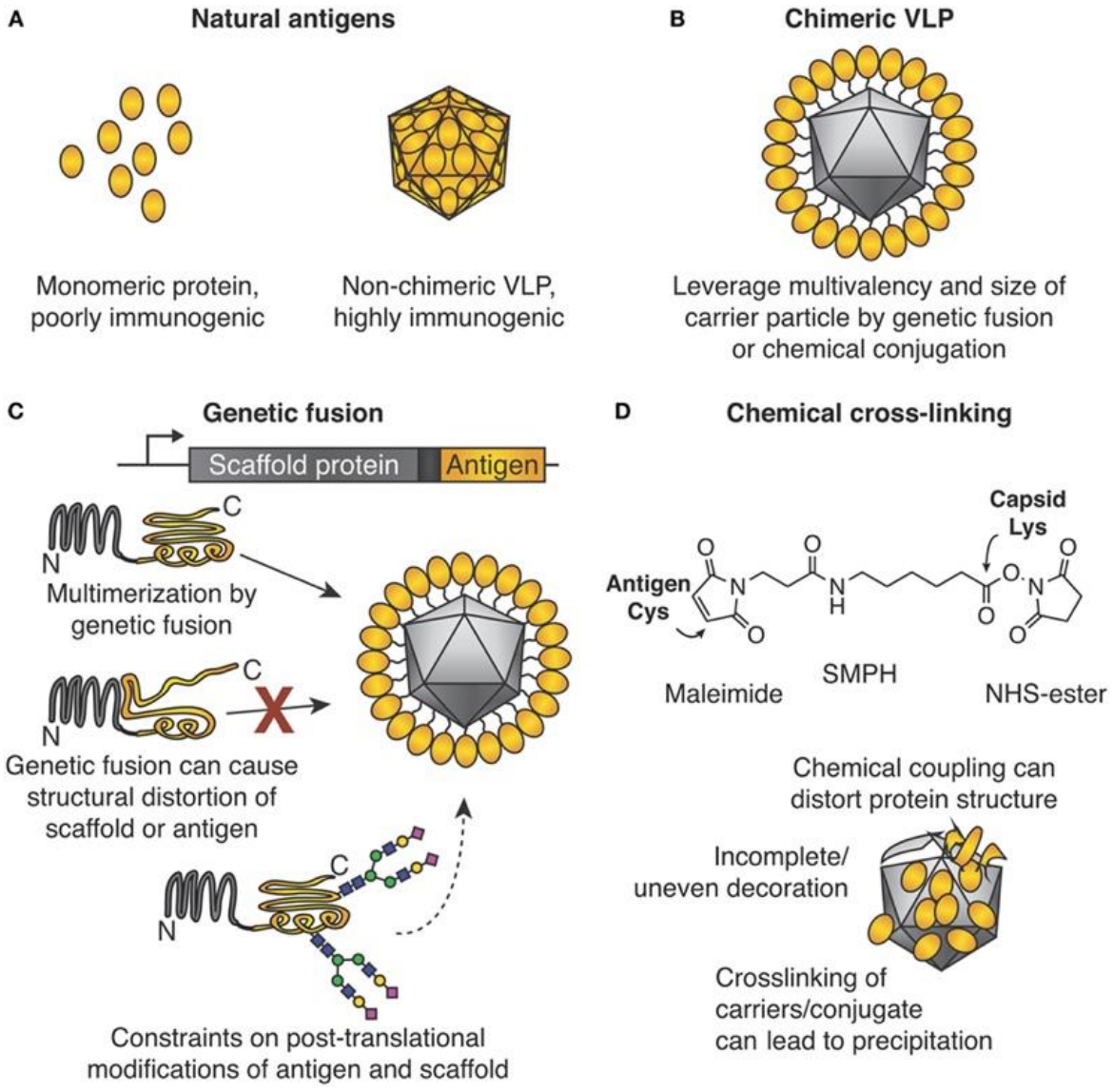


Figure 6: Chimeric virus-like particles (VLPs) and classic approaches for their decoration. (A) Importance of the particulate state for immunogenicity. (B) Chimeric VLPs leverage the multimeric nature of a scaffold for increased immunogenicity. (C) Genetic fusion for

chimeric VLP assembly. The gene encoding an antigen of interest is fused to the gene encoding the multimerizing protein. Self-assembly leads to multimerization and ordered display of the antigen. Problems encountered may be (i) structural distortion of antigen or scaffold, which may lead to failed VLP assembly or induction of ineffective antibodies, or (ii) post-translational modification by the host may not be ideal for both antigen and multimerization platform. (D) Chemical cross-linking for chimeric VLP assembly. Side chains on the antigen and VLP are connected by a cross-linker, e.g., SMPH [succinimidyl 6-(β-maleimidopropionamido)hexanoate]. Problems encountered can be (i) distortion of structure of antigen or scaffold from uncontrolled conjugation, (ii) uneven decoration of VLP with antigen, and (iii) inter-particle cross-linking and subsequent impaired solubility. Adopted from (Brune & Howarth, 2018).

An interesting idea was presented by Garg et al. They achieved to synthesize a multivalent VLP-based prophylactic vaccine against four arthropod-borne viruses – Chikungunya, Japanese encephalitis, Yellow fever and Zika virus. The VLPs are secreted by 293T stable cell lines and generate a high amount of nAbs for all viruses in mice experiments. Such an approach is preferably for both manufactures and populations sensitive to these viruses, as it is a more economic technology than the production of LAV and can protect against four viruses, minimizing the vaccine administrations (Garg et al., 2020). Another appealing procedure is the formation of chimeric VLPs. Chimeric VLPs can be produced either by genetic fusion or by chemical conjugation (Brune & Howarth, 2018). SpyCather-SpyTag methodology is an innovative decoration of VLPs via the spontaneous isopeptide bond formation, as presented in **Figure 7**.

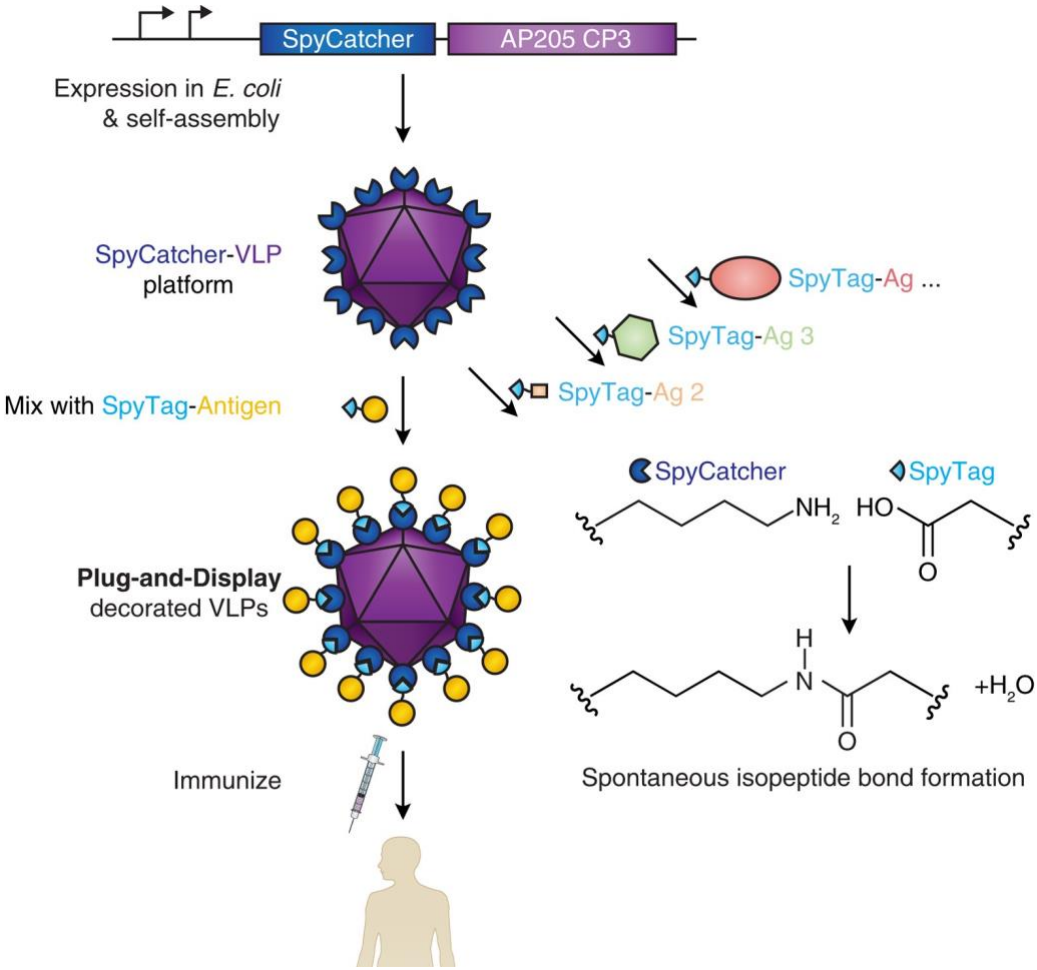


Figure 7: Overview of Plug-and-Display VLP assembly. SpyCatcher is genetically fused to the AP205 phage coat protein (AP205 CP3) and expressed in E. coli. Self-assembly of monomers generates SpyCatcher-VLPs. Upon mixing, SpyTag-antigen forms a spontaneous isopeptide bond with SpyCatcher-VLPs, yielding decorated particles for immunization. Adopted from (Brune et al., 2016).

Recently, a vaccine of this type was synthesized, utilizing as a VLP platform the core-capsid protein of AP205 phage. The platform contained antigens of both *P. falciparum* (VAR2CSA epitope) and HPV (L2 RG1 epitope) for protection against both malaria and HPV infection and its' in-vitro results were encouraging for the production of combinational vaccines via the use of a single VLP-scaffold (Janitzek et al., 2019).

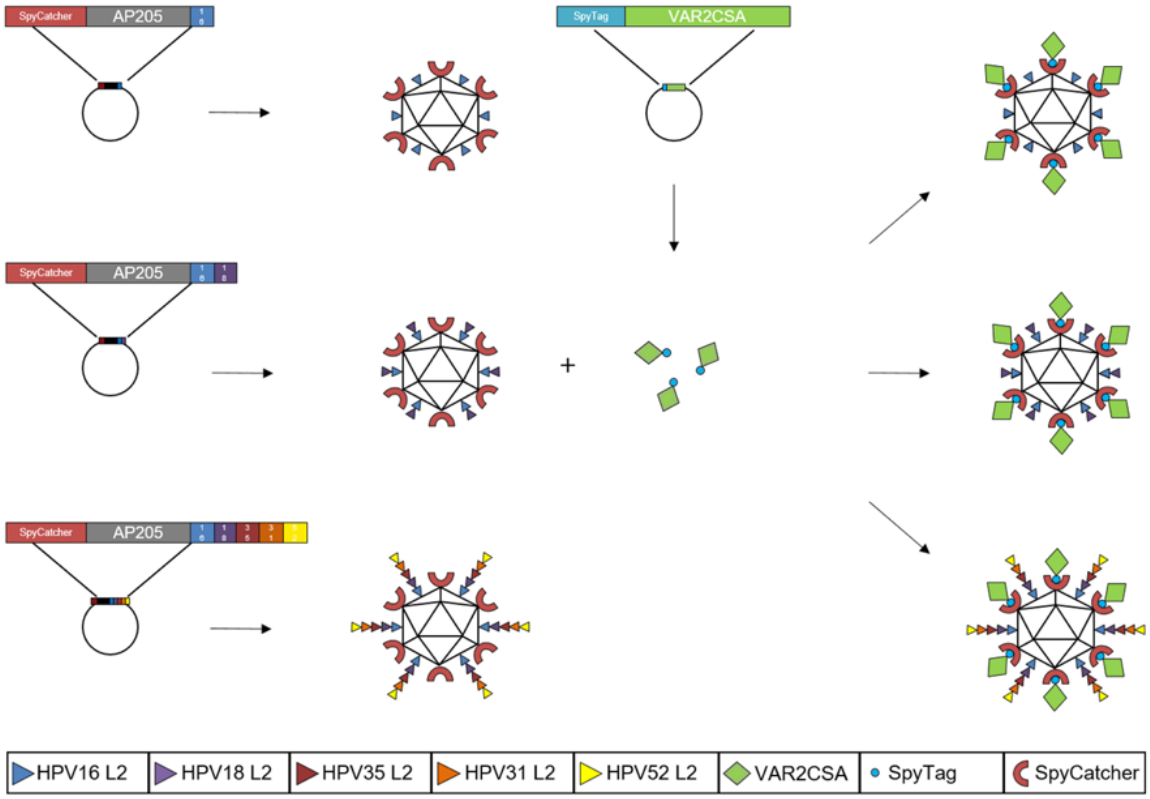


Figure 8: Schematic representation of the method used to create the combinatorial HPV and PM VLP vaccines. Three combinatorial HPV and PM VLP vaccines were created. Specifically, the AP205 capsid protein was genetically fused to SpyCatcher at the N-terminus whereas the C-terminus was genetically fused to either one (HPV16), two (HPV16 and 18) or five (HPV16, 18, 35, 31, 52) concatenated peptides derived from the highly conserved, cross-reactive epitope of the HPV L2 minor capsid protein (amino acid 17–38). Recombinant expression in E. coli resulted in formation of three distinct VLPs each displaying 180 L2 polypeptides and SpyCatcher proteins. Subsequent mixing of the PM antigen, VAR2CSA (genetically fused to SpyTag at the N-terminus) with VLPs resulted in covalent attachment of VAR2CSA to the surface of the VLPs. Adopted from (Janitzek et al., 2019).

5.2 Liposomes – Virosomes

The word liposome comes from two Greek words: ‘lipos’ (λίπος) meaning fat and ‘soma’ (σώμα) meaning body. The word liposome thus describes a system (body) that consists of lipid molecules, specifically phospholipids. Liposomes are lipid bilayers and can be uni- or multi-lamellar. Except phospholipids, they may also contain cholesterol, other lipids, and

polymers (Demetzos, 2016). They were discovered by Bangham in 1964 (Bangham & Horne, 1964) and, in 1974 they were first mentioned as possible adjuvants in vaccine formulations by Allison and Georgiadis (Allison & Georgiadis, 1974). In 1995 the first liposomal product was approved by the FDA with the trade name Doxil® for the treatment of patients with Kaposi's sarcoma (Barenholz, 2012). Nowadays, many other liposome-based formulations have been approved. Among them, there are two vaccines, Inflexal® and Epaxal®, both from Crucell Berna Biotech, Switzerland, for the prevention of Influenza virus and Hepatitis A virus, respectively (Bulbake et al., 2017).

Under the right environmental pressure, phospholipids are organized into pseudo-spherical architectures, whose properties are highly connected with the biophysical behavior of the building blocks (Demetzos, 2016). Hence, liposome size, lamellarity, surface charge, and bilayer fluidity vary depending on the physicochemical characteristics of the monomers alone, and their combination (Watson et al., 2012). The bilayer has an amphiphilic character, as the polar heads of phospholipids are oriented toward the water molecules and the hydrophobic chains are placed on the internal area of the membrane (Demetzos & Pippa, 2014). The hydrophobic interactions of the hydrocarbon chains in an aqueous medium seems to be the driving force for the liposomal structure.

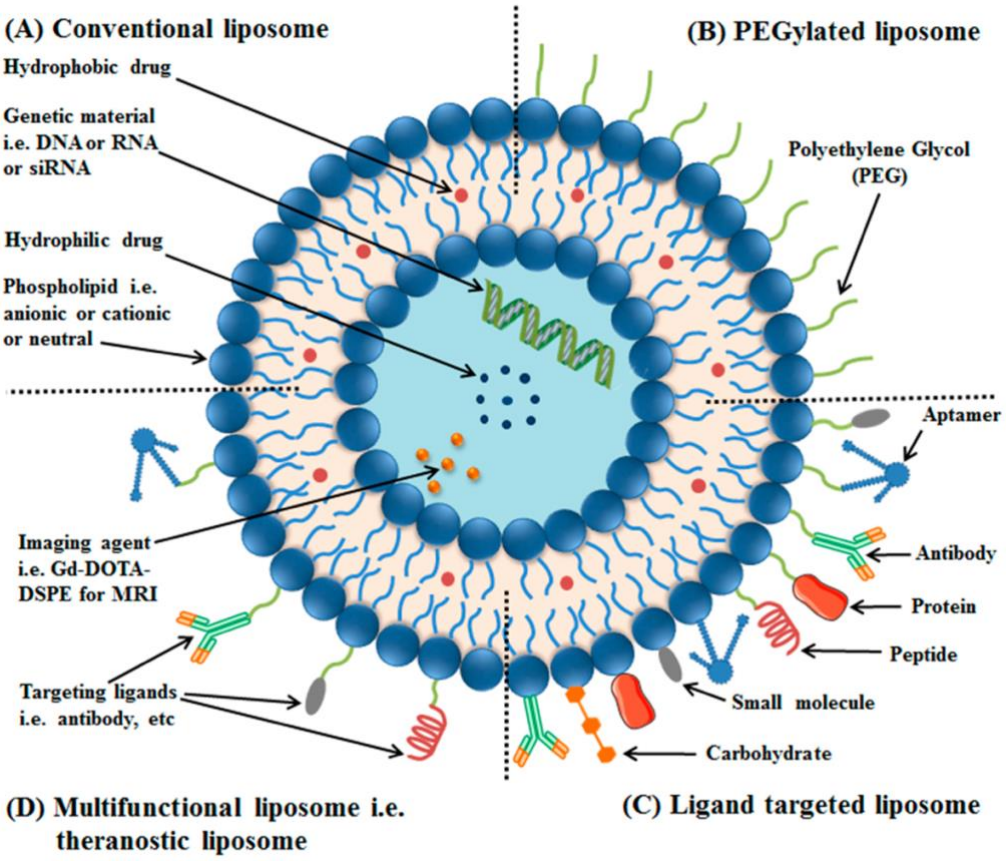


Figure 9: Conventional liposomes are made of phospholipids (A); PEGylated/stealth liposomes contain a layer of polyethylene glycol (PEG) at the surface of liposomes (B); targeted liposomes contain a specific targeting ligand to target a cancer site (C); and multifunctional such as theragnostic liposomes, which can be used for diagnosis and treatment of solid tumors (D). Adopted from (Riaz et al., 2018).

A main advantage of the liposomes is that because of their conformation, they can transfer both hydrophobic molecules (incorporated in the membrane) and hydrophilic ones (encapsulated in the aqueous core) (Metselaar & Storm, 2005). Furthermore, as phospholipids are the basic component of the cell membrane, liposomes have biomimicking properties and are well tolerated and low/non-toxic platforms (G. Yang et al., 2019). They are biodegradable and usually, they do not bio-accumulate after administration. As they are the most studied nano-systems for clinical use, they are safer than other vesicles and their major issues and problems are already known hence, they can be more easily studied. Finally, they can transfer more than one antigen and with the right surface functionalization, they can slowly release their cargo, thus utilized as systems for enhanced, controlled-release (Riaz et al., 2018). It is also, notable that some drawbacks of liposomal formulations as drug delivery systems (DDS) can approve to be beneficial for their use as vaccine colloidal dispersions. For example, we know that when in-vivo administrated, liposomes get quickly recognized by complement molecules and the mononuclear phagocytose system (MPS), leading to fast recognition from cells of the immune system (Riaz et al., 2018). Hence, several techniques have been proposed for the development of stealth liposomal DDS, the most famous of which is the PEGylation (Bulbake et al., 2017). In contrast, this property is desirable when we are thinking of a vaccine. As a result, all the above are beneficial for the development of innovative vaccine platforms.

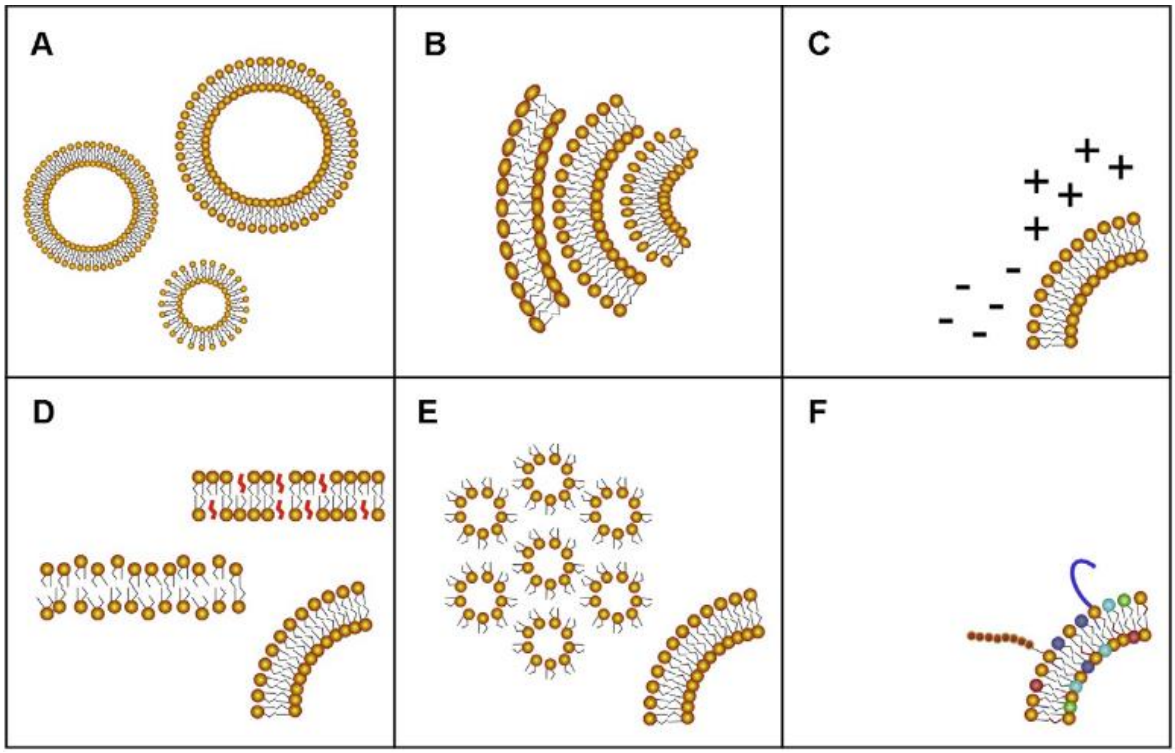


Figure 10: Liposome composition parameters that influence immune responses. Biophysical formulation parameters that influence adjuvanticity of liposomal vaccines include (A) vesicle size, (B) lamellarity, (C) membrane surface charge, (D) bilayer fluidity (as examples, cholesterol-rich liquid ordered and cholesterol-free liquid crystal phases are shown), (E) propensity to undergo lamellar-hexagonal bilayer phase transition, and (F) presence of immunostimulatory lipids. Adopted from (Watson et al., 2012).

Regarding the immunological concepts, liposomes proved to have enhanced immunomodulatory properties and activate both $CD4^+$ (MHC class II) and $CD8^+$ (MHC class I) T-cell pathways as well as, B-cell responses (Bulbake et al., 2017). By tailoring their morphology and physicochemical characteristics, different innate and adaptive immune responses may be possible. Indeed, sometimes their signals to the immune system are so strong that liposomal formulations are used only as adjuvants and not as antigen carriers as well (the topic of adjuvant NPs is described below in more detail). Several studies showed that when antigenic proteins or peptides are conjugated to the lipid membrane, the activation of defensive mechanisms is more intensive than when they are encapsulated in the aqueous area (Blom et al., 2017; Serre et al., 1998). According to vaccine glossary, liposomes with conjugated antigenic epitopes on their surface are characterized as virosomes. More specifically, virosomes are produced with the appropriate process (e.g. ultracentrifuge) of

the virus we are interested in, isolation of the membrane components and mix with other pharmacologically inactive molecules, e.g. lecithin or phospholipids (Mischler & Metcalfe, 2002). In this way, virosomes have better bio-mimicking properties of pathogens' infection mechanism. Another important characteristic is the surface charge. Cationic liposomes interact more easily with plasma proteins, e.g. albumin, leading to opsonization, and stimulation of APCs, e.g. dendritic cells and macrophages, due to electrostatic forces with the anionic plasma membrane. Moreover, cationic liposomes have the ability to disrupt the endosomal and/or phagosomal membrane because of its' durability to proton influx (Henriksen-Lacey et al., 2011; T. Li et al., 2018). Size, also play a key role as larger liposomes (≥400 nm) induce T_H1 type immune response while the smaller ones (100 nm) induce T_H2 type response (Badiee et al., 2012). These may result in a significant difference in the immune response as T_H1 are involved mostly with the cell-mediated immunity and phagocyte response while T_H2 is connected with humoral immunity (Romagnani, 1999). In conclusion, membrane fluidity affects the immunogenicity of the liposomes. More rigid liposomes, contained of saturated lipids and low concertation of cholesterol have proved more immunogenic than liposomes with lower transition temperatures and higher cholesterol ratio (Kaur et al., 2014; Watson et al., 2012).

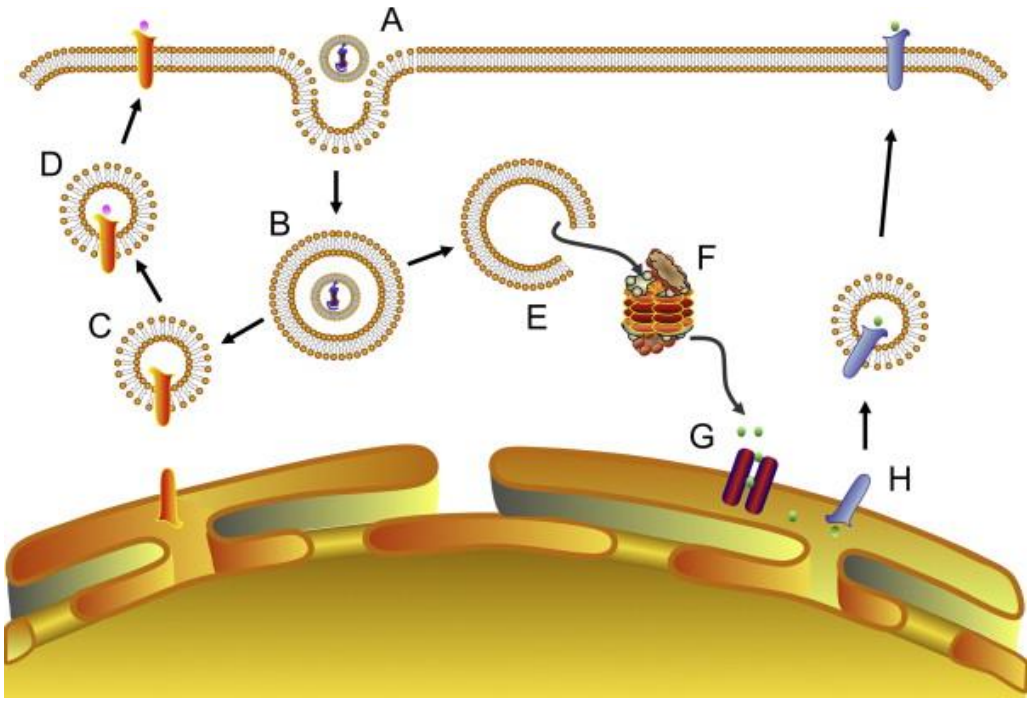


Figure 11: Intracellular processing of liposomal antigens. Intracellular antigen processing events influenced by liposome properties include (A) cell binding, (B) internalization, (C)

fusion with MHC II-containing organelles, (D) loading onto MHC II molecules followed by trafficking to the cell surface for antigen presentation, (E) escape from endosomes to the cytosol, (F) proteasomal degradation, (G) transit to the ER, and (H) loading onto MHC I molecules followed by trafficking to the cell surface for antigen presentation. Adopted from (Watson et al., 2012).

5.3 Polymeric NPs

Several polymeric NPs have been used in nanovaccinology either to entrap/conjugate the antigens or to act as adjuvants. Polymeric materials can well cooperate with many other biomaterials such as liposomes or inorganic NPs to create sophisticated nanostructures with the ability to "smart-response" when in vivo administrated.

One of the most well-studied polymeric biomaterial, utilized in vaccines is poly(lactide-co-glucolic acid) (PLGA). PLGA is a biodegradable and biocompatible material that has been approved as a vesicle from both FDA and EMA for human use. Its excellent safety profile as well as the ease of surface modification and size distribution allow the formation of unique systems with different properties (Allahyari & Mohit, 2016). PLGA formations have been studied for their ability of prolonged release of their cargo, a beneficial property for the enhanced activation of the immune system. Dhakal et al. have extensively researched the use of PLGA for the formation of effective vaccines (Binjawadagi et al., 2014; Dhakal et al., 2017; Hiremath et al., 2016). They have created innovative platforms against respiratory syndrome virus, H1N1 and H1N2 influenza virus. The results after intranasal administration in pigs show that both cytotoxic T-cells and T-helper cells can induce immunity and the memory mechanisms against the above pathogens. This is an interesting outcome as intranasal route may be desirable in some cases as they could be stored at room temperature and there is no need for refrigerators. Another effort for the development of a mucosal vaccine was presented by Tallabaka et al. They developed a PLGA based immunostimulant covalently conjugated with a C5a receptor agonist, EP67. The modified PLGA NPs present an enhanced T-cell long-lasting mediated protection in mice population (Tallapaka et al., 2019). Finally, some studies have evaluated the value of PLGA NPs for the dual role to deliver both the antigen and the adjuvant. A PLGA nanoparticulate formulation was synthesized to encapsulate *Anjelica sinensis* polysaccharide (ASP) as an adjuvant and Ovalbumin (OVA) as a model antigen. BALB/c mice vaccinated subcutaneously with those

systems, presented improved lymphocyte proliferation and enhanced Th1 and Th2 response, resulting in promising cellular and humoral immunity (Gu et al., 2019).

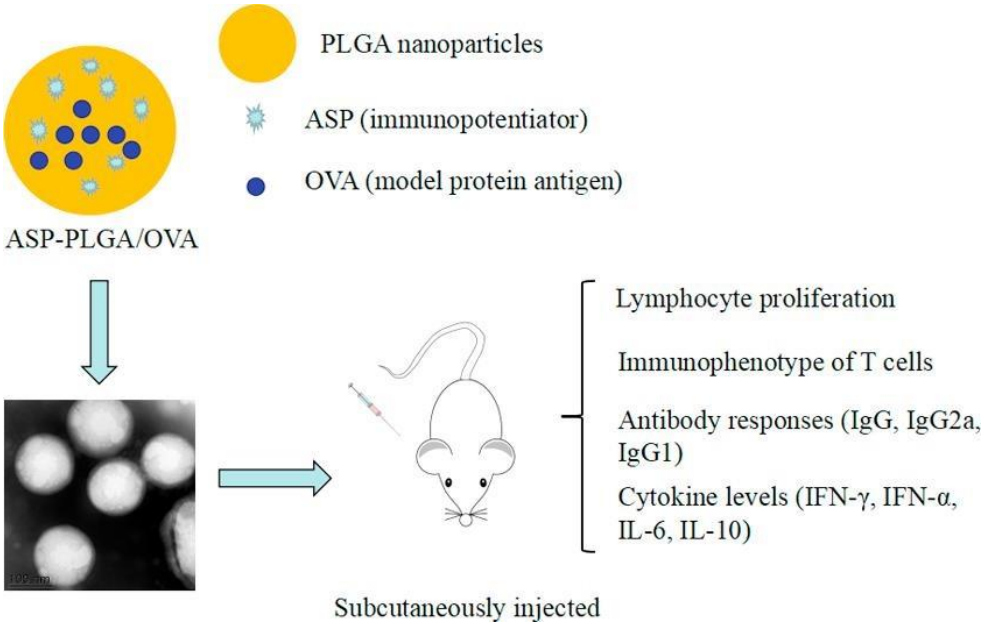


Figure 12: Graphical abstract of Gu et al. experiment. Adopted from (Gu et al., 2019).

Apart from synthetic linear or grafted polymeric formulations, another group of polymeric nanoparticles has gained attention in the last years. Self-assembled protein (or peptide) NPs (SAPNs) are excellent vaccine platforms due to their biocompatibility and their special morphological characteristics. SAPNs architecture was inspired by the viral capsids and the VLP technology as they are demonstrated to mimic viral particles and present repetitive, highly immunogenic epitopes on their surface (Doll et al., 2015). Peptide monomers assemble into oligomers, which then form NPs, usually with an icosahedral conformation (Raman et al., 2006). The main difference with VLPs is that in the case of SAPNs, not the antigens but other peptides, which interact with the antigens, have the ability to self-assemble. Thus, SAPNs can be synthesized as scaffolds of antigenic epitopes that cannot self-organized in particulate systems alone. Hence, SAPN development is a rational bottom-up technique that takes advantage of our knowledge in structural biology and biophysics science to create sophisticated engineered proteins, capable to self-assemble via hydrophobic interactions (Karch & Burkhard, 2016). The protein monomers usually contain two coiled coin motifs connected with an intermediate short linker. The antigenic part can then be added either in the N- or C-terminal of the monomer. At this time, the monomers

organized into dimers, trimers, or pentamers and spontaneously form more complex morphologies, e.g. icosahedrons (Raman et al., 2006).

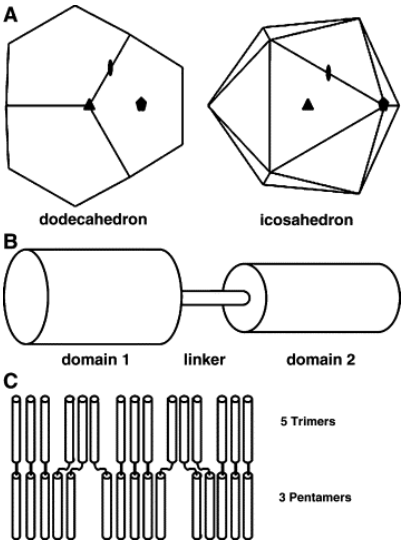


Figure 13: Basic concepts of the design. A) Possible regular polyhedra built from two-, three-, and five-fold symmetry elements. The symmetry elements are denoted as black symbols. The dodecahedron and the icosahedron have the same internal symmetry elements and are built from 60 identical 3-dimensional building blocks (asymmetric units). B) Architecture of the monomeric building block for self-assembly into regular polyhedra. The building block is composed of an oligomerization domain 1, a linker domain, and a second oligomerization domain. C) Even units consisting of trimers and pentamers. The number of monomers (building blocks) is defined by the least common multiple (LCM) of the oligomerization states of the two domains 1 and 2 of the building blocks. Adopted from (Raman et al., 2006).

Many antigens have been incorporated with SAPNs such as HIV-1 (Karch et al., 2019; Wahome et al., 2012), *Plasmodium falciparum* (Burkhard & Lanar, 2015; Kaba et al., 2018, 2012; Seth et al., 2017) and bronchitis virus (J. Li et al., 2018) antigenic epitopes. Some experts support that the nature of these systems give them the privilege of highly antigenic systems that do not need an extra adjuvant. Thus, simplest systems with more predictable behavior could be produced. On the other hand, the history of VLP structural formations do not confirm such hypothesis. Already licensed VLP vaccines provide higher titers of antibodies when combined with adjuvants, despite the repetitive presentation of the antigenic epitopes, as mentioned above. Based on these data, scientists have already started to study the incorporation of their SAPNs with the right adjuvant systems to provide a safe and effective stimulation of the immune system. One of these approaches, supported by the US military research programs, supports that the presentation of HIV-1 V1V2 loop on the surface of SAPNs can be increasingly outraged by the addition of extensively studied liposomal adjuvant conformations (Karch et al., 2019).

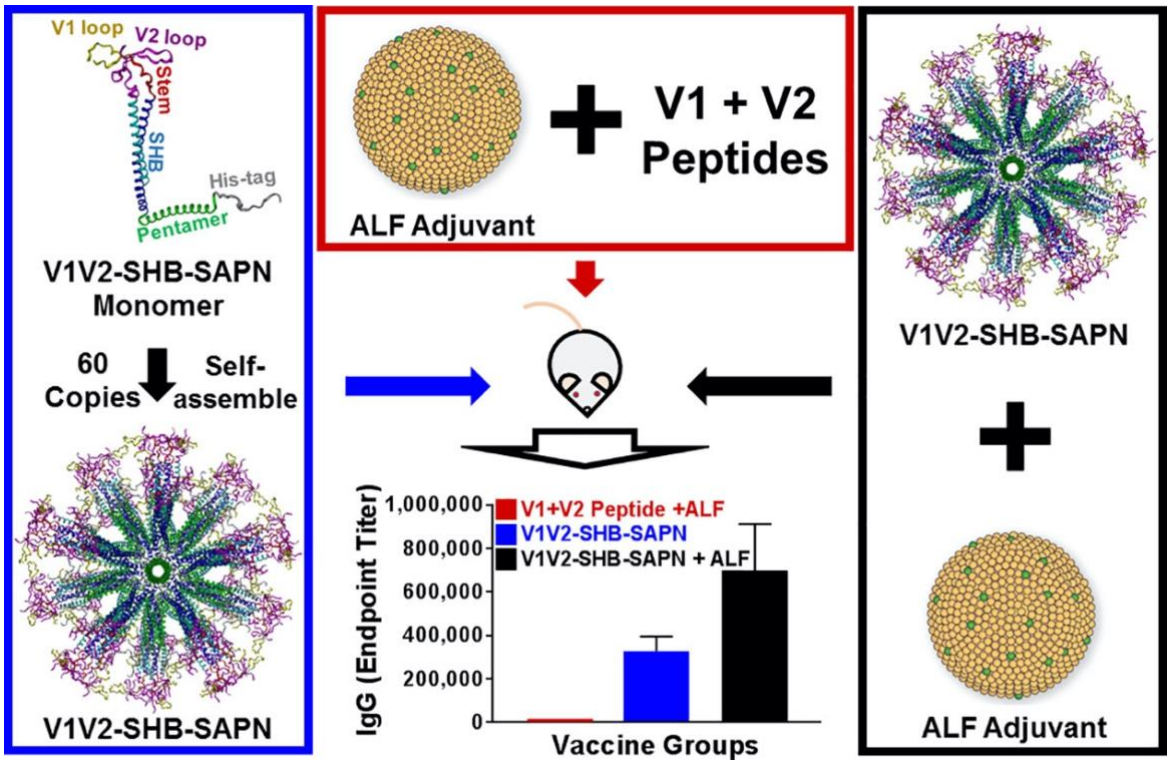


Figure 14: Self-Assembling Protein Nanoparticle monomers with the V1V2 loop of HIV-1 Env protein self-assemble into a three-dimensional nanoparticle (V1V2-SHB-SAPN) that displays 60 copies of the V1V2 loop forming 20 trimers in a native-like conformation (blue box). Mice were vaccinated with the V1V2-SHB-SAPN (blue box), V1V2-SHB-SAPN adjuvanted with Army Liposome Formulation (ALF) (black box), or V1 + V2 peptides adjuvanted with ALF (red box). V1V2-SHB-SAPN vaccines induced significantly higher IgG titers than mice vaccinated with V1 and V2 peptides. Adopted from (Karch et al., 2019).

5.4 Inorganic Nanoparticles

Inorganic nanoparticles have not only been studied as drug delivery systems but also as vaccine platforms. Gold, calcium, and silica NPs are the most famous ambassadors of this category. Until today no inorganic nanoparticulate-based vaccine has been approved neither for therapeutic nor for prophylactic reasons, although experiments show beneficial results for their use as antigen platforms or adjuvants. Their “clean” and stable morphology as well as their capability of high antigen payloads are some of their basic advantages. On the other hand, their major drawback is their toxicity issues. As they are non-biodegradable materials, they may bio-accumulate in target organs and trigger unwanted immune responses and inflammatory cascade. Thus, enhanced toxicity studies remain extremely important for the understanding of their excretion mechanisms after in-vivo administration.

5.4.1 Gold Nanoparticles

Gold NPs (GNPs) size range between 2-100 nm and can be synthesized in various shapes as spheres (Gregory et al., 2012), rods (Tazaki et al., 2018), cubes (Niikura et al., 2013), nanocages, stars, prisms (R. Kumar et al., 2015) and nanoclusters (H. Wang et al., 2016). All these different morphologies have been utilized for the preparation and evaluation of many prophylactic vaccines against viruses, bacteria, and parasites. The physicochemical characteristics of GNPs allow the easy conjugation with both the antigen and adjuvant via simple and sometimes, almost one step procedures (Tao et al., 2017). Quach et al. associated the immunostimulation of GNPs with their size and concentration, concluding that larger chimeric particles (80 nm) showed a better efficacious and toxicological profile for vaccination against dengue virus than the smaller ones (20 and 40 nm), after subcutaneous administration in BALB/C mice. Both $CD4^+$ and $CD8^+$ T cell activation induced, as well as, promising nAbs titers were produced (Quach et al., 2018)

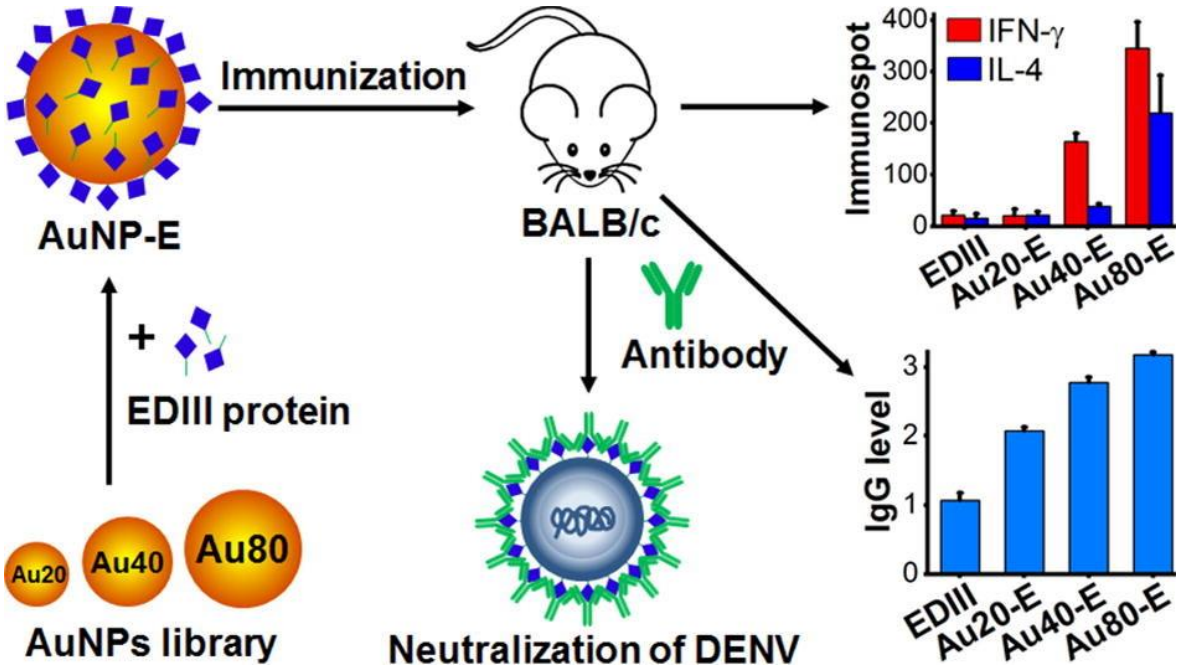


Figure 15: Dengue subunit vaccine (AuNP-E) formed from gold nanoparticles (AuNPs) and domain III of envelop glycoprotein (EDIII) elicit T-cell response, characterized by the number of IFN-γ and IL-4 producing splenocytes, and the generation of antibody that specifically binds to EDIII and neutralizes dengue virus (DENV) in a manner dependent on AuNP's size and concentration. Adopted from (Quach et al., 2018).

Moreover, another interesting example is the synthesis of an AuNP-M2e-sCPG formulation as a universal vaccine against Influenza A serotypes (Tao et al., 2014). As M2e is a highly conserved N-terminal extracellular portion of M2- ion channel protein, the use of such a vaccine could provide protection on a variety of Influenza A viruses. Furthermore, the addition of CPG adjuvant could additionally enhance the immunogenicity of the vaccine. Indeed, this formulation has proved to be effective after intranasal administration to mice, by inducing high titers of IgG antibodies and memory B-cells, even in elderly mice (Bimler et al., 2019).

5.4.2 Calcium Phosphate Nanoparticles

Calcium phosphate (CaP) is also a promising adjuvant. Many applications of calcium phosphate ceramics have shown good behavior as components of bone implants and other regenerative medicinal applications (Buschmann, 2016). Its' major advantage, in comparison with the other inorganic NPs, is that it is a natural component of human body (Dorozhkin, 2012) and thus, it is well tolerated, bio-degradable and non-toxic in logical concentrations (Lin et al., 2017). However, nanoparticulated CaP may have different toxicological and physicochemical characteristics due to the special properties of NPs, such as high surface-to-volume ratio. Hence, additional safety studies are necessary for the approval of CaP NPs in clinical practice (Demetzos et al., 2020). Until the appropriate Medicine Agencies provide the guiding principles for the evaluation of nanoparticulated inorganic adjuvants, many pre-clinical studies present promising results. In comparison with alum salts, the most common adjuvant in vaccines, CaP NPs have better immunostimulating properties as both T_H1 and T_H2 responses are triggered. Many different types of vaccines have been developed with the use of CaP NPs as adjuvants. For example, this type of adjuvant is suitable for the adsorption of both antigenic proteins and nucleic acid (pDNA or RNA), after the essential surface functionalization. As recent examples, subunit prophylactic vaccine has been produced against the Influenza A (H5N1) virus (Morcol et al., 2019) and pDNA vaccine against Hepatitis B virus (Rojas-Sánchez et al., 2020). As a result, CaP NPs have shown to be attractive alternatives of a safe and effective adjuvant although more research should be done in this area.

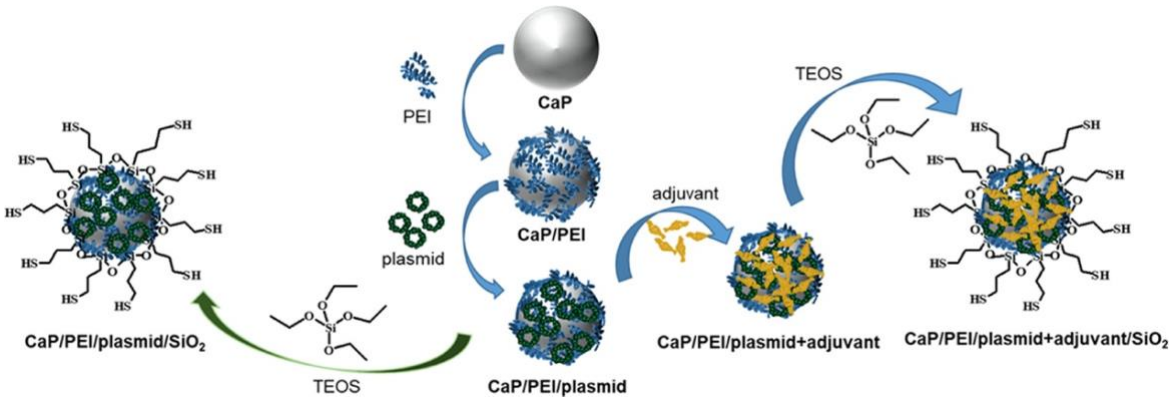


Figure 16: Schematic representation of the synthesis process for CaP/PEI/plasmid/SiO2 and CaP/PEI/plasmid + adjuvant/SiO2 nanoparticles. Adopted from (Rojas-Sánchez et al., 2020).

5.4.3 Silica Nanoparticles

Finally, silica nanoparticles (SiNPs) can be used in nanovaccinology to both carry an antigen and induce immune response due to their unique properties. SiNPs can form either core-like, non-porous spherical structures (Thalhauser et al., 2020) or mesoporous morphologies (Ferreira Soares et al., 2020). Concerning the solid SiNPs, the antigenic protein can be either adsorbed on the surface of the particle or conjugated via covalent bonds, while in mesoporous SiNPs the antigen is encapsulated into the porous and can be stabilized via electrostatic or hydrophobic forces (Huang et al., 2020). Additionally, positive charge appears to further improve the cellular uptake of the SiNPs from APCs (Amin & Boateng, 2020). As unfunctionalized SiNPs are negatively charged due to the silanol groups on their surface (Huang et al., 2020), additional positively charged moieties could be added (Amin & Boateng, 2020). A promising study from Bai et al. showed that hollow mesoporous silica nanoparticles loaded with VLPs for the prophylaxis against foot-and-mouth disease virus (FMDV) presented better immunostimulating results in comparison with the use of VLPs immunomodified with Freund's complete adjuvant. High antibody titers as well as INF-γ and proliferation of T-cells were induced (Bai et al., 2019). Moreover, Huang et al. assume that mesoporous SiNPs of 200-400 nm have the best size and pore diameter for the activation of the innate cellular immune response against *Corynebacterium diphtheria*. Diphtheria toxoid was utilized as a proof of concept material and not as an antigenic factor with special characteristics (Huang et al., 2020). Mahony et al. reported that amino functionalized mesoporous SiNPs of 90 nm diameter presented better humoral and cellular immune response against ovalbumin (OVA) in comparison with a higher quantity of OVA subunits adjuvanted with QuilA, a famous immunomodulator saponin mixture. The 3 doses of each

vaccine injected to mice via the intramuscular route and the finding showed that the functionalized mesoporous SiNP formulation except promising adjuvant and delivery platform properties did not present any morphological changes of high-risk organs and tissues (kidneys and spleen) (Mahony et al., 2013). Finally, Bavandpour et al. compared mesoporous silica NPs with mesoporous carbon NPs as prophylactic oral vaccines against *Vibrio cholerae*. The first ones presented higher anti- (cholera toxin subunit B) IgG and IgA titers than the carbon ones. Questions remain of whether silica formulations provide better protection than already approved oral vaccine (Karimi Bavandpour et al., 2020).

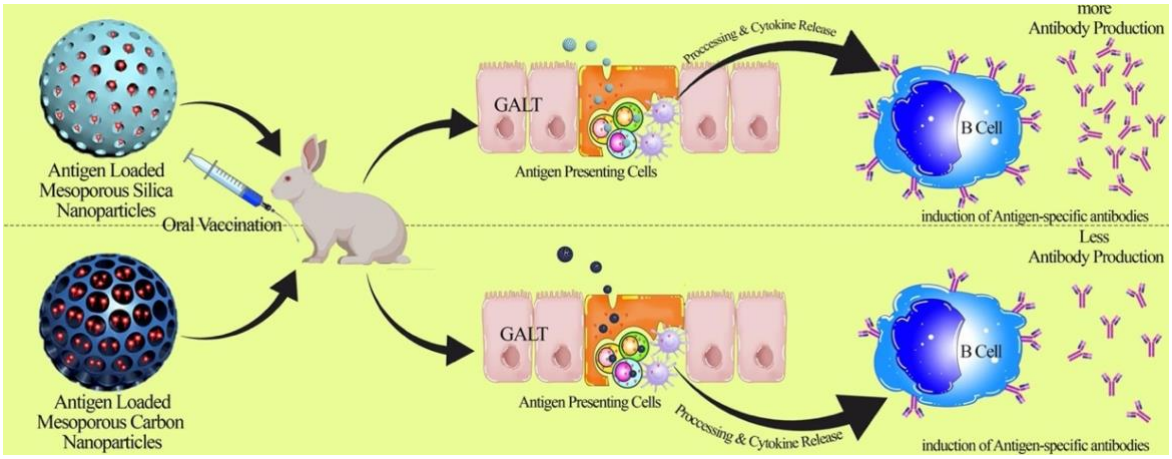


Figure 17: Antibody production after oral vaccination with CTB-MSN and CTB-MCN. Adopted from (Karimi Bavandpour et al., 2020).

5.5 Viral Vectors

The utilization of viral vectors for vaccine development has a dual role as they function both as antigen delivery systems and adjuvants. The first effort to create such a vaccine started from Moss and colleagues in 1984 for the protection of HBV infection using the vaccinia virus (Moss et al., 1984). Today, after the authorization of two viral vector-based vaccines, and the subscription of many such formulations in the phase of clinical trials, the use of these vaccines remains a taboo. Causatives for the aloofness are safety issues, as recombinant viruses, attenuated or not, promotes the immunity against another pathogen via the infection of the host cells. Many modern technologies and different viral species were tested to verify the safety and efficacy of these formulations.

Live viral vectors, either replicating (usually attenuated) or non-replicating, expose to certain genetic engineering processes so that they encode heterogenous antigens. This technique is mainly achieved via the insertion of the desirable antigenic genes and the deletion of the

possibly harmful ones. Certain pathogen associated molecular patterns (PAMPs) present in the surface of the viral platforms, result in a more effective recognition from the cells of the immune response than the use of a subunit antigen. Thus, the addition of an adjuvant is usually unnecessary, decreasing, in this way the complexity and the cost of the vaccine (Ewer et al., 2016). In many cases, the antigen delivered by those platforms is not expressed in the virus but, it is expressed after the infection of the host cell by the translational and post-translational mechanism of the second one via the virus replication cycle. The major advantage of the above is the correct protein conformation and glycosylation that ensures an effective presentation motif and a potent immune response (Rauch et al., 2018). After the absorption of the virus from the host cell and the expression of the immunogenic protein, the protein can be presented via the MHC I and activate the $CD8^+$ T-cell pathway (Ewer et al., 2016).

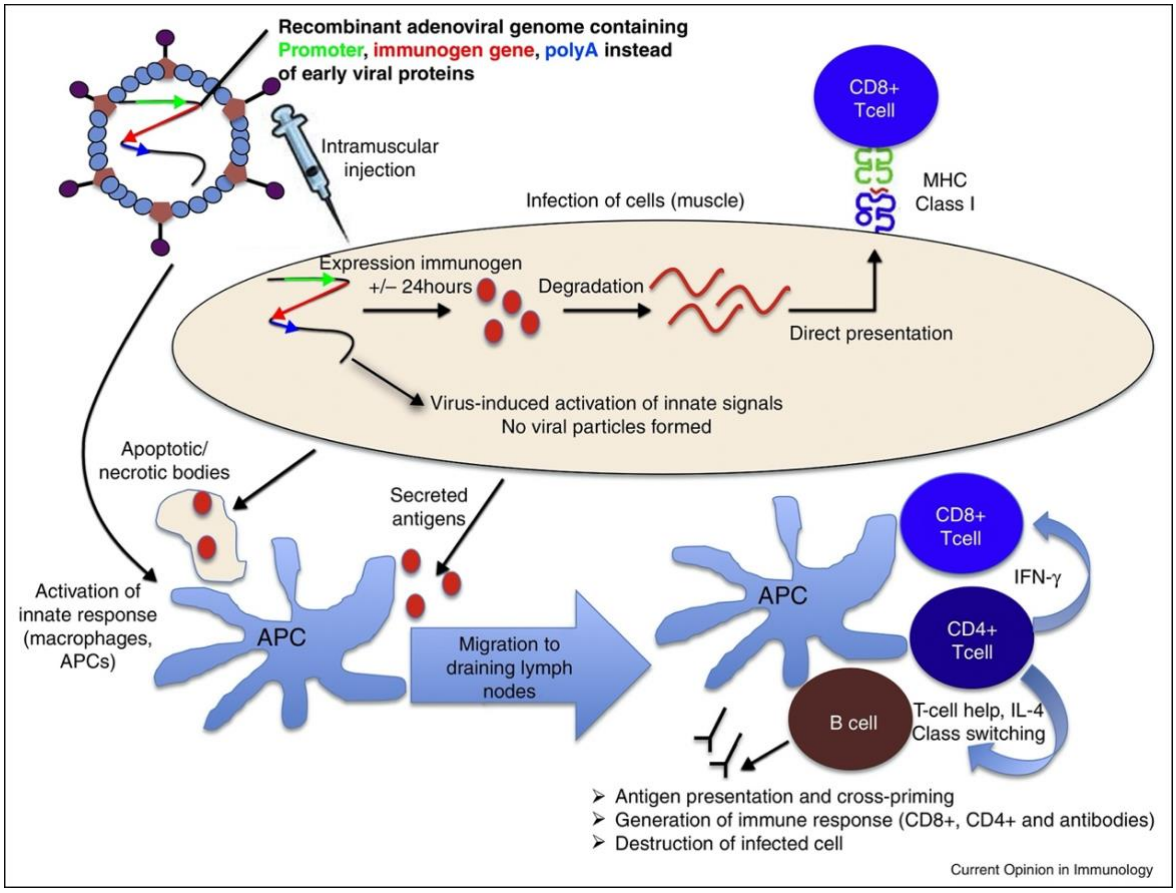


Figure 18: Mechanism of induction of transgene-specific cellular and antibody responses by replication-defective viral vector vaccines. Administration of a recombinant adenovirus vaccine by intramuscular injection results in infection of muscle cells (non-productive in the case of replication-defective viral vectors) followed by expression of the transgene within

24 hours, together with triggering of innate immune responses via interactions between viral nucleic acids and pathogen recognition receptors. Expressed proteins undergo proteasomal degradation and presentation to CD8+T cells in association with MHC class I molecules or may be secreted and taken up by professional antigen presenting cells (APC). APC may also acquire vaccine antigens as apoptotic or necrotic bodies or may be directly activated by interaction with the viral vector. Antigen-loaded APC migrate to draining lymph nodes where they are able to prime CD8+, CD4+ T cells and B cells. Adopted from (Ewer et al., 2016).

Despite all these unique properties of the viral vectors, there are remain some serious concerns about their mechanism of action and subsequently, their safety. One of the most important drawbacks of viral vectors is the fact that the virus itself can induce the immune response to synthesize neutralizing antibodies against its parts. As a result, in the case of human viruses, a high human seroprevalence for certain strains concludes in quick recognition and inactivation of the virus before the promotion of the immunity against the desirable antigen. This problem was clear in the case of human adenoviruses (Ad). Ad are non-enveloped icosahedral dsDNA viruses (**Figure 19a)**, capable to produce robust immune response, mainly by MHC I antigen presentation (**Figure 19b**) (Coughlan, 2020). They are classified into two main categories, human Ad (HAd) and non-human primate Ad. Ad5 is a HAd that has extensively studied as a viral vector with encouraging results for the prevention of many pathogens due to the ease of its' genetic modification. For example, the replacement of the E1A or E1B gene with the antigenic expression cassette would lead to the formation of a replication-deficient virus that encodes the antigenic protein. Sometimes E3 and E4 gene areas can be deleted, reassuring the potent immunostimulation (Humphreys & Sebastian, 2018; Rauch et al., 2018). One the other hand, Ad5 is a common virus and large human populations already appear to be Ad5-seropositive, decreasing the efficacy and the predictability of this platform (Buchbinder et al., 2008). Hence, rarer HAd, such as Ad35 (Crank et al., 2016) and chimpanzee Ad (ChAd), like ChAd63 (malaria – Univerity of Oxford, and leishmaniasis – University of York, prophylactic vaccines) (Osman et al., 2017; Tiono et al., 2018) and ChAdOx1 (many active clinical trials i.e. against SARS-CoV-2, NCT04444674) have been evaluated. In some cases, different viral vectors are used for the first and the boost-dose to reassure the activation of the immune response (Crank et al., 2016).

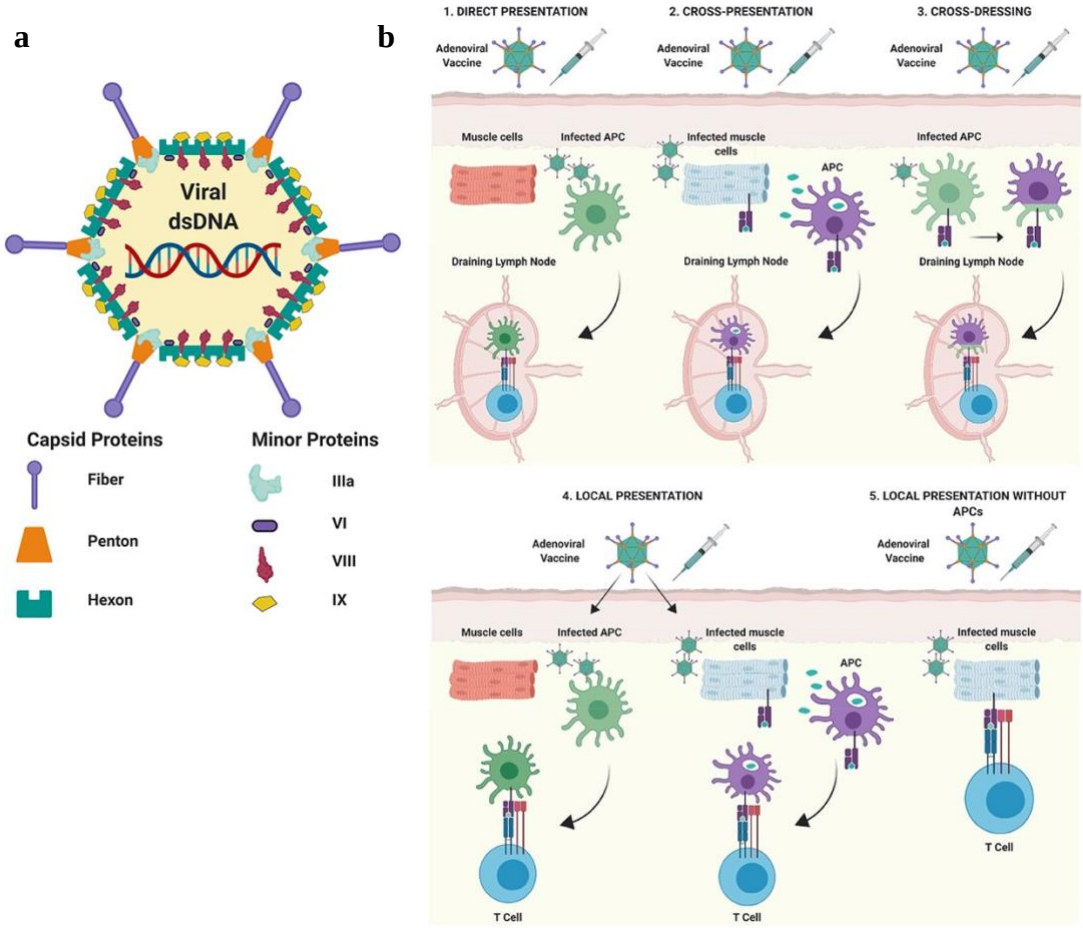


Figure 19: a) Schematic adenovirus structure. b) Mechanisms of antigen presentation after intramuscular immunization with adenoviral vectored vaccines. (1) Direct-presentation: Adenoviral vaccine transduces APCs at the site of injection. APCs migrate to draining lymph nodes (dLNs) where they present processed vaccine antigen to T cells. (2) Cross-presentation: Vaccine antigen debris from Ad vaccine transduced cells is phagocytosed by professional APCs at the site of injection, transferred to dLNs by APCs and presented to lymphocytes. (3) Cross-dressing: Peptide: MHC complexes from Ad-transduced APCs may be transferred to naïve APCs by a process of membrane gnawing called trogocytosis. (4) APCs present at, or infiltrating into the site of injection, can present antigen directly to T lymphocytes. (5) Non-professional APCs such as parenchymal cells at the site of injection (muscle cells shown as an example) can present antigenic peptide on MHC I to infiltrating CD8+ T lymphocytes, outside of secondary lymphoid organs. Adopted from (Coughlan, 2020).

The history of viral evolution has shown that their behavior is sometimes unpredictable and certain mutations may lead to a significant alteration of their immunogenicity. Hence, even if the studied vectors have a safe profile, mutations may be evoked after the genetic

modifications. As they are new candidates for antigen delivery, the Medicine Agencies, should be extremely careful with the authorization of those systems. Certain EU regulations are active at the moment for the evaluation of viral vehicles to reassure the safety of both the vaccinees and the environment (Baldo et al., 2013). The recent example of an authorized by FDA and EMA vaccine against dengue disease shall remind us that these innovative platforms have not been studied for a long period and thus, extensive trials are necessary. Dengvaxia® (Sanofi Pasteur) contains a yellow fever live attenuated viral vector that is genetically modified to contain proteins from the dengue virus. Four types of chimeric yellow fever dengue viruses are present in its vaccine that protects against dengue virus serotypes 1-4 (EMA, 2020a). In 2015, Dengvaxia® licensed in the Philippines for protection of 9-45 years old people against dengue. In 2017 the vaccination program enrolled by the Philippines government was terminated after suspicions that Dengvaxia® caused increased danger for aggressive infection from the dengue virus (Halstead, 2018). Indeed, post-hoc clinical trials and samples re-analysis from Sanofi verified the concerns. Dengvaxia® proved to increase the risk of severe dengue and dengue hospitalization in seronegative populations, mostly for children, and it has been related to some deaths (Sridhar et al., 2018). After these events, Dengvaxia® licensed from FDA and EMA in 2018 only for people 9-45 years of age who live in areas where the disease is epidemic and have already been infected once from the virus in the past (EMA, 2018). Although the vaccine may lead to benefits of the living standard in these areas, the negative results in the Philippines concluded in vaccine hesitance of a large percentage of the natives. Hence, more strict regulations may be needed for the evaluation of innovative vaccines, especially when they concern dangerous viruses and administrations in young populations. Unlike Dengvaxia®, Imojev® (Sanofi Pasteur), the first viral vector-based vaccine seems to have great efficacy and safety results. It is a modified yellow fever virus (YFV17D) that despite the YFV protein encodes two envelope proteins of the JE SA 14-14-2 strain. The purpose of the vaccine is the protection against infection from the Japanese encephalitis virus (JEV). JEV belongs to the Flaviviruses, can cause Japanese encephalitis, a serious CNS disease, and is epidemic in many Asia-Pacific regions (Appaiahgari & Vrati, 2010). Imojev® is currently licensed in Australia, Malaysia, Philippines, Thailand, and most recently in South Korea and Taiwan (H. S. Kim et al., 2020; Ma et al., 2020; WHO, 2013). Interestingly, live attenuated JE SA 14-14-2 vaccine is now evaluated for the ability of cross-protection against other similar mosquito-borne flaviviruses. Wang et al., support that after mice vaccination, the subjects developed cross-protection against the Zika virus via activation of the cellular mediated immune response (R.

Wang et al., 2020). Such a result could be extremely positive as many of the flaviviruses co-exist in epidemic dangerous areas.

To conclude, viral vectors are promising vaccine platforms as they can not only transfer the antigen into the host cells but also, provide that the desirable antigenic genome will be expressed and the final protein will have the right conformation. As this process is made in the cytoplasm, MHC I presentation of the antigen is promoted, resulting in the activation of $CD8^+$ T-cells and the cellular immune response. This methodology could be beneficial for flaviviruses (Zika, Japanese encephalitis, Yellow fever, and Dengue viruses)

5.6 Vaccine Adjuvants

The word “adjuvant” comes from the Latin “adjuvare”, which means “to help”. According to EMA: “A vaccine adjuvant is a component that potentiates the immune responses to an antigen and/or modulates it towards the desired immune responses” (EMA, 2005b). Live attenuated vaccines do not require adjuvant to be efficient, but subunit and particulate vaccines induce a weak immune response and as a result, an adjuvant is an essential component of the formulation. Therefore, efforts to develop highly effective and safe vaccines are of significant interest. Depending on the type of the pathogen different categories of adjuvants can be used to provide the best result (Petrovsky & Aguilar, 2004). Adjuvants alone do not have an immunogenic ability, but when co-administrated with an antigen, they can activate the innate mechanisms of the immune system and improve the efficacy of the vaccine. The activation is triggered via the recognition of adjuvant domains from the cellular pattern recognition receptors (PRR). According to the active EMA guidelines, even if an adjuvant does not present serious adverse effects, its’ use must, also, be beneficial and improve the safety and efficacy profile to be approved (EMA, 2005b). Below, we review some of the most common and promising innovative adjuvants.

5.6.1 CpG oligodeoxynucleotides

CpG oligodeoxynucleotides (CpG ODN) are short single-stranded DNA molecules that comprise CG motifs (cytosine phosphate guanidine). These motifs get recognized and bind with a certain endosomal receptor, Toll-like receptor 9 (TLR9), of antigen-presenting cells (Chatzikleanthous et al., 2020). Depending on the way they induce B-cells, CpG ODN are classified into three classes, A, B, and C with different morphological and functional

properties as presented in **Figure 20** (Bonam et al., 2017). CpG ODN were studied as vaccine adjuvants due to their ability to stimulate the T_H1 pathway, biasing cytokines and chemokines, and in this way, elicit a strong $CD8^+$ T-cell response (Chatzikleanthous et al., 2020; Vollmer & Krieg, 2009).

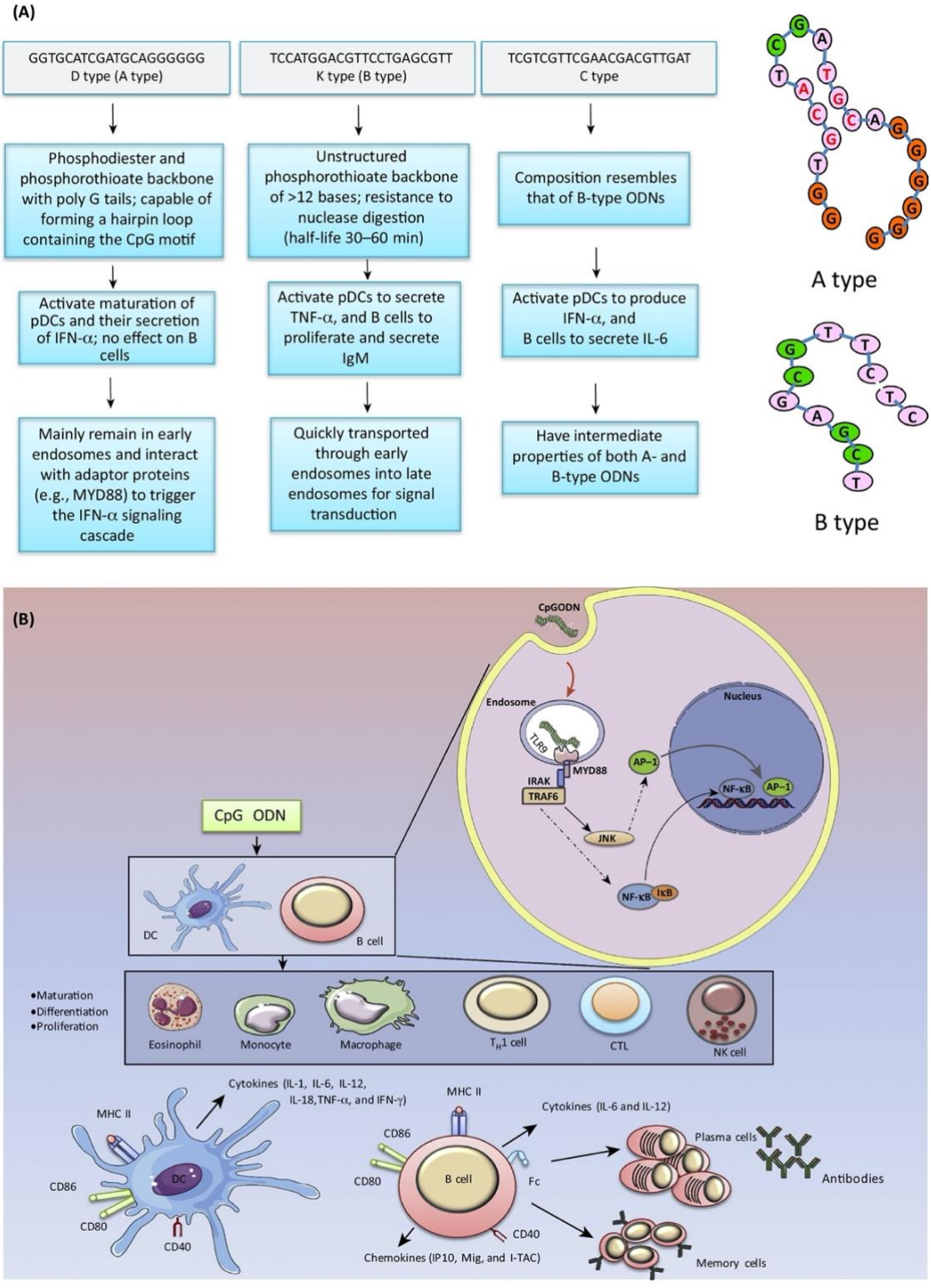


Figure 20: Structure and Mode of Action of CpG Oligodeoxynucleotides (CpG ODNs). (A) The three different types of CpG ODN (A, B, and C), their structures, and properties. (B) CpG ODNs modulate innate and adaptive immune responses in several ways. (A) The CpG ODN–TLR9 signaling pathway. TLR9 receptors are present on the endosomal membrane. After internalization, CpG ODN activates elements of the MyD88/IRAK/TRAF6 pathway, leading to the simultaneously activation of two kinase pathways (MAPK/c-JUN and NF-κB) and the AP-1 and NF-κB promoter genes. (B) CpG ODN activates directly DCs and B cells acting as APCs. Activated DCs and B cells also activate other immune cells, such as neutrophils, monocytes, macrophages, Th1-type CD4+ T cells, CTL, and NK cells, which then mature, differentiate, and proliferate. APCs construct a base by forming co-stimulatory signals and provide strong memory responses. Abbreviations: APCs, antigen-presenting cells; Fc, fragment crystallizable region; IFN, interferon; IP10, IFN-γ-inducible protein 10 (or CXCL1); I-TAC, interferon-inducible T cell alpha chemoattractant (or CXCL11); JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MHC-II, class II major histocompatibility complex; Mig, monokine-induced by IFN-γ (or CXCL9); pDC, plasmacytoid dendritic cells. Adopted from (Bonam et al., 2017).

Although these molecules seem to provide effective results, concerting the activation of the immune system, concern remains about their safety. Lymphadenopathy and enlargement of lymph nodes, trigger of autoimmunity and systemic inflammation were observed in human subjects/ participants in clinical trials. The most serious adverse effects manifested for immunosuppressed participants, e.g. HIV infected and populations with cancers (Scheiermann & Klinman, 2014). Innovative formulations for eliminating these adverse effects have been presented in the literature. One of these was the effort to combine the promising properties of CpG ODN and increase the safety profile via its' conjugation with cationic liposomes. Interestingly, although CpG ODN are assumed to be Th1 inducers, the CpG-cationic liposome system stimulated both Th1 and Th2 pathways. The authors hypothesized that the above might be the result of the dual role of cationic liposomes as vaccine delivery systems and immunomodulators (Chatzikleanthous et al., 2020).

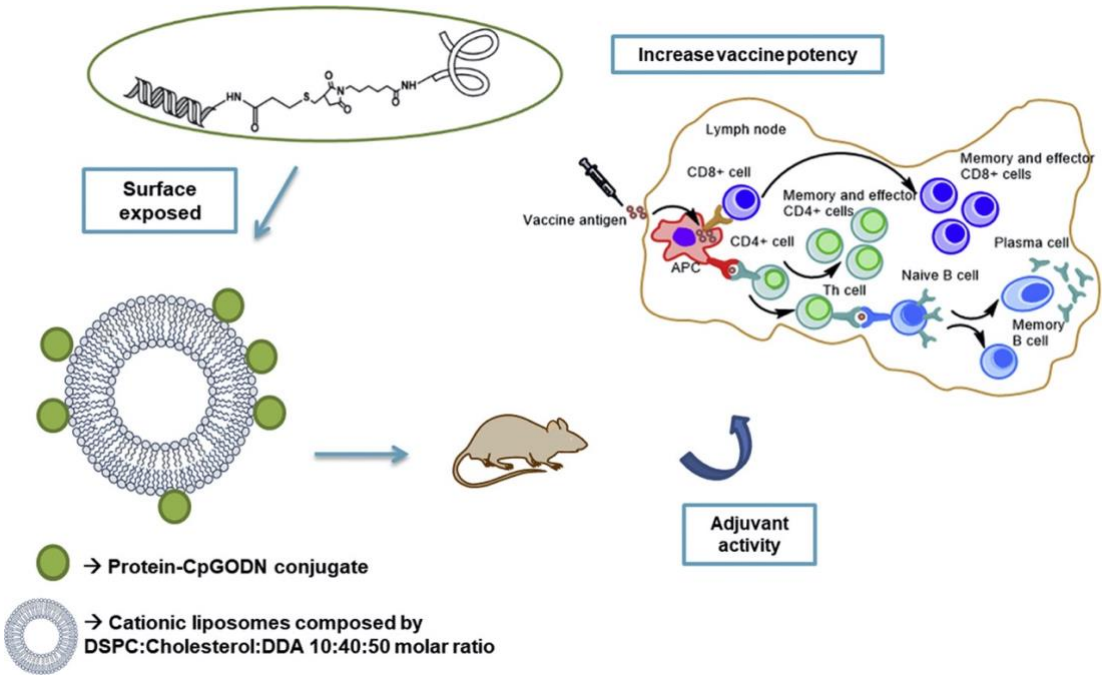


Figure 21: Graphical abstract of Chatzikleanthous and team experiment. Adopted from (Chatzikleanthous et al., 2020).

In 1999 the first human clinical trial took place for the evaluation of CpG ODN as a vaccine adjuvant and until today, many trials have been completed and some are in progress. Among them, NuThrax® (Emergent BioSolutions), a prophylactic vaccine against anthrax is in phase 2/3 (NCT03877926, NCT03518125) are active, evaluating whether the benefit of CpG-7909 addition is worthy. Moreover, the NCT00100633 trial showed that better protection of HIV patients against HBV achieved after a boost dose of CpG-7909. Such a funding is extremely interesting as studies calculate that only 50% of HIV-infected individuals induce the desirable, protective levels of anti-HBV antibodies after the completion of the scheduled vaccination program of commercial vaccine (Vollmer & Krieg, 2009). Additionally, a phase 2 trial of a CpG-10104 adjuvanted vaccine against hookworm infections (NCT03172975) and a phase 1 trial for Influenza virus types A and B protection (NCT03945825). The second trial compares the safety, reactogenicity, and immunogenicity of commercialized quadrivalent influenza vaccines, Fluzone® or Flublok®, with and without the use of adjuvants. One of the adjuvants is Advax-CpG55.2. Advax-CpG55.2 is a mixture of Advax™, a semicrystalline, delta inulin polysaccharide (~1-2 μm diameter) (Counoupas et al., 2017), and CpG55.2, which, as mentioned before, is a TLR9 agonist. The interesting part here is that both Advax™ and CpG55.2 alone have immunomodulatory properties. Finally, a clinical trial phase 1 is active by Clover Biopharmaceuticals AUS Pty Ltd for protection

against SARS-CoV-2 (NCT04405908). This vaccine is a recombinant subunit vaccine and its' immunogenicity will be evaluated with the use of adjuvant (AS03 or CpG 1018). The results of all these human trials will provide interesting results about the benefit of utilizing CpG ODN as a safe and efficient vaccine adjuvant.

5.6.2 QS-21

Figure 22: Quillaja Saponaria draft
(Martinic, https://www.behance.net/gallery/77484821/Quillay-%28Quillaja-saponaria%29?tracking_source=search_projects_recommended%7Csaponaria)

QS-21 is a saponin derived from the bark extract of the Chilean tree Quillaja saponaria (Quillajaceae). Quil A, an enriched extract from Q. saponaria contains more than 20 soluble triterpene glucosides. Triterpene glucosides were first mentioned to present adjuvant properties in 1925 by Ramon and indeed, Quil A is now known to highly stimulate both humoral and cellular immune responses (Lacaille-Dubois & Wagner, 2017). QS-21 is the major triterpene glucoside of Quil A and a very promising adjuvant. QS-21 is a mixture of two isomers, QS-21 Api and QS-21 Xyl. Each isomer consists of four domains: a branched trisaccharide, a triterpene, a linear tetrasaccharide and a glycosylated, pseudodimeric fatty acyl chain, as presented in **Figure 23** (Schijns & Lavelle, 2011).

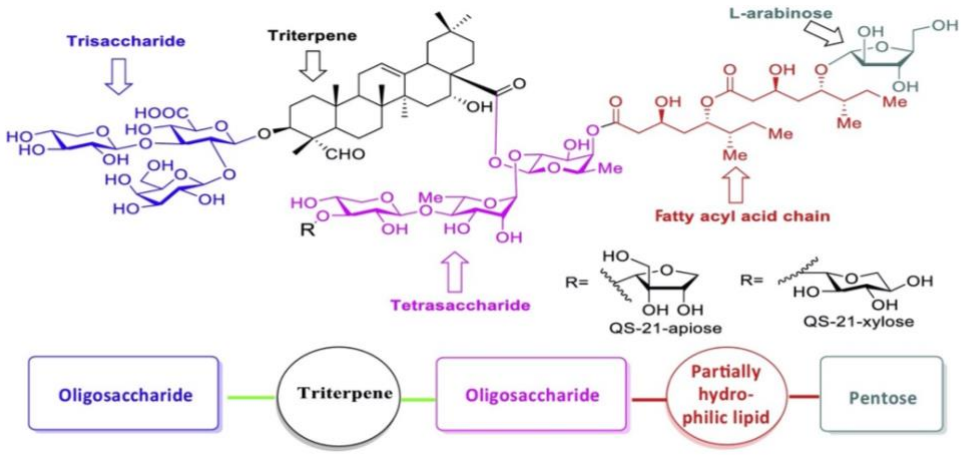


Figure 23: Structural elements of QS-21. Adopted from (Bonam et al., 2017)**.**

Until today the mechanism of action of QS-21 is not yet fully understand, although some interesting hypotheses have been proposed. QS-21 is a potent adjuvant that induces Th1/Th2 immunity via interaction with T-cells and APCs, like DCs. For the binding with T-cells, the aldehyde group of the molecule plays a key role, as it forms a Schiff base with amino groups on the surface of T-cells. On the other hand, the interaction with the DCs, do not include a certain receptor-protein and might occurs via cholesterol-depended endocytosis. For this activity, the amphiphilic character of the saponin QS-21 is of high importance. The C-type lectin receptors (CLTs) on the surface of DCs may also have a role in the activation of Th2 mediated response. A QS-21 synthetic analog, named QT-0101, produced from the diacylation of the fucosyl pyranose residue, approved to induce sole Th2 response without activation of the Th1 pathway. 3- and 4- hydroxyl groups of the fucopuranosoyl residue lead to DC-SIGN receptor, which is a CLT receptor responsible for Th2 response (Marciani, 2018). Finally, a novel mechanism of action is proposed by inducing the caspase 1-depended NLRP3 inflammasome (Lacaille-Dubois, 2019).

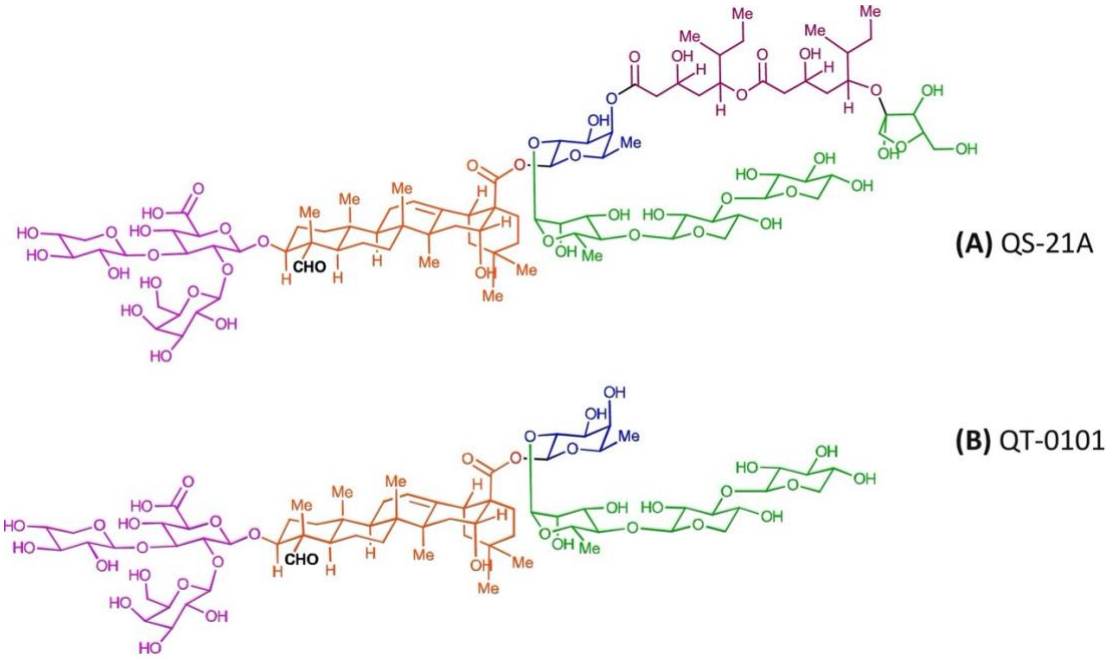


Figure 24: Panels A and B correspond to QS-21A and its deacylated derivative QT-0101, respectively. The triterpene aglycone is shown in red, C-3 oligosaccharide in purple, C-28-bound fucosyl pyranose in blue with the rest of that oligosaccharide in green. The galactosyl pyranose is shown in violet. Adopted from (Marciani, 2018).

The amphiphilicity mentioned above, explains the disruption of lipid bilayers and should be associated with a serious adverse effect of QS-21 that is the hemolysis presented after administration of high doses (Lacaille-Dubois, 2019). Moreover, the chemical instability of QS-21 should also be taken into consideration as the pure mixture of the saponins is thermo- and pH-sensitive, leading to hydrolytic diacylation. Finally, an environmental issue is manifested, as high quantities of the bark extract would be a threat to the plant. Even with the current needs cause worries about the ecological damage (Ragupathi et al., 2011). It is not the first time such a concern appears for a natural product. The story of Paclitaxel (Taxol) is one the most representative of this issue in the recent history of natural derived pharmaceutical products. All these reasons driven to the search of alternatives such as the production of synthetic analogs, incorporation of QS-21 with other adjuvants, so lower quantities of the extract are needed or development of large scale plant cell-culture methods (Lacaille-Dubois & Wagner, 2017; Marciani, 2018; Ragupathi et al., 2011). Among complex hybrid adjuvants, the most studied and promising ones, are ISCOMS® AS01 and AS02. These two adjuvant categories are further described below.

5.6.3 MPL

3-*O*-desacyl-4'-monophosphoryl lipid A (MPL) is a derivative of the liposaccharide (LPS) of *Salmonella minnesota*. The original LPS consists of a hydrophilic polysaccharide domain and a hydrophobic lipid portion, called lipid A **(Figure 25)**. Lipid A is responsible for the toxicological effect caused by Gram-negative bacteria (endotoxic activity). MPL has weakened the endotoxic intensity of lipid A via the removal of a phosphate group and increase by one the acyl chains **(Figure 26)** (Reed et al., 2009). MPL is a TLR4 agonist and in this way, it strongly stimulates the $CD4^+$ T-cell response, activates the NF-kB pathway leading to secretion of several pro-inflammatory cytokines (Bonam et al., 2017; Villarreal & Casale, 2020). MPL is approved by both EMA and FDA as a vaccine adjuvant but is mostly used in complex with other adjuvants.

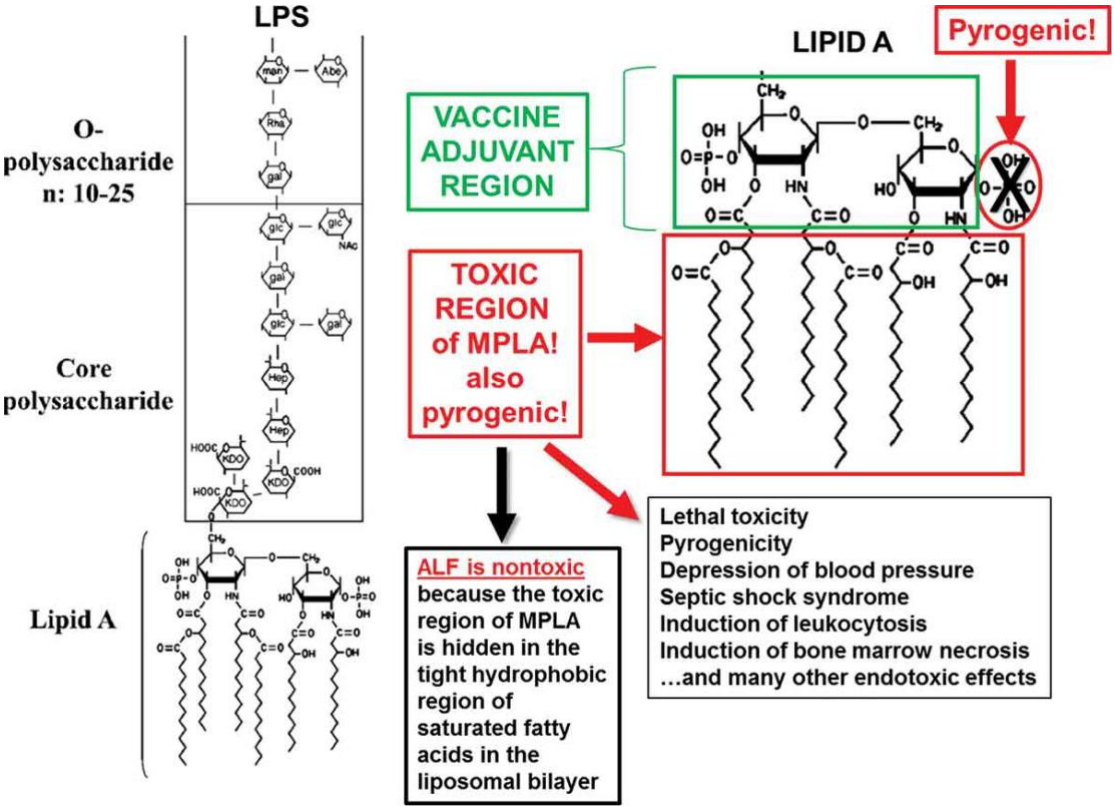


Figure 25: Structure-function relationships of lipid A derived from Gram-negative bacterial lipopolysaccharide (LPS). Adopted from (Alving et al., 2020).

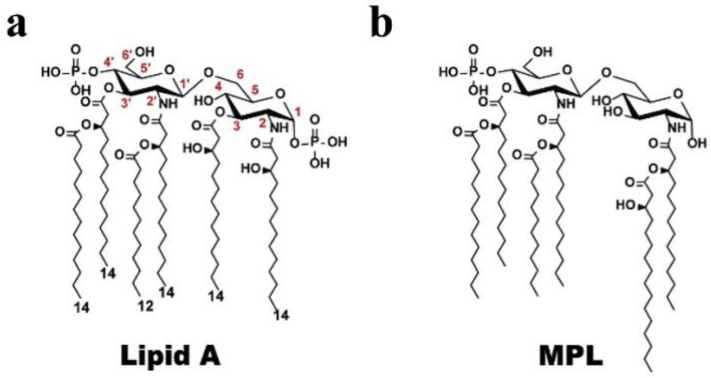


Figure 26: (a) Lipid A structure. (b) A representative structure of MPLAsm (hexa-acylated MPL). Adopted from (Ji et al., 2020).

5.6.4 Immune-Stimulating Complexes (ISCOMs) and ISCOM-matrix

ISCOMs were first described by Morein and colleagues in 1984 and since then an evolution in the adjuvant technology arrived (B Morein et al., 1984). They are spherical cage-like structures of an approximately 40 nm diameter and they consist of cholesterol, phospholipids, specific saponins from *Quijalla saponaria*, and incorporated antigen (Reimer et al., 2012). Later, it was noted that the incorporation of the ISCOM formulation with the antigen is not necessary for the immunomodulatory character of the adjuvant and empty ISCOM particles were developed. These particles are now called ISCOMATRIX and their main representative is Matrix-M™. Matrix-M™ consists of two different types of NPs, Matrix-A and Matrix-C. These NPs differ in the saponin fraction (Fraction-A and Fraction-C respectively) (Magnusson et al., 2018). DPPC phospholipid has been used for the formation of Matrix-M™ NPs. ISCOPREP™, used for the synthesizing of the final ISCOMATRIX™ structures, has a molar ratio of Matrix-A: Matrix-C 7:3 (Hu et al., 2005; Skene et al., 2008). A negative surface charge is present due to the glucuronic acid of the *S. saponaria* saponins (see **Figure 24**, light purple trisaccharide domain). This charge is useful for electrostatic interactions with positively charged antigens and also provides the physicochemical stability of the systems conformation (Pearse & Drane, 2005).

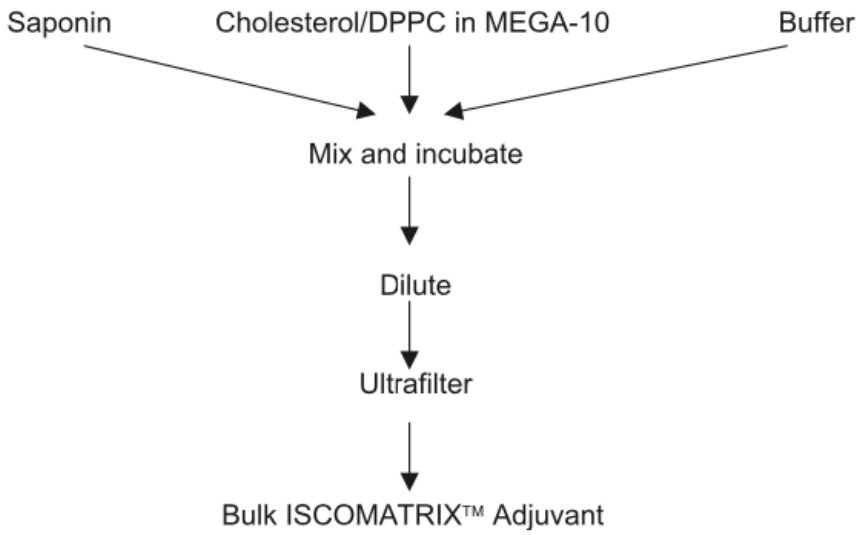


Figure 27: Flow chart showing the ISCOMATRX adjuvant manufacturing process. MEGA-10 (Decanoyl-*N*-methylglucamide) is a non-ionic detergent. Adopted from (Pearse & Drane, 2005).

As early as 1997, it was proposed that ISCOMs induce both Th1 and Th2 mediated immune response (Magnusson et al., 2018), while in 2012, a study took place for the evaluation of the mechanism of action of Matrix-M™ in murine. The results showed an increase of leucocytes and DCs in lymph nodes in a murine model (Reimer et al., 2012). ISCOMATRIX formulation promotes high levels of both humoral (high titers of specialized antibodies) and cellular (Ag-specific $CD8^+$ T-cells), while a plethora of chemokines and cytokines contribute to generate a potent, robust and long-lasting immune response (Morelli & Maraskovsky, 2017).

The above platforms were developed by Isconova (now Novavax) and clinical trials sponsored by big pharmaceutical companies have been performed. At this moment, 10 studies involving Matrix-M™ are active. Eight of them concern prophylactic vaccines against malaria. Another one concerns NanoFlu®, an influenza nanostructured vaccine (NCT04120194), and finally, the last one is for prophylaxis against SARS-CoV-2 (NCT04368988 – phase 1/2 & NCT04533399 – phase 2b).

5.6.5 Oil-in-water emulsions

Many oil-in-water (o/w) emulsions have been evaluated as immunostimulants. In this paper, we analyze the most studied and promising ones, MF59, AS02, AS03 and AS04.

MF59 is an o/w nano-emulsion adjuvant utilized in seasonal and pandemic influenza vaccines. The first MF59 adjuvanted vaccine was licensed from FDA in 1997 by the trade name Fluad® (Seqirus Inc.) as an inactivated influenza vaccine for elderly populations. During the 2009 H1N1 pandemic, two more MF59 adjuvanted vaccines approved (Focetria® and Celtura®) (Kommareddy et al., 2017). It is notable that these formulations were licensed for use in pregnant women and young children, older than 6 months. Finally, MF59 adjuvanted Aflunov® is also licensed for protection against H5N1 influenza virus. Nowadays (March 2020), the Committee of Medicinal Products for Human Use (CHMP), adopted a positive opinion about Fluad Tetra, a tetravalent inactivated influenza vaccine, which is the evolution of the previous trivalent formulation.

MF59 contains squalene (4.3% w/w) in citric acid buffer, and the surfactants Tween 80 (0.5% w/w) and Span 85 (0.5% w/w) and the size of the droplets is approximately 160 nm (O'Hagan, 2007). Squalene is a natural component of human body, as it is necessary for the biosynthesis of steroid hormones and cholesterol, while Tween 80 and Span 85 are widely used both in the pharmaceutical and cosmetic industry. As a result, the nano-emulsion is biocompatible, bio-degradable, and non-toxic (Kommareddy et al., 2017).

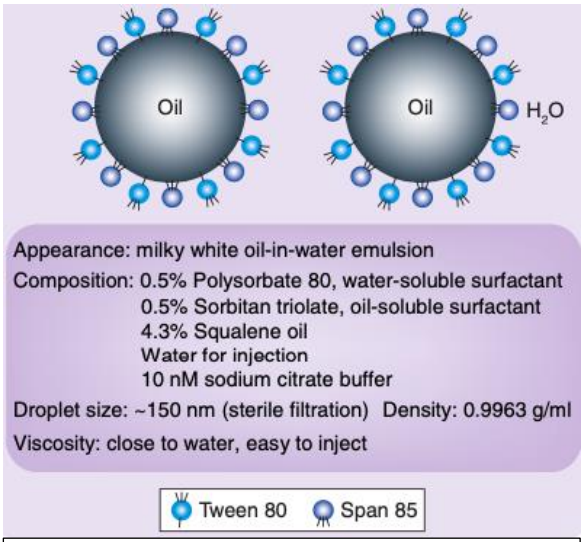


Figure 28: The composition of MF59 adjuvant emulsion. Adopted from (O'Hagan, 2007).

As to MF59 mechanism of action, it promotes mainly a Th2 response. Th2 response is also induced by alum, but studies showed that MF59 produced a more intensive response and had better immunomodulating results in mouse models. The potency of MF59 is caused by the development of a local immunostimulating environment. A plethora of chemokines and cytokines, such as CCL2 monocyte chemoattractant, lead to an increase of monocytes and granulocytes populations, which results in influx of APCs in the site of injection. Then, the APCs transfer the antigen into the lymph nodes to activate the adaptive immunity and the production of high antibody titers (O'Hagan et al., 2012; Villarreal & Casale, 2020). As MF59 does not activate the Th1 mediated mechanism, studies should be proposed for the incorporation of the safe MF59

with a Th1 inducer such as the CpG ODN that is mentioned above (O'Hagan, 2007; S. Wang et al., 2020).

Adjuvant Systems 02, 03 and 04 (AS02, AS03, AS04) have also been studied extensively as immunostimulating o/w emulsions. AS03 and AS04 are licensed for human use.

AS03 is a squalene based emulsion, as MF59, but with the difference that AS03 additionally contains α-tocopherol (Vitamin E) (Del Giudice et al., 2018). AS03 has been utilized in vaccines against avian influenza (H5N1) and H1N1 influenza pandemic (2009). During the pandemic, two AS03-containing vaccines were authorized, one in Europe (Pandermix) and one in Canada (Arepanrix). One year after, Pandermix was associated with narcolepsy syndrome in the adolescent population and until 2015 more than 1300 cases of the appearance of the certain adverse effect had been reported in the EMA EudraVigilance database. Although the mechanism of this adverse action might correlate with the production of an antibody for an influenza nucleoprotein and not with the existence of AS03, more research is necessary on the subject (Ahmed et al., 2018; Johansen et al., 2018). It is also of exceptional importance to research whether the same result would appear after immunization of a non-adjuvanted vaccine or with the use of another similar adjuvant, as AS03 may not be the major causative of narcolepsy but the driving force.

AS04 is composed of aluminum salt and MPL. Specifically, MPL gets adsorbed on the surface of the nanoparticulate form of alum. Cervarix and FENDrix are two licensed vaccines, manufactured by GSK, against HPV and HBV respectively, which contain AS04. These two vaccines have been widely used, concluding that AS04 is not just effective, but, as well, safe (Garçon & Mechelen, 2006). AS04 induce local NF-κB activity and cytokine production and thus, APCs such as DCs mature and accumulate in the draining lymph nodes to stimulate adaptive immunity. T- and B-cells are not directly activated but finally, AS04 leads to enhanced adaptive immune response and production of memory cells. The adjuvants' mechanism of action is mostly driven by MPL while alum prolongs the local cytokine production (Arnaud M Didierlaurent et al., 2009).

Finally, AS02 is an o/w emulsion containing both MPL and QS-21. In a recent study, Chen et al. compared the role of four different adjuvants (MF59, $Al(OH)_3$, AS03 and AS02) in the immunogenicity of a prophylactic vaccine against Streptococcus pneumoniae. The vaccines were tested in mice and the results showed that AS02 vaccine-induced cross-protection against different bacterial strains. High titers of antigen-specific IgG_1 and IgG_{2A} were produced leading to activation of a mixed Th1/Th2 pathway. In contrast, MF59 and $Al(OH)_3$,

only induced a Th2 response. As a result, AS02 could be a promising adjuvant in such a vaccine (Chen et al., 2019). Nevertheless, GSK has decided to replace AS02 with AS01 adjuvant due to its' better immunogenicity and safety profile (Ansong et al., 2011; Moris et al., 2018). At this moment no active or programmed clinical trial is on schedule according to ClinicalTrial.gov.

5.6.6 Liposomal Adjuvant Systems

After the evaluation of QS-21 and MPL possibilities and dangers, GSK and US. Army developed liposomal platforms to incorporate the above constituents. These platforms called AS01 and $AS01_B$ is a patent of GSK while $AS01_E$ was studied by both GSK and the US. Army. Two vaccines containing $AS01_B$ have been approved. The first one, under the trade name Shingrix (GSK), is licensed from FDA and EMA for prophylaxis of adults >50 years from herpes zoster (Alving et al., 2020). The second one, Mosquirix (GSK), received a positive scientific position from EMA for vaccination outside EU and now post-authorization studies in children are in progress. According to the EMA risk-management plan, updated in 2019, one of the adverse effects that have been observed and associated with administration of Mosquirix is febrile convulsion in subjects 5-17 months and it shall further be investigated (EMA, 2015, 2019b). At this moment, 73 clinical trials containing AS01 evaluation have been submitted to ClinicalTrials.gov, from which 21 are still ongoing, concerning malaria, HIV, and respiratory syncytial virus infections.

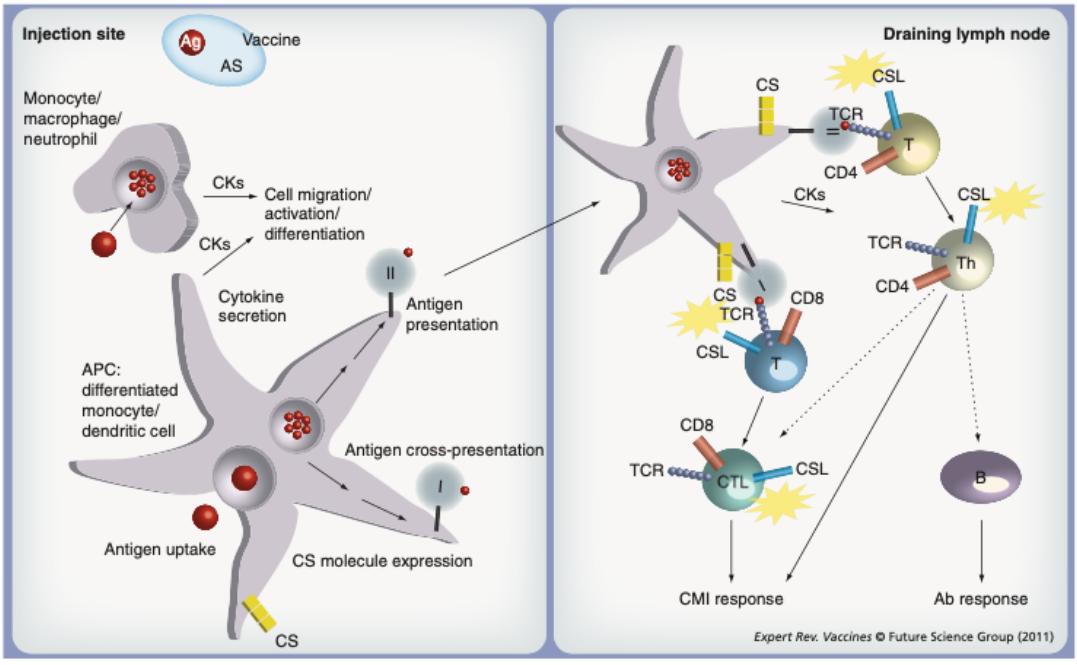


Figure 29: Schematic model describing how Adjuvant Systems-adjuvanted vaccine potentially affects antigen-presenting cells and the subsequent adaptive immune response. The adjuvanted vaccine at the muscle injection-site stimulates immune cells to transiently release CKs. These CKs can promote the recruitment of more immune cells. APCs take up and process antigen fragments to be presented at the cell surface, complexed with MHC class II molecules (II). 3-O-desacyl-4'-monophosphoryl lipid A can promote the cell surface expression of CS molecules. QS-21 may also promote antigen presentation by MHC class I molecules (I). At the draining lymph node, the antigen-loaded APCs potentially interact with naive T cells. These CD4+ and CD8+ T cells recognize antigen fragments presented by MHC class II and MHC class I molecules, respectively, via TCRs, and CD4 and CD8 coreceptors, respectively. Concomitant interaction of CS molecules on the activated APCs and their ligands (CSLs) on the T cells ensures T-cell activation. The secretion of CKs by the APCs can also affect the type of T-cell activation and can promote the production of antibodies by B cells. Activated antigen-specific CD4+ T cells (Th cells) can support adaptive antigen-specific antibody responses and cell-mediated responses.
Ab: Antibody; Ag: Antigen; APC: Antigen-presenting cell; AS: Adjuvant Systems; CK: Cytokine; CMI: Cell-mediated immune; CS: Costimulatory; CSL: Costimulatory ligand; TCR: T-cell receptor; Th: T-helper cell. Adopted from (Garçon & Van Mechelen, 2011).

What is achieved with the liposomal formulation of these materials is the decrease of the hemolytic danger caused by QS-21. As mentioned above, high doses of QS-21 can cause lysis of the plasma membranes of erythrocytes. This action is correlated with the interaction of QS-21 with the plasma cholesterol, concluding in the formation of pores and defects of the membrane bilayer. Hence, the production of an adjuvant system with a liposomal structure that contains cholesterol, orientates QS-21 to interact with the cholesterol of the liposomes rather than the cell membrane cholesterol. AS01 as well as a similar system developed by US Army, called ALFQ (Army Liposome Formulation containing QS-21 and MPL), can develop sophisticated architectures that protect from hemodialysis and orientate the eight sugars of QS-21 in a conformation visible for interaction with the lectin receptors, present on the surface of the cells of innate immune system **(Figure 30)**.

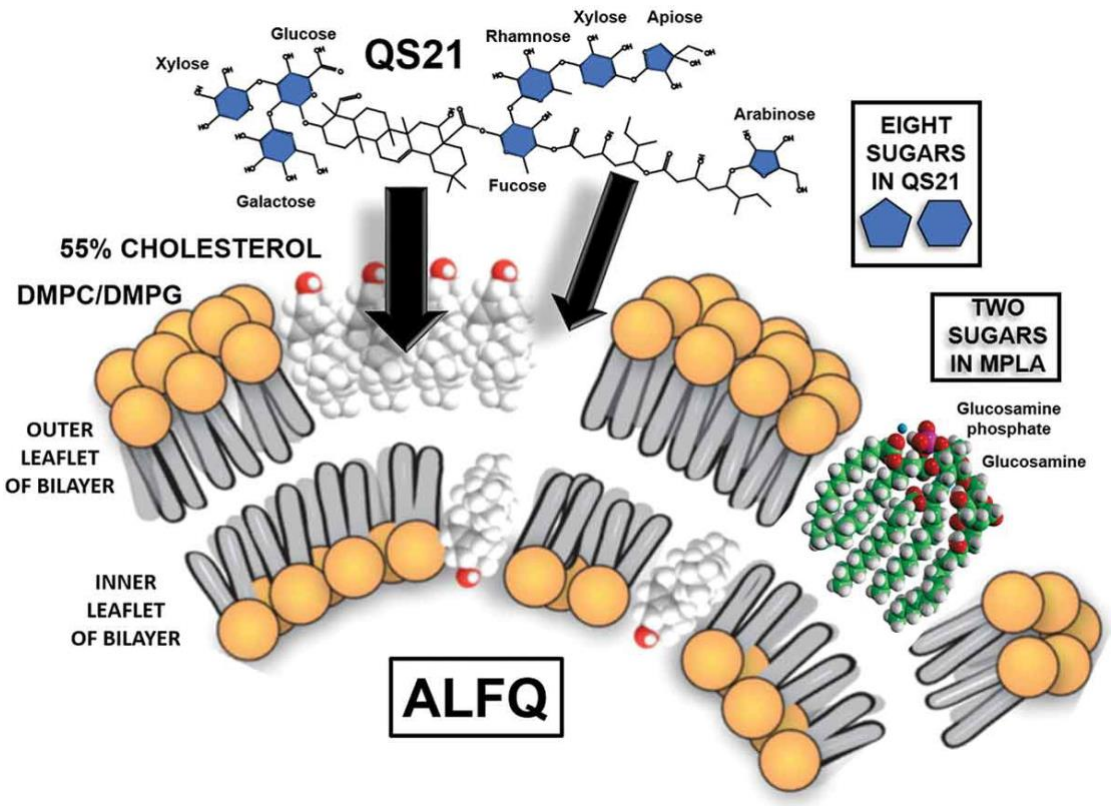


Figure 30: Theoretical formation of a 'sugar lawn' of 10 sugars on the surface of ALFQ by interaction of QS21 with ALF liposomes containing Figure 5. Theoretical formation of a 'sugar lawn' of 10 sugars on the surface of ALFQ by interaction of QS21 with ALF liposomes containing 55 mol% cholesterol compared to phospholipid. Adopted from (Alving et al., 2020).

The major differences between AS01 and ALFQ are the saturation degree of the phospholipids and the ratio of cholesterol in the final conformations. AS01 contains the unsaturated phospholipid dioleoyl phosphatidylcholine (DOPC), while ALFQ contains the unsaturated dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) (Alving et al., 2020; A M Didierlaurent et al., 2017). Concerning the cholesterol ratio, it is 33.7 mol% for AS01 and 55 mol% for ALFQ. These characteristics can alter the behavior of the adjuvant systems. To understand the difference in the conformation of the liposomes, it is enough to see their self-assembly behavior after the addition of QS-21. AS01 liposomes retain their hydrodynamic diameter in the nanoscale while the ALFQ present an increase of their size from 50-100 nm to approximately 30,000 nm. This size differentiation of the systems, obviously, results in alterations of the way the immune system recognizes and interacts with the adjuvants. Whether a size increase promotes a better or a worst immunity remains in question and the appropriate studies should be carried out.

Table 2: Lipid compositions of AS01B and ALFQ liposomes. Adopted from (Alving et al., 2020).

AS01B liposomes (per 1.0 ml human dose)	Weight	Molarity
Dioleoyl phosphatidylcholine (DOPC)	1,000 µg	1.272 mM
Cholesterol	250 µg	0.65 mM
Cholesterol mol%		33.7
MPLA (3D MPL®)(Native 3-deoxy MPLA)	50 µg	N/A*
MPLA/phospholipid ratio		N/A*
QS21	50 µg	0.025 mM
ALFQ liposomes (per 1.0 ml human dose)	Weight	Molarity
Dimyristoyl phosphatidylcholine (DMPC)	6986.27 µg	10.305 mM
Dimyristoyl phosphatidylglycerol (DMPG)	788.73 µg	1.145 mM
Cholesterol	5,413 µg	14.0 mM
Cholesterol mol%		55
MPLA (3D PHAD®)(Synthetic MPLA)	200 µg	0.13 mM
MPLA/phospholipid ratio		1/88
QS21	100 µg	0.05 mM

*The molecular weight and molarity of native 3-deoxy MPLA are undetermined because it contains more than one congener.

6 Case studies of nanovaccines in clinical practice

6.1 Hepatitis B virus vaccine

Hepatitis B virus (HBV) is a member of the Hepadnaviridae family and can infect both mammals and birds. There are 8 infectious genotypes (A-H) for humans, differ by at least 8%, and can be further divided into at least 24 subgenotypes, which differ more than 4% (Schaefer, 2007). HBV is an enveloped, ~42 nm virus, containing an icosahedral nucleocapsid and its genetic material is a 3.2 kb relaxed circular DNA (rcDNA). When the virus enters the host cell, the viral nucleocapsid transfer the rcDNA into the nucleus, where it is converted by the host DNA repair machinery to a double-stranded covalently closed circular DNA (cccDNA) in a form of mini-chromosome (Fanning et al., 2019; Jain et al., 2015).

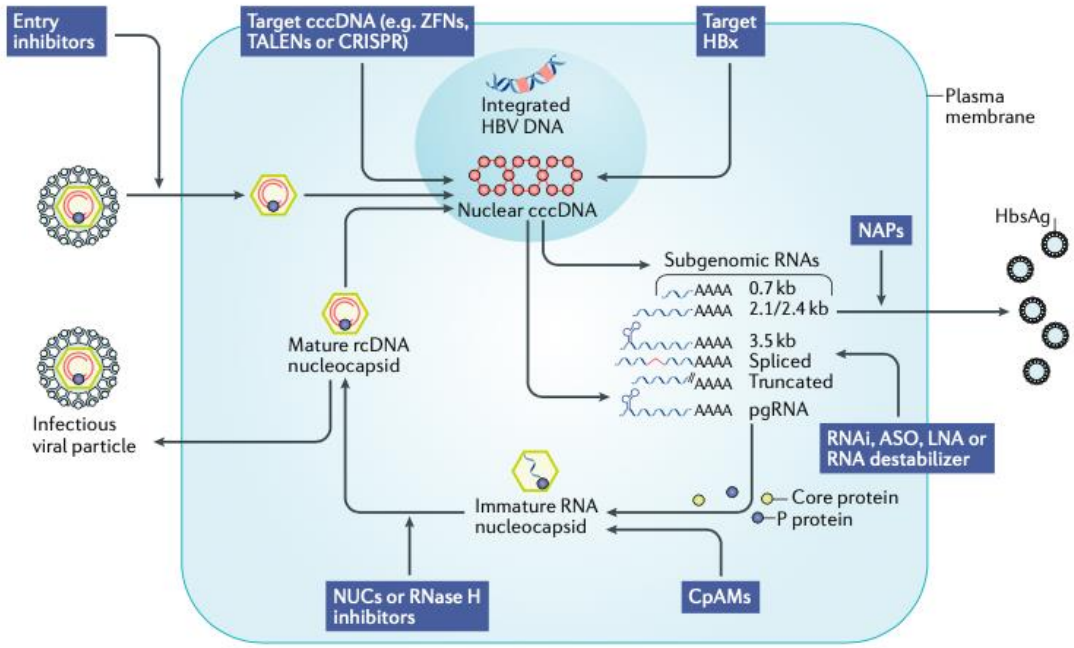


Figure 31: The hepatitis B virus life cycle and novel therapeutic interventions. The incoming virus (top left) binds first to heparin sulfate proteoglycans and then binds with higher affinity to the sodium–taurocholate cotransporting polypeptide (NTCP) receptor on the host via the pre- S1 domain of hepatitis B surface antigen (HBsAg), entering the cell via receptor-mediated endocytosis. Entry can be inhibited by antibodies to HBsAg or by peptides or compounds, such as Myrcludex B, that compete with the virus for binding to NTCP. In the endosome, the viral envelope is removed and the nucleocapsid interacts with importin- α or importin- β to be transported to the nuclear pore complex. Nucleocapsids pass through the

nuclear pore complex and release the hepatitis B virus (HBV) genome into the nucleus. The relaxed circular DNA (rcDNA) is converted by the host DNA repair machinery into episomal double- stranded covalently closed circular DNA (cccDNA). This replication intermediate serves as the template for HBV mRNA and pregenomic RNA (pgRNA). cccDNA can be cleaved by gene- targeting strategies such as transcription activator- like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), or CRISPR–Cas9. Hepatitis X protein (HBx) maintains expression of cccDNA, without which the cccDNA is silenced by the structural maintenance of chromosomes protein 5 (SMC5) and SMC6 complex. Therefore, HBx is a potential target for new drugs. Six RNA species are transcribed from cccDNA, including a pgRNA that binds HBV polymerase. These RNA species can be targeted by small interfering RNA or antisense approaches. The pgRNA- bound polymerase binds a hexamer consisting of three core dimers that then nucleates the self- assembly of the nucleocapsid enveloping the polymerase and pgRNA — a process that can be disrupted by core protein allosteric modulators (CpAMs), resulting in either empty nucleocapsids or aberrant nucleocapsid structures. CpAMs also bind to the capsid and inhibit the release of rcDNA into the nucleus. The polymerase synthesizes rcDNA from pgRNA in the nucleocapsid by reverse transcription, a process that can be inhibited by nucleoside or nucleotide drugs (NUCs), to form mature nucleocapsids. The mature nucleocapsids can be recycled to the nucleus unless they are bound by large HBsAg, in which case the nucleocapsids are redirected to budding sites. The virus then acquires an envelope, first via the endosomal sorting complex required for transport proteins and completed in the multivesicular bodies before budding. Budding can be halted by nucleic acid polymers (NAPs). ASO, antisense oligonucleotide; LNA, locked nucleic acid; P, polymerase; RNAi, RNA interference. Adopted from (Fanning et al., 2019)

cccDNA contains 4 open reading frames and is the template for transcription of the viral RNAs. cccDNA encodes 7 proteins, from which, 3 are the viral envelop proteins -large (L), medium (M) and small (S)- and each of them has its own mRNA (Seeger & Mason, 2015). All envelop proteins are expressed by the same ORF. S-protein is translated by the S-domain of this ORF (226 aa), M-protein by S-domain and an additional pre-S2-domain (281 aa) and L-protein by S-, pre-S2 and pre-S1- domains (389 or 400 aa depending on the genotype). pre-S2 and pre-S1 are N-terminal extenders of S-domain (Bruss, 2007). These surface proteins are glycosylated, partially or fully, in certain positions when produced in eukaryotic cells, and they bind with the neighbor proteins, on the envelope of the infectious virions, via

disulfide bridges between cysteine amino acids, developing stable morphologies (Bruss, 2007; Joe et al., 2020). A bind between the pre-S1-domain of L-protein and sodium taurocholate co-transporting polypeptide (STCP), in the membrane of hepatocytes, leads to viral entry in the cell (Fanning et al., 2019). Hence, the viral infection depends on the functionality and tertiary order of the L envelope protein and more specifically, its last N-terminal amino acids.

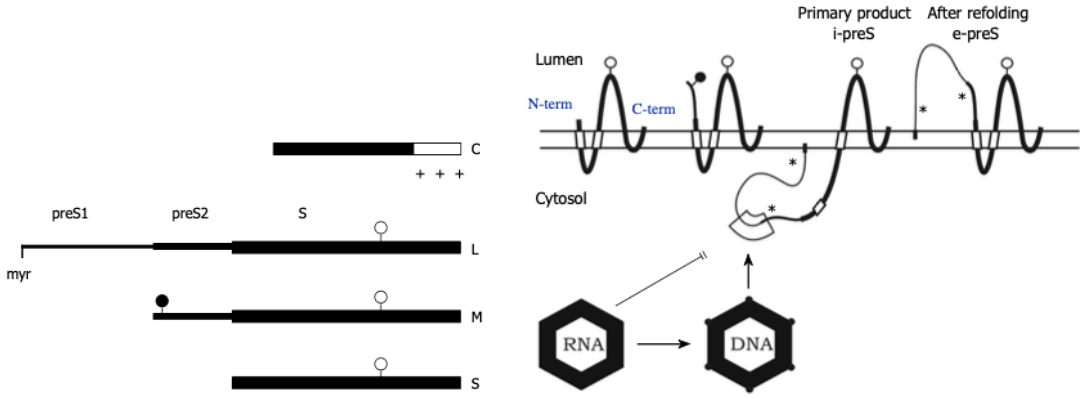
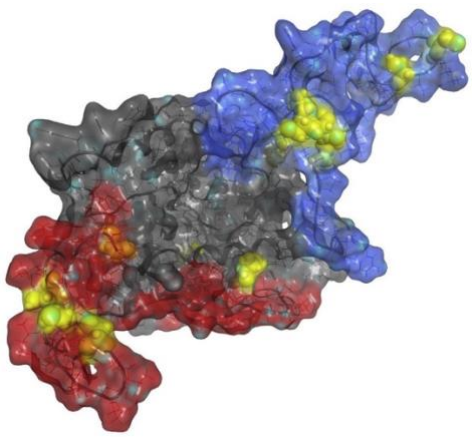


Figure 32: Transmembrane topology of the HBV envelope proteins and model for envelope-capsid interaction. The transmembrane folding of the S protein is determined by an N-terminal and an internal signal shown as open boxes. The C-terminal domain is hydrophobic and probably embedded in the lipid bilayer (horizontal open bar). The C terminus is oriented towards the ER lumen. The folding of the M protein is similar to S. The preS2 domain of M (thinner line) is located in the ER lumen. In the initial folding of the L protein, the preS domains are located in the cytosol (i-preS). Whether the N-terminal myristate group (filled box) is inserted into the membrane as shown here is unknown. After refolding approximately half of the L chains expose the preS domains at the luminal side of the membrane (e-preS). Open and filled circles: see Figure 1. Asterisks indicate potential but unused N-glycosylation sites in preS of L. A domain in i-preS (boxed area) and in the cytosolic loop of S may interact with the capsid during budding. Immature capsids containing pregenomic RNA are not capable to bud. During viral DNA synthesis, the capsid shell changes (indicated by filled circles at the edges) and becomes competent for envelopment. Adopted from (Bruss, 2007).

During the viral life circle, the envelop proteins are overexpressed and mixed with host lipids, they form sub-viral particles, which do not contain the nucleocapsid or genetic material and are non-infectious (Martinot-Peignoux et al., 2013). These particles, also known as HBsAg (Hepatitis B surface antigens) particles, are self-assembled into quasi-



spheres (~20nm octahedral particles) or filament-like particles of various sizes. The different morphologies are based on the amounts of L-protein. A higher percentage of L-proteins leads to filament-like particles, while the spheres are mainly composed of S-proteins. The percentage of host lipids in the sub-viral particles is approximately 25%, indicating that a liposome-like formation is not probable. After their formation, HBsAg particles are present in host plasma, in a 10,000-fold higher concentration than infectious virions (Bruss, 2007).

Pharmaceutical industries and scientists took advantage of the mechanism of action of the non-infectious but highly immunogenic sub-viral particles for the development of an HBV vaccine. The first-generation vaccine (Heptavax-B®), approved in 1981 in the U.S, used purified HBsAg particles isolated by the plasma of patients with chronic hepatitis B. Although the vaccine gave promising results, issues such as the safety of blood products and the coverage of large quantities, resulted in the evolution of new technologies (Zhao, Li, et al., 2013). Today, recombinant Virus-like particle HBV vaccines have almost fully replaced the first-generation plasma-derived particulate vaccine. The most common HBV vaccines in the developed world are Recombivax HB® by Merck & Co. and Engerix-B® by GSK (Qian et al., 2020). In fact, Recombivax HB® was the first recombinant human vaccine (Zhao, Li, et al., 2013). Since then, more than 10 other VLP-based HBV vaccines have been approved worldwide. All of these vaccines are administrated intramuscularly in three doses. The basic characteristics of these vaccines is that the HBsAg particles are produced using the expression system of yeast cells (e.g. S. cerevisiae, P. pastoris and H. polymorpha) via the technology of recombinant DNA and are adjuvanted with aluminum hydroxide (Jain et al., 2015; Kushnir et al., 2012).

Figure 33: Model of the monomeric HBs protein obtained after I-TASSER modelling. The AGL sequence (D99-F180), which is very flexible, is exposed to the solvent and colored in blue. The a-helix core, which forms the rigid central domain, is shown in grey. The HCL (R23-C90), which is internalized in the particle, is colored in red. The cysteines of each domain are represented by yellow spheres. Disulfide bonds (C107-C138, C137-C149 and C139-C147) are used as constraints in the I-TASSER software and were created using the MOE software. Adopted from (Berthier et al., 2020).

The domain of the viral DNA which is used for this procedure is the gene sequence that encodes the S-envelop protein of HBV. The S-protein has the ability to spontaneously self-assemble with a lipid matrix, donated by the producer cells, into sphere formations of approximately 20nm diameter (Jain et al., 2015). According to the literature, two sub-populations of HBsAg particles (18-20 nm and 22-23 nm) appear in both, human blood and physicochemical stability evaluations of the vaccines (Berthier et al., 2020; Jain et al., 2015; Mulder et al., 2012). It is assumed that the size diversity might be the result of measurements at different times. The unmatured particles seem to have a slightly smaller size and, via the maturation process, a more rigid and bigger conformation takes place. The mature structure is stabilized by S-S bridges between cysteine amino acids of the S-protein molecules. Recent research in molecular dynamics of these sub-viral particles shows that the S-proteins may assemble in tetramers (two dimers) while, discontinuous phospholipid membranes are present, mostly near the hydrophobic a-helix of S-protein area. An aqueous environment is observed in the internal part of HBsAg particles, where lipids, triglycerides or solvent molecules of the producer cells can be found in several assemblies (Berthier et al., 2020; Greiner et al., 2010). Phospholipids or other lipidic molecules of the particles' core might play a key role in the maturation process and the formation of the "molecular diaphragm" and, consequently, in the stability and antigenicity of HBsAg formulation utilized in vaccine manufactory (Berthier et al., 2020).

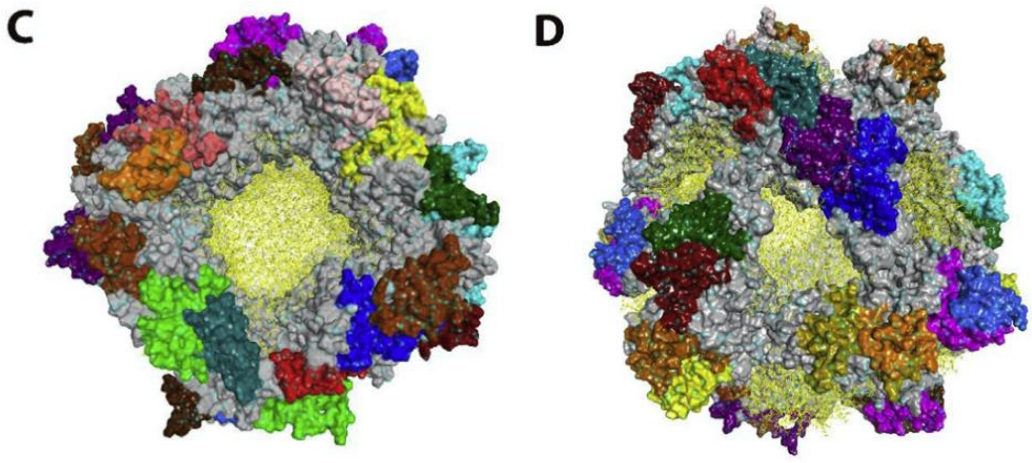


Figure 34: Atomic model of small (C) and large (D) particles. The solvent surface for proteins was computed and colored in grey, except for the 48 AGL loops (99e178) which

were colored by chain. DOPC molecules were displayed as yellow lines. Adopted from (Berthier et al., 2020).

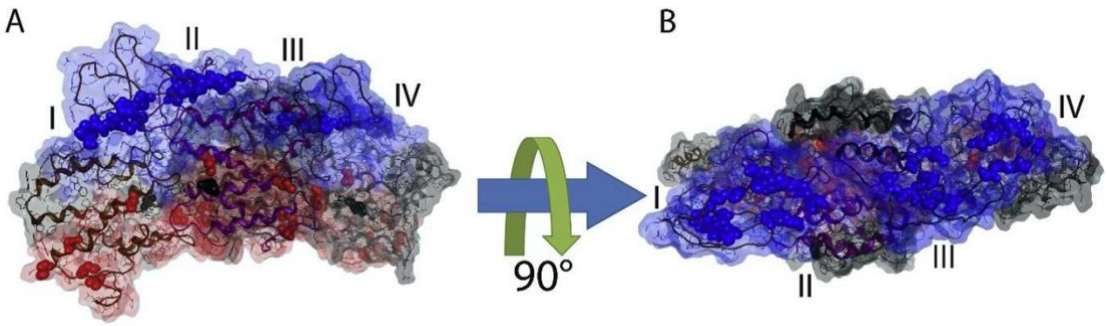


Figure 35: Tetrameric HBs beam (2 dimeric asymmetric units). Blue: external surface, Red: internal surface. Adopted from (Berthier et al., 2020).

Taking as an example the Engerix-B® summary of product characteristics by European Medicine Agency (EMA), it is worth noting that HBsAg VLPs are presented to self-assemble spontaneously, without any chemical process, in spheres of 20 nm diameter (EMA, 2000), which corresponds to the small-size population. On the other hand, Recombivax HB® contains ~22 nm VLPs, belonging to the large-size population. These VLPs undergoes spontaneous chemical maturation after purification. The maturation leads to a 2 to 3-fold higher immunogenicity in comparison with the premature particles (Zhao et al., 2011). According to the above, information of whether the HBsAg particles of Engerix-B® retain their size or mature after the vaccine administration to a more rigid and immunogenic conformation into the human organism, is of particular importance. To the best of the authors' knowledge, no such information was been provided until today.

Apart from HBsAg VLP vaccines produced in yeast cells, there are also prophylactic VLP vaccines produced in mammalian (Chinese Hamster Ovary – CHO) cells, referenced as third-generation vaccines against HBV (Shouval et al., 2015). The main difference of the HBsAg particle formulation processes is that mammalian cells produce both non-glycosylated and glycosylated particles, while, yeast cells form only non-glycosylated ones, as they lack post-translational modification abilities (Mohsen et al., 2017; Qian et al., 2020). The main representative of third-generation vaccines is Sci-B-Vac®, manufactured by SciVac Israel Ltd. This vaccine is licensed in Israel and other countries of East Asia. Sci-B-Vac® is glycosylated in certain positions (gp27, gp33, gp36 and gp42) and, moreover, it contains HBsAg particles formed by all three (S-, M- and L-) enveloped HBV proteins adsorbed onto aluminum hydroxide. The above factors result in faster and higher

seroprotection against HBV, as additional protective antibodies for PreS1 and PreS2 domains of L- and M-proteins are also induced (Mohsen et al., 2017). In this way, it is more probable for the population non-responsive to the conventional second-generation vaccines, such as immunocompromised or people with renal failure, obesity, and diabetes mellitus to reach the favorable anti-HBs titer of ≥10 mIU/mL. (Shouval et al., 2015).

The need for the production of safe and effective vaccines for the non-responsive population is remarkable when considering that a hemodialysis patient infected by HBV has 60% chance to develop chronic hepatitis while a healthy person has 5-10% (EMA, 2005a). Except for the third-generation vaccines, another approach for the immunization of sensitive groups against HBV is the change of the adjuvant. Fendrix® (GSK Bio) and Heplisav-B® (Dynavax) are based on this strategy. The first one approved in 2005 by EMA for patients with renal insufficiency and utilize AS04C (3-*O*-desacyl-4´-monophosphoryl lipid A - MPL and aluminum phosphate) as an adjuvant system. It is licensed for people aged at least 15 years old and it is effective when 3-4 doses, depending on the patients' health situation, are administrated (EMA, 2005a, 2017a). Heplisav-B® is licensed for adults (≥18 years old) by FDA for hyporesponsive populations and includes synthetic cytosine phosphoguanine oligonucleotide, CpG sequence 1018, as an adjuvant. It is the only approved HBV vaccine that needs only two doses and encouraging results are provided for people with diabetes mellitus, HIV infected or elderly populations (Farooq & Sherman, 2019; Hyer & Janssen, 2019).

6.2 Hepatitis E virus vaccine

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus, belonging to the genus Orthohepevirus in the Hepeviridae family (Kamar et al., 2017). Hepatitis E is usually self-limited acute hepatitis (Facciolà et al., 2019) but also, the main cause of acute liver failure in developing regions (Yin et al., 2020). Although the mortality rate in healthy patients is 1-3%, it dramatically increases for pregnant women to 10-50% accompanied by a high risk of abortion and stillbirth (Yin et al., 2020; Shrestha et al., 2003). HEV genotypes 1 to 4 (HEV1, HEV2, HEV3, and HEV4) are infectious for humans and HEV1 is the most common in human populations (Kamar et al., 2017). The virus genome comprises three open reading frames (ORFs) from which ORF2 encodes the HEV capsid protein. ORF1 encodes a non-structural protein, whilst ORF3 is involved in the egress of HEV from the infected host cells. The capsid protein consists of 660 amino acids and it is divided into three

domains: the shell, the middle and the protruding domain (Kamar et al., 2017). The protruding domain or E2s (aa 459-606) contains five conformational neutralizing epitopes (Zhao, Zhang, et al., 2013).

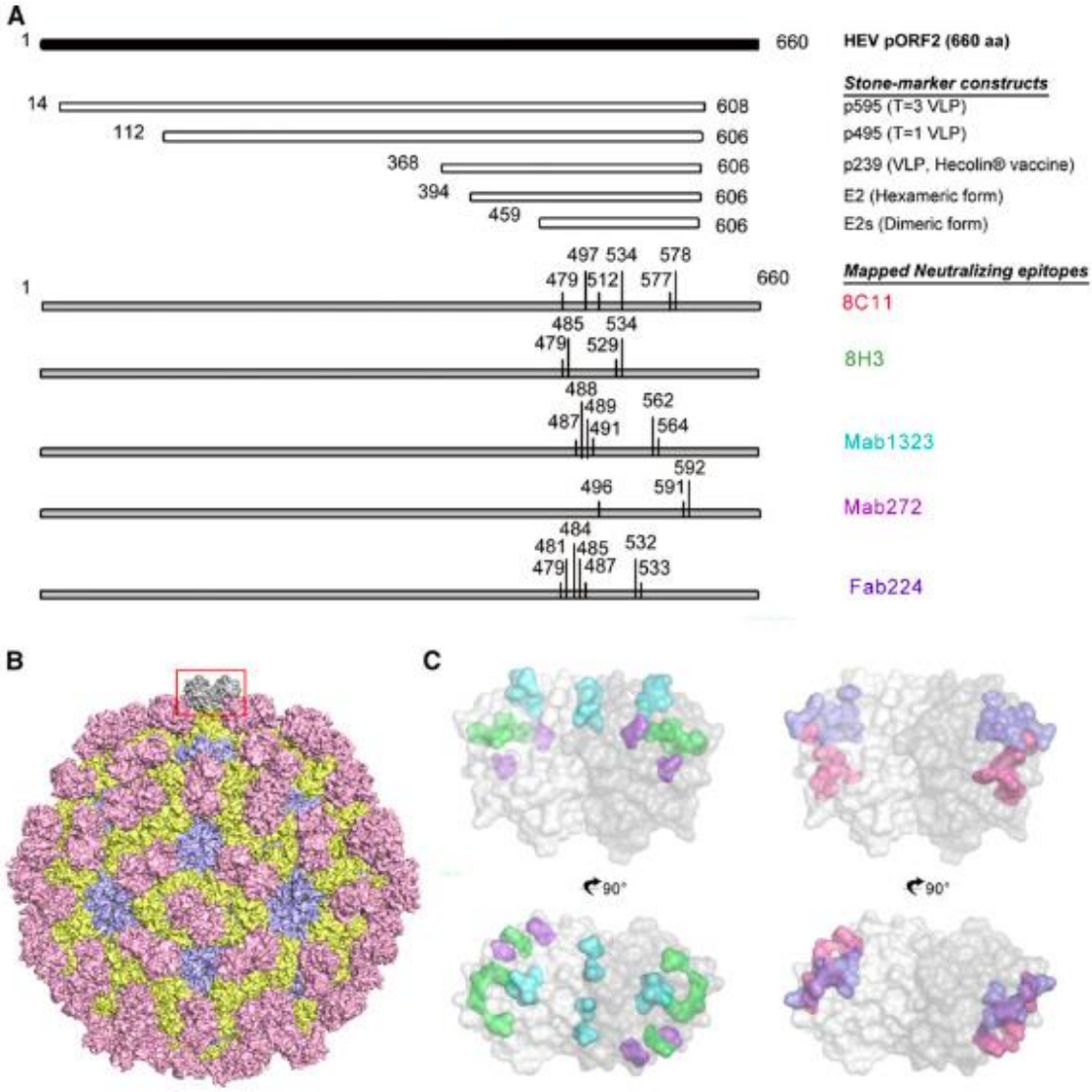


Figure35: Key antigenic determinants on HEV pORF2. A | Antigenic and assembly properties of truncated pORF2. B | Crystal structure of HEV capsid protein. Crystal structure of T = 1 HEV-VLP (PDB:2ZTN). Mesh representations of dimeric E2s domains highlighted the neutralizing epitopes against several neutralizing monoclonal anti- bodies. C | Key neutralizing epitopes on pORF2. All the identified neutralizing sites were mapped in E2s domain**.** Adopted from (Zhao, Zhang, et al., 2013)

Due to poor yields and other difficulties in the culture of HEV, the production of a live-attenuated or inactivated vaccine has not been possible. Hence, many efforts to develop HEV vaccines have focused on the production of a recombinant vaccine with the use of Virus-

Like Particles (VLPs) technology (Facciolà et al., 2019). One of these efforts led to the approval of the first HEV vaccine in December 2011 in China with the trade name Hecolin® by Xiamen Innovax Biotech Co., Ltd. Hecolin® is available as a pre-filled syringe (0.5mL), containing aluminum hydroxide-adsorbed antigen suspended in phosphate buffer saline (pH 7.4) in a molar ratio 800μg adjuvant (aluminum hydroxide): 30μg antigen (Yin et al., 2020). The antigen contained in Hecolin® is a VLP, constructed by a domain of the HEV genotype 1 capsid protein encoded by ORF2 (aa 368-606 of capsid protein), named p239. p239 includes the key binding sites with neutralizing monoclonal antibodies 8C11 and 8C12 (Innis & Lynch, 2018). The VLP is prepared utilizing a recombinant Escherichia coli expression system via recombinant technology and has 23-29 nm hydrodynamic diameter (S. W. Li et al., 2015). The vaccine is administrated intramuscularly in three doses, 0, 1 and 6 month, and has over 99% efficacy in the prevention of HEV genotype 4 infection for at least 13 months after the administration of the last dose (WHO, 2015). There is no available data from human clinical trials that Hecolin® provides prophylaxis from HEV1, HEV2, and HEV3 infections although murine and rhesus macaques experiments show protection against all HEV genotypes (WHO, 2015). Until today Hecolin® is not approved elsewhere from China and is the only licensed vaccine against the Hepatitis E virus for people 16-65 years old (Yin et al., 2020). In May 2015 World Health Organization (WHO) issued a position paper for Hepatitis E vaccine and acknowledged the need for more extended research of Hecolin® in pregnant women, patients with chronic liver disease and immunosuppressed persons, as the immunogenicity of the vaccine had not been evaluated in persons aged <16 years, >65 years and in populations at higher risk of developing severe Hepatitis E disease (WHO, 2015). In 2019 the WHO Expert Committee on Biological Standardization (ECBS) published the authoritative, harmonized guidelines and recommendations for quality, safety and efficacy of recombinant Hepatitis E vaccines via the corresponding technical report (WHO, 2019). Six clinical trials phase IV for Hecolin® have been completed from 2015 till May 2020, based on WHO guidelines targeting to the vaccine approval for human use world widely (*Results @ Clinicaltrials.Gov*, n.d.).

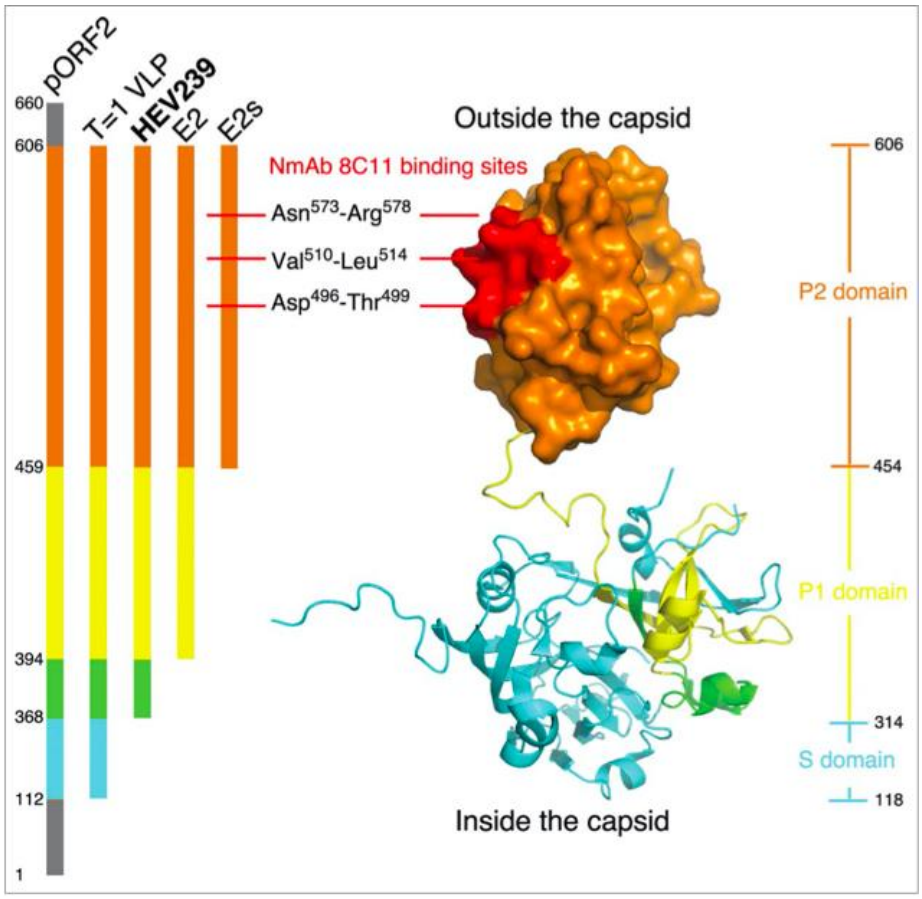


Figure 36: Structural interpretation for HEV 239 vaccine. The structure of HEV ORF2 aa112–606 is render- ing in surface mode (P2 domain) and cartoon mode (P1 and S domain), colored by key regions for several HEV historic constructs (left panel), i.e., aa112–367 in cyan, aa368–393 in green, aa394–458 in yellow and aa459–606 in orange. To elucidate the spatial configuration, 3 crystal structures were in superimposition to demonstrate major neutralization sites (Neutralizing mAb 8C11 binding sites colored in red, by PDB (protein database) no. 3RKD), outmost protrusion domain (E2s domain in orange surface, by PDB no. 3GGQ) and P1&S domains (contributing for virus capsid assembly, by PDB no. 3HAG). The figure was pre- pared by the program PyMol. Adopted from (S. W. Li et al., 2015).

Except Hecolin®, two other efforts led to vaccine candidates in clinical trials. The first one is a vaccine originally developed by the National Institutes of Health (NIH) in the USA with the cooperation of DynCorp (now Novavax) and sponsored by GlaxoSmithKline and U.S. Army Medical Research and Development Command (WHO, 2014; Shrestha et al., 2007). This vaccine, called rHEV or p495-based vaccine, utilize as an antigen the 56 kDa domain of the HEV genotype 1 capsid protein, amino acids 112-608, which is self-assembled into a VLP formation (J. Zhang et al., 2016). rHEV produced in insect cells with the help of a

baculovirus expression system (Li et al., 2020). A phase two clinical trial completed in 2005 (NCT00287469) providing encouraging results for the safety and efficacy of rHEV and since then no other studies programmed. According to Innis and Lynch, the discontinue of this project occurred due to a lack of interest of other partners/ sponsors and the existence of more serious health issues in regions with a high danger of HEV epidemic (Innis & Lynch, 2018). Last but not least, a recombinant VLP-vaccine based on the p179, amino acids 439-617 of HEV genotype 4 capsid protein, was manufactured by Changchun Institute of Biological Products Co., LTD (Li et al., 2020). The vaccine completed in 2016 a clinical trial phase 1 (NCT02603055) resulting that 30µg dosage formulation is safe and well tolerable (Cao et al., 2017). All these three vaccines are manufactured using the technology of Virus-like particles, a platform analyzed before and are adjuvanted with aluminum hydroxide.

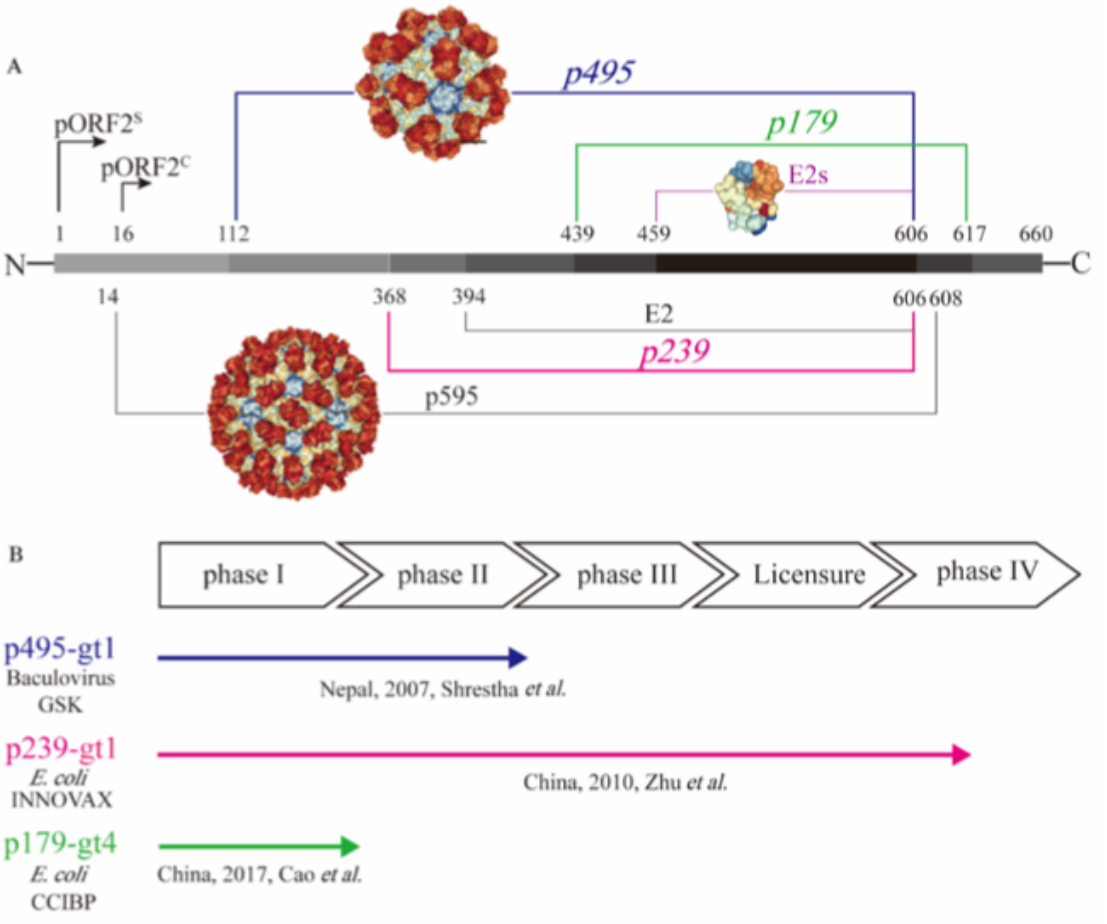


Figure 37: Presentation of different truncated versions of hepatitis E virus (HEV) pORF2. (A) shows the molecular structure of truncated pORF2, and (B) shows three existing HEV vaccines, which have been studied in clinical trials. HEV pORF2 consists of 660 amino acids. HEV p595 (aa 14–608) can form a virus-like particle (VLP) that is similar to the native

virion. The structure of p595 was demonstrated by cryo-EM. HEV p495 (aa 112–608) can form a VLP, and the structure has been determined by X-ray. HEV p495 was used as a vaccine antigen manufactured by GSK, which showed good safety and efficacy in a phase II clinical trial. HEV p239 (aa 368–606), named Hecolin®, has been licensed in China. The HEV p179 (aa 439−617)-based vaccine, which was manufactured by Changchun Institute of Biological Products Co., Ltd. (CCIBP), was safe and well tolerated in a phase I clinical trial. E2 was a useful candidate for diagnostic reagents and was able to form hexamers in solution. The structure of E2s (aa 459–606), the shortest version to form a dimer harbouring the major neutralizing epitopes, was determined at a high resolution. Adopted from (Y. Li et al., 2020).

6.3 Human Papilloma Virus vaccine

Papilloma viruses, belonging to the Papillomaviridae family, are non-enveloped DNA viruses with a size of approximately 55 nm (Zheng & Baker, 2006). Until 2020 more than 400 different genotypes have been submitted in genome databases from which 228 cases concern viruses that affect human populations, also known as human papillomaviruses - HPVs (*https://pave.niaid.nih.gov*; *https://www.hpvcenter.se*). HPVs are of special interest as they have been connected with various mucosal epithelial cancer types, while almost 5% of cancers have been linked with HPV infections (D. Wang et al., 2020) and 640,000 cancer cases are estimated to be contributed to HPV (WHO, 2017a).

HPVs are non-enveloped DNA viruses and they are classified in five genera, α-, β-, γ-, mu- and nu-papillomavirus genus. The division is based on the DNA analysis of the open reading frame (ORF) area that encodes the L1 capsid protein of HPVs. Two human papillomaviruses must have more than 60% similarity of the L1-ORF nucleotide sequence to belong on the same genera (Bzhalava et al., 2015). β- and γ-papillomaviruses are typically harmless or evoke benign cutaneous warts. Some of α-papillomaviruses seem to be more aggressive than others in the same genera. These types have been characterized as high risk or (probably) carcinogenetic types (HPVs type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). Additionally, another group of possible carcinogenic HPVs is known (HPVs types 26, 30, 34, 53, 66, 67, 69, 70, 73, 82 and 85). Finally, HPV-6 and HPV-11 are only responsible for genital warts (Roden & Stern, 2018). Many other HPVs of all genera have not been found to be responsible for cell-death or any disease, leading to the result that they shall be primitive viruses with a long history. The long-lasting relationship with their host (human)

contributed to the development of the “smartness” for both organisms, virus and host, to coexist without being harmful to each other (Doorbar et al., 2012).

The DNA of HPVs is an ~8 kb circular DNA, which is divided into three main domains: the early (50% of the viral genome), late (40% of the viral genome) and non-coding regions (10% of the viral genome). The first one includes ORFs encoding non-structural proteins (E1-E8), while the late region ORFs are responsible for the expression of the structural proteins, major capsid protein L1 and minor capsid protein L2 (Zheng & Baker, 2006). These two proteins are assembled to form a T=7 icosahedral capsid (Prasad & Schmid, 2012; D. Wang et al., 2020). The L1 proteins assemble spontaneously into 72 pentamers (capsomeres), which then organized to the final capsid formulation. In the center of some capsomeres an L2 protein is encrypted (12-72 L2 monomers). The L2 capsid proteins are not necessary for the formation of the virions but they do play a key role in the infectious rate and the proliferation velocity of the viruses (Yadav et al., 2020).

The HPV infection occurs and is limited within the squamous epithelium, without inducing systemic phenomena (Roden & Stern, 2018). The virus enters the epithelium via an abrasion and binds to the basement membrane with L1- heparin sulfate proteoglycan (HSPG) interactions (K. M. Johnson et al., 2009). Lysine residues of 3 sites in L1 loops, exposed on the surface, play a key role in the conjunction of the virus with the basement membrane (Richards et al., 2013). This results in a conformational alteration of the virus capsomer and the revelation of some L2 areas. Then, the minor L2 protein subject to proprotein convertase, furin, cleavage that leads to exposure of the N-terminal L2 epitope (amino acids 17-36)(Kines et al., 2009; Schellenbacher et al., 2013). Thus, the virus can insert into the basal keratinocytes and continue its life-circle, taking advantage of the wounding mechanisms of cutaneous or mucosal epithelium (Kines et al., 2009; Roden & Stern, 2018).

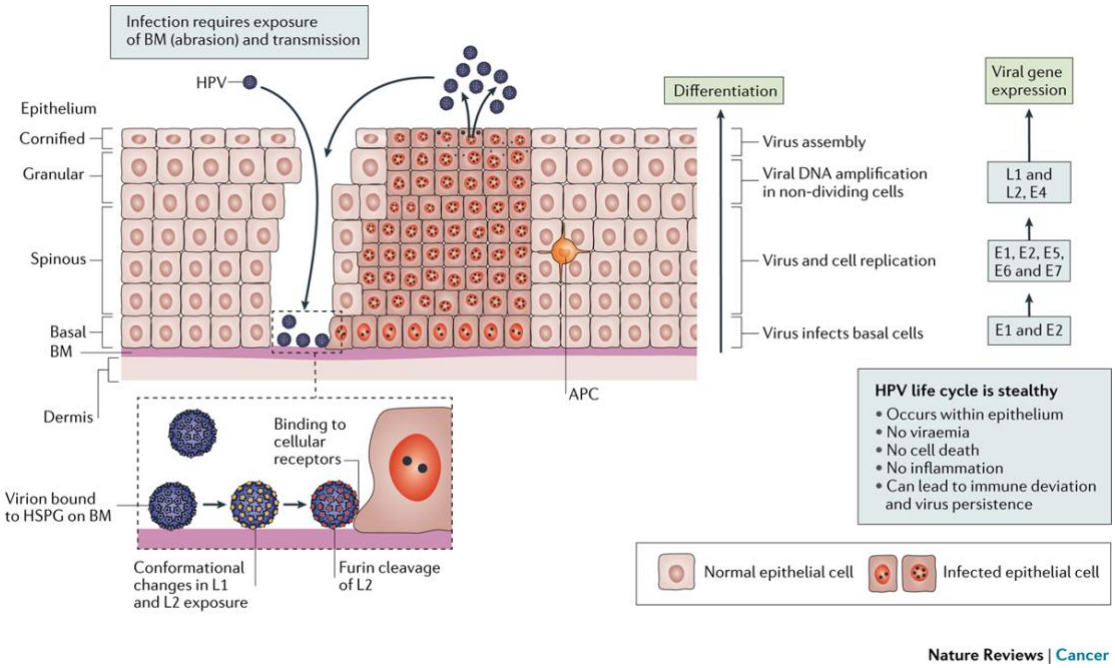


Figure 38: The life cycle of HPV. Abrasion, which leads to denudation of the basement membrane (BM) from epithelial cells, provides access to the basal keratinocytes. During the course of human papillomavirus (HPV) infection, the virus binds to heparin sulfate proteoglycans (HSPGs) and/or laminin 5 on the BM through the major capsid protein L1. This triggers conformational changes in the capsid that further expose the minor capsid protein L2, including a conserved site on the L2 amino terminus that is susceptible to cleavage by extracellular furin. Furin cleavage of L2 reveals several conserved protective epitopes of L2, including residues 17–36, on the capsid surface and is critical to infection. This is followed by virus uptake into the target basal keratinocyte. Several uptake pathways have been implicated, none of which are necessarily mutually exclusive. In the infected basal cells (which might include stem cells), the viral genome replicates and establishes ~50 HPV episome copies, which then segregate between the daughter progeny as the cells undergo cell division. The early viral proteins E6 and E7 are key to stimulating the continued proliferation and milieu for E1 and E2-driven vegetative viral genome replication to a very high copy number. Terminal differentiation of infected cells in the upper epithelial layers activates the expression of E4 and then L1 and L2 to package the very high copy numbers of the viral genome. The virions are released as E4 disintegrates the cytokeratin filaments, and the keratinocyte remnants are sloughed off the epithelial surface. Thus, the viral life cycle is completed without directly causing cell death and without systemic viraemia or apparent inflammation to avoid alerting the local immune responses. Adopted from (Roden & Stern, 2018).

Due to the fact that high-risk HBVs (hrHPVs) are implicated in the appearance of cervix - 99% of cervical cancers contain HPV strains (Roden & Stern, 2018) -, penis (51%), vulva (15-48%), anus (88%) and oropharynx (13-60%) cancer (WHO, 2017b), efforts have been made for the development of safe and effective prophylactic vaccines against these virus strains. Knowing that HPV-16/18 are associated with at least 70% of cervical cancers (Serrano et al., 2015) and 80% of non-cervical HPV-related cancers (WHO, 2017a), these strains were considered as the major HPV types for the manufacture of a cost-effective vaccine. As a result, the first HPV (HPV 6/11/16/18) and second VLP-based vaccine approved by FDA and EMA in 2006 with the trade name Gardasil® by Merck (EMA, 2007b; Qian et al., 2020). Gardasil® is a quadrivalent formulation that contains 4 different virus-like particles (VLPs), each of whom assembled by the L1 surface protein of one type HPV (6, 11, 16 or 18). The VLPs are produced in yeast cells (Saccharomyces cerevisiae) via the recombinant DNA technology and have an average diameter of 60 nm. It contains 20 μg of type 6 and type 18 HPV L1 proteins and 40 μg of types 11 and 16 L1 proteins. Moreover, Gardasil® is adjuvanted with amorphous alluminiun hydroxide sulphate - AAHS (0.225 mg Al) (EMA, 2017b). This vaccine fully protects against exposure to HPV 16 and 18, main causatives for a plethora of anogenital malignancies as well as from HPV 6 and 11, responsible for the manifestation of anogenital warts (Patel et al., 2013). Cervarix®, licensed in 2007 (EMA and FDA) from GSK is a bivalent prophylactic vaccine for HPV 16 and 18 (EMA, 2007a). The L1 VLPs of Cervarix® are produced using a Baculovirus expression system in insect (Trichoplusia ni) cells (WHO, 2017b). 20 μg of HPV-16 and HPV-18 L1 (40μg of L1 protein in total) are included in the 0.5 mg suspension. AS04 [3-*O*-desacyl-4'-monophosphoryl lipid A or MLP (50μg) are adsorbed on aluminum hydroxide, hydrated (0.5mg of Al^{+3})] is utilized as an adjuvant (EMA, 2019a).

Recently, Gardasil® has been replaced by Gardasil-9®, a nonvalent evolution of the first one, as it provides protection against 5 more hrHPV stains (HPV 31/33/45/52/58). Gardasil-9® uses the same adjuvant and expression system as Gardasil® and the quantities of each L1 protein range between 20-40 μg (EMA, 2020b). All these three vaccines should be storage in 2-8ºC and are administrated intramuscularly. Two (females 9-14 years old) or three (females ≥15 years old) doses are needed for the build of an effective immunogenicity (IgG titers) for a remarkable period of time (WHO, 2017b). After the successful completion of a safety and efficacy phase 3 clinical trial (Qiao et al., 2020), Cerolin®, manufactured by Xiamen Innovax, approved by China National Medical Products Administration (NMPA) in

December 2019 as a prophylactic recombinant HPV vaccine. Cerolin® technology uses Escherichia coli as an expression system of HPV-16 and HPV-18 L1-VLPs (Wong et al., 2020). Moreover, it is an adjuvanted vaccine with aluminum hydroxide (Qiao et al., 2020).

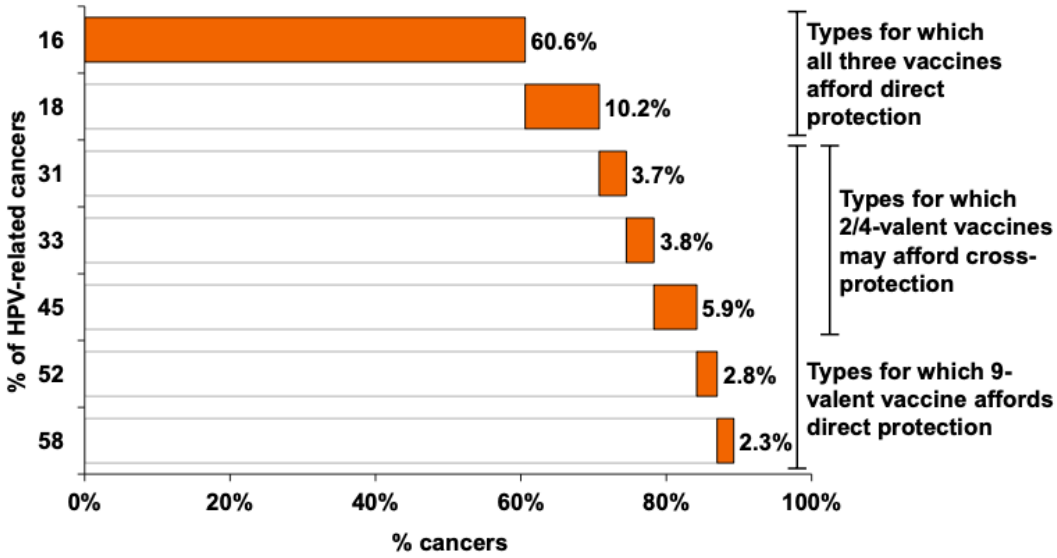


Figure 39: Relative contribution of different viral types to cervical cancer. Adopted from (WHO, 2017a).

Since the first two vaccines are on the market for over 10 years, some important meta-analysis results have been held. A notable observation is that although Cervarix® contains lower VLP concentration than Merck vaccine, 5 years after vaccination, the subjects how had vaccinated with the bivalent formulation (2vHPV) presented higher titers of neutralizing antibodies (nAbs) for HPV 16 and 18 than those received the 4vHPV vaccine. The variation was more obvious for elderly people, as the decrease of titers was faster. In spite of the difference of nAbs amount, both vaccines presented an excellent seroprotection (100% for Cervarix® and >95.7% for Gardasil®) (Einstein et al., 2014). The differences in immunogenicity may occur due to their adjuvants. Gardasil® is adjuvanted with aluminum salts, which activate T_H2 cells via extension of antigen presentation. On the other hand, Cervarix®, except for alum, also utilize MLP that is a TLR4 agonist. In this way, it provides an enhanced activation of innate immune response and cytotoxic T lymphocytes, associated with T_H1 route(Herrin et al., 2014; Smith et al., 2013).

Another important issue is whether these vaccines can be prophylactic for other similar HPVs of the same species. For example, HPV-31 and HPV-33 are very similar to HPV-16 and HPV-45 to HPV-18. Indeed, nAbs against infections of the above hrHPVs are observed,

although their titers are noticeably lower (Kavanagh et al., 2017; Malagón et al., 2012; Mesher et al., 2016). Until today we have not verified data of the antibodies' threshold needed for an effective immunogenicity, but there are promising results for the existence of cross-protection as these three viruses are linked with another 13% of cervical cancer cases (WHO, 2017b). Gardasil-9® seems to have an even better prophylactic profile associated with the different types of L1-VLPs it contains (WHO, 2017a). However, as the number of HPV types increases, so does and the complexity of the formulation and as a result the manufacturing process and the vaccine price (Roden & Stern, 2018). In addition, some serious efforts are made for the evolution of a next-generation HPV vaccines that contain L2 chimeric or hybrid systems (Yadav et al., 2020). Taking into account that L2 amino acid 17-36 cross-neutralizing domain remains conserved for different HPV types, higher titers of nAbs could be produced by these formulations in comparison with the conventional L1-VLPs. L2 monomers alone cannot assemble into VLP and do not provide the desired quantities of nAbs, but, for example, their addition to the L1-formulations would have encouraging results, as these chimeric VLPs have a greater chance for biomimicking the exact conformation of the virions (Schellenbacher et al., 2013). Such vaccines may also provide prophylaxis against β-Papillomaviruses that cause cutaneous warts (Schellenbacher et al., 2009).

7 Future Perspectives

As it is analyzed above, the mammalian immune system is a very complex system. The immune cells can identify and fight any pathogenic stimuli that occurs from both external stimuli, such as pathogens, or internal signals from dysfunctional host cells. According to the very interesting procedure by Cohen and Efroni, we shall not correlate its function with a binary self-not-self algorithm. The computation of each immune cell that accepts certain information from its environment as well as the interaction with other cells of the immune system is the reason for the production of an effective response. The excellence of this system to receive and respond immediately to a plethora of different messages is remarkable, noting that immune cells are blind to recognize information that does not activate its receptors. Interestingly, the researchers correlate our immunity mechanism of action with terms from computer science, especially from the section of artificial intelligence, such as crowd wisdom and machine learning (Cohen & Efroni, 2019).

We would like to go the Cohen and Efroni observations a step further and connect them with quantum mechanics. Principles such as the impossibility to differentiate the input and output as well as the hardware (chemistry) from the software (bio-information) -talking with computational terms - and the immediate communication and interaction of a population (crowd wisdom) are basic phenomena observed in the science of quantum computation (Davies, 2004). Although such an approach may sound weird at first, it is reasonable if we think that several important decisions of the immune system are based on events that happen in the nano-scale, where quantum effects are proven to exist (McFadden & Al-Khalili, 2018). Quantum coherence, tunneling or even superposition are some of the principles that might explain contemporary questions in the area of immunology.

Quantum biology is inextricably connected with the fathers of quantum mechanisms and especially Erwin Schrödinger and a series of his lectures entitled “What is life? The physical aspect of the living cells” (Schrödinger, 1944). Nowadays, some interesting works have been made in this area, although they are still on baby steps. The lack of the right equipment and the extremely complicated biological systems are the basic obstacle for its evolution. To conclude, we propose that if scientists try to understand the human immune system as a computational system that interacts with bio-information it would be useful to connect it not with conventional binary system computers but, with quantum computers or even quantum super-computers if we accept that a single cell behaves like a quantum computer.

Moreover, regarding that human immune response depends on interactions in the nano-scale, it is beneficial to study nano-formulations as vaccine platforms. The results of their use in vaccine technology can be classified into three axes: a) economy, b) effectiveness, and c) safety. Firstly, nanotechnology can be useful for the development of innovative platforms that can be administrated via alternative routes than the usual ones. For instance, nasal (Marasini et al., 2017; Nakahashi-Ouchida et al., 2018) intradermal (Al-Zahrani et al., 2012; Caucheteux et al., 2016) or mucosal (Faruck et al., 2020; L. M. Johnson et al., 2020; Y. Zhang et al., 2018) formulations can be produced by utilizing nanoparticles. Although these sophisticated formulations might seem expensive, the final immunization per person cost can be decreased in comparison with classic formulations. This can be explained due to the minimization of doses that are needed to build immunity, the ability to combine more than one antigenic epitopes and development of multifunctional chimeric vaccines (cross-protection), or the lower storage cost (e.g. in many cases innovative formulations can be durable to a wide temperature range and there is no need for cooling systems, a problem that low-income countries are usually worried about).

Secondly, due to the special immunogenic properties of nano-formulations, scientists are making an effort to develop vaccines against pathogens there is not protection with classical formulations. Due to the high surface-to-volume ratio of nanovaccines, enhanced immunogenicity of both innate and adaptive immunity can be produced. The enrichment of such formulations with the right adjuvant that will activate certain pathways of the immune response can target to the certain key cells. HIV-1 (Brinkkemper & Sliepen, 2019), Zika (Shanmugam et al., 2019) and Ebola (H.-W. Yang et al., 2017) are some of the viruses that nanovaccines have shown promising results. Today such formulations are in clinical phases and the next years we assume that some of them will be approved by the international medicine agencies.

Safety is one of the biggest issues when new technologies are discussed and nanovaccines are not an exception. Toxicological issues about nanotechnological products have not yet been fully understood and more research is needed to conclude in some general results. Currently, each vaccine is evaluated as a unique formulation because there are no central instructions. Some structures such as certain VLPs have been used for a remarkable time and their safety is approved, whereas some others as inorganic nanoparticles enhance serious questions about their bio-distribution. Even in the case of VLP-based vaccines scientists have not conclude in some standard tests that are needed to develop effective and safe vaccines. Apart from safety for vaccinees, the production of nanotechnological products can

be dangerous for the staff and the environment too. Nanoparticles that are diffused in the atmosphere could be responsible for great undesirable effects in animals, mainly based on the bio-accumulation in living tissues. Hence, pollution of the air, water, and earth with nanoparticulate effluents could conclude in toxic effects in the animal population, food chain disorder or even the disappearance of endangered species. Fortunately, the evolution of nanotechnology in other areas, such as the one of electronic devices, led the governments to adopt a legislative framework about the ecological safety. This framework contains the nanomedicinal products, although they have not been extensively used yet.

In-vivo trials as well as human trials are necessary today for the development of immunogenic and safe vaccines. Due to ethical issues, scientists are trying to find effective methods to decrease the number of subjects that are necessary not only in vaccine but also in drug development. We hypothesize that systems biology and 3D-bioprinting would have a promising contribution to accomplishing the above purpose. Over the last years, it has been evident that a disease is not attributed to a single factor (e.g. a gene) but several complex processes are taking place at the same time. Thus, the approach of single molecular targets is obsolete and more holistic processes have been developed. Systems biology uses big datasets of the 'omics' - transcriptomics, metabolomics and proteomics - and with the help of computer science predict the behavior of a biologic system, for instance, a cell, tissue, organ, or even a living organism to certain stimuli (Schneider & Klabunde, 2013). Developing in silico models that could forecast the systemic effect of the administration of an active substance could minimize the number of in-vivo trials. However, nowadays, this technology is not ready to been exploited by pharmaceutical companies for early-stage drug or vaccine development as specialized models are developed with very strict parameters so that the model leads to reliable conclusions. Measurement of critical process parameters and optimization of quality by design (QbD) processes would be the first fields of pharmaceutical manufacture that will take advantage of systems biology (Richelle et al., 2020). Along the way, we assume that more advanced systems will be developed that could provide us reliable results via in silico tests.

3D-bioprinting is another innovative idea for minimizing in vivo preclinical animal tests or human clinical trials. 3D-printing is mainly known for the printing of 3D structures with complex architectures. The materials that can be printed are thermo-responsive that allow the materials' phase transition from stable to liquid or gel phase and vice versa. 3D-bioprinting utilizes smart thermo-responsive bio-materials, mainly polymers, that are bio-degradable to synthesize highly hierarchical structures of medicinal implants, tissues, or

organs (H. Lee & Cho, 2016). Except for the designing of complex scaffolds or extracellular matrixes (ECM), modern bioprinters can also deposit cells. Organs-on-a-chip is a promising idea based on microfluidic technology. Microfluidic organ-based platforms could be manufactured by high-technology bioprinters with the prospect of mimicking the functionality of real organs. Thus, scientists can study the effects of administrating a certain formulation (e.g. a vaccine) in a human-like chip of only some micrometers diameter (Sun et al., 2020). Recently, chips that connect more than ten compartments (organs) have been produced and are their ability to correctly predict the real pharmacokinetic and/or pharmacodynamic in vivo behavior in different stimuli is in evaluation stage (Berthier et al., 2020; Novak et al., 2020).

According to all the above, we propose that universal medicine agencies, such as EMA and FDA, should recognize the benefits of utilizing nanotechnology for the development of high quality effective, safe, and economically affordable vaccines and adopt the right legislative framework. In this way, not only high but also low-income countries can have access to the most important human privilege: human health. Utilizing standard well-characterized safe vaccine platforms (as in the case of nucleic acid vaccines) or adjuvants, as well as eliminating the cost for clinical trials via new technologies, will lead in a decrease of the vaccination cost and increase of immunogenicity. To achieve the above, we propose that extensive research for understanding the pathogen-host cell interaction and the mechanism of action for the nanovaccine formulations is necessary. Thus, the study of the quantum effects in the area of nano-vaccinology seems to have a key role and further, specific research will lead to more general and applicable conclusions/suggestions.

8 Conclusion

Nanomedicine is currently one of the most quickly evolving areas of health sciences. Smart nano-formulations are licensed as drug or antigen delivery systems. Moreover, in vaccinology, nanostructures have also been utilized as adjuvants. Due to their special unique properties, these platforms provide benefits in assembling into highly immunogenic formations that can present the desirable epitopes to the cells of the immune system. A variety of properties such as size, shape, surface charge and bio-materials construct particulate platforms that enhance human defense mechanisms with a completely different way. Thus, each class of nano-platforms provides certain advantages and disadvantages, the knowledge of whom would conclude in the right nanoparticle selection for every pathogen. Nanovaccines advantages in comparison with classic formulations is useful for the fast development of safe and effective formulations that could be available worldwide. Although some nanovaccines are licensed nowadays, universal medicine agencies and the world health organization shall provide certain directives for the development and the authorization of such products. Some industry-friendly processes to examine the critical physicochemical and pharmacological properties are mentioned. We believe that the next years will be the peak point for the study and production of novel nanovaccines against infectious diseases and epidemics.

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