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Chapter 7

Summary and perspectives
7.1 – Summary

The best form of protection against influenza is vaccination, in terms of efficacy to protect individuals and reduction of the social impact of epidemics on our human societies. Chapter 1 of this thesis details the current influenza vaccines available and their lack of efficacy, and the current need for new adjuvanted influenza formulations. Pathogens are often particles and formulating antigens into nanoparticles (NP) results in systems that resemble the pathogens in terms of size, and notably can promote antigen uptake by dendritic cells (DC). The principal aim of the research in this thesis was to investigate how NP systems can act as an adjuvant for subunit influenza vaccine.

To achieve this aim two types of NP are described: peptide polymer NP and cationic liposomes, and the following three sub-aims were defined:

- to explore different nanoparticulate systems in order to modulate the immunogenicity of a subunit influenza vaccine;
- to study the impact of the composition, charge and preparation of these systems on their adjuvant effect;
- to investigate the co-delivery of HA antigen with immune-modulators, encapsulated into nanoparticulate systems.

First, the potential of polymer-peptide block copolymer NP as adjuvant for seasonal influenza vaccine was studied (chapter 2-4). For this purpose we customized the constituents and the preparation method. The poly(γ-benzyl L-glutamate)-E (PBLG-E) represents the first of a new class of peptides: polypeptide-b-peptides. These compounds are versatile regarding their chain length and functionality. Different methods are available to produce polymer-peptide based NP, but each of them has limitations and might not be suitable for vaccine delivery. Therefore, in chapter 2 we have developed a new method for producing nanovesicles, also known as polymersomes, from polypeptide-b-peptides PBLG36-E, using a detergent removal method that has been used for many decades to produce liposomes. The method was adapted to be suitable for use with block copolymers, which have different assembly characteristics than lipids. The detergent aided polymersome preparation utilizes detergent molecules (sodium cholate) to molecularly disperse the block copolymer in aqueous solution, a role that is usually taken by organic solvents. The shielding effect of the detergent on the block copolymer is then reduced such that the intrinsic morphology of the block copolymer particles, i.e. polymersomes, emerges. This method has the advantage of not requiring organic solvent or a high energy input (e.g., sonication), known has factors which can denature biomolecules.

Chapter 3 presents a study of the adjuvant effect of polymersomes loaded with a seasonal influenza subunit vaccine (H3N2 A/Wisconsin strain). The polymersome’s building material was PBLG50-K block copolymer and the water-addition solvent-
evaporation method was used to produce the nanovesicles. The block copolymer was dissolved in tetrahydrofluran (THF) and the solution was quickly added to an aqueous phase, allowing the THF to evaporate in a couple of minutes. The PBLG50- K was shown to be assembled into polymersomes with an average size of 250 nm and a negative zeta potential. Then different amounts of vaccine (the purified viral membrane protein hemagglutinin (HA) were mixed with the NP, resulting in a raise of the average size and polydispersity. The physical association of HA and polymersomes in these aggregates was confirmed by transmission electron microscopy (TEM). The immune response induce by the polymersomes was investigated in vivo in a mouse model. The polymersomes succeeded to enhance significantly both total serum IgG and hemagglutination inhibition (HI) titers, compared to non-adjuvanted antigen. However, the polymersome formulation induced a high IgG1 response and a low IgG2a/c response, which is indicative of strong Th2 response, while the Th1 response was rather low.

To optimize the efficacy of the peptide polymer NP toward a Th1 response, two major changes were introduced, as described in chapter 4: firstly, a new peptide polymer copolymer (PBLG30-TAT), based on the amino acid sequence derived from the cell-penetrating TAT peptide; second, an immune modulator (CpG) was encapsulated into the NP. After a comprehensive physicochemical characterization of these new systems, the immunogenicity was tested in vitro with human DC, and the formulations showed the ability to induce an upregulation of maturation markers (MHC-II and CD86) when codelivered with CpG (NP/HA+CpG). Furthermore, after intramuscular vaccination in mice, the NP/HA+CpG formulation elicited stronger HI titers compared to non-adjuvanted NP/HA and the Al(OH)3/HA control. Besides, NP/HA+CpG provoked significantly higher levels of IgG2 a/c antibodies compared to all other formulations.

In chapter 5 & 6, cationic liposomes’ adjuvant mechanism was investigated, using HA as a model antigen. The different immunological effects induced by cationic liposomes, when they are used for vaccine formulation, suggest that not only their cationic charges initiate their adjuvant abilities by increasing antigen protection and delivery, but also that possible specific effect of the lipids or liposomes exist. However, previous studies used liposomes that are not directly comparable (e.g., due to the use of variable antigens, different administration routes, etc.). Therefore, in chapter 5 we studied how the content and the physicochemical properties of the positively charged compound influence the adjuvant effect of cationic liposomes. In order to enable the focus on the liposome content, we prepared cationic liposomes made of different cationic compounds (DDA, DPTAP, eDPPC ) but with similar physicochemical characteristics (size, zeta potential, bilayer rigidity, etc.), loaded via adsorption with HA. We also included DC-Chol based liposomes in our comparison, while recognizing that their characteristics will differ from the other cationic compounds with respect to both the head group and the hydrophobic tail. In a mouse model, HA adjuvanted with the DC-Chol/DPPC
liposomes elicited significantly higher total anti-HA antibodies (IgG1 and IgG2a/c) and HI titers compared to the other liposomal HA formulations and non-adjuvanted HA. However, it was not clear whether the cholesterol backbone or the tertiary amine head group of DC-Chol was responsible for this.

Therefore, in chapter 6 the influence of cholesterol in the lipid bilayer of cationic liposomes on the immunogenicity of adsorbed HA was studied. For this purpose, liposomes consisting of a neutral lipid (DPPC or cholesterol) and a cationic compound (DDA, or eDPPC) were produced and characterized. They generally showed comparable size distribution, zeta potential and HA loading. Furthermore, in vitro studies of the formulations with monocyte-derived human DC and immunization studies in C57BL/6 mice showed that the incorporation of cholesterol in the bilayer of cationic eDPPC liposomes enhances the cellular uptake and also their adjuvant effect in vivo. Moreover, to further improve the immunogenicity of HA-loaded DC-Chol liposomes, they were loaded with CpG or imiquimod. Whereas encapsulation of imiquimod did not seem to have any impact on the immune response, encapsulation of CpG in DC-Chol liposomes enhanced significantly the IgG2a/c titers against adsorbed HA compared to HA adsorbed to non-adjuvanted DC-Chol liposomes or Alhydrogel, and showed increased IFN-γ production by restimulated splenocytes.

7.2 - Perspectives

This thesis has set out to study the impact of nanoparticulate adjuvants on the immune response against the antigen, HA. In the next sections several important aspects of adjuvanted influenza vaccine design are discussed and recommendations for future development are provided.

7.2.1 - Further investigation of the immune response induced by nanoparticulate adjuvanted influenza vaccines

Several aspects concerning nanoparticulate influenza vaccine formulations have been studied in this thesis, in particular the characterization of the formulations and their immunological effects (in vitro and in vivo). The results obtained showed that both cationic liposomes and peptide polymer NP have good adjuvant properties, enabling the raise of the immune response against HA. Importantly, IgG2a/c antibodies were significantly raised when HA was formulated in NP (chapter 4 & 6), which reflects the induction of a Th1 response (confirmed by the increase in IFNγ secretion), and likely a CTL response which is more efficient at eliminating influenza infected cells. In order to evaluate the protective effect our best formulations, a challenge in mice (or ferrets) should be performed to determine if this increased
immunogenicity results in a better protection against influenza infection. Moreover, the dose-sparing ability of our adjuvant systems should be studied.

Subunit influenza vaccines might be less efficient at inducing cell-mediated immunity normally induced by natural infections. Subunit influenza vaccines are “clean vaccines”, purified and with a low content of influenza nucleoproteins, and rich in HA protein which is the most variable region of the virus. However, if the vaccines induce a humoral and a cell-mediated immune responses directed to conserved regions of influenza virus, they are more likely to induce protective immunity to a large variety of influenza viruses, including drift variants and viruses of novel subtypes. For instance, MF59 can both improve the antibody responsiveness to influenza and redirect the quality of the antibody response against influenza antigens. This oil-in-water emulsion induced more cross reactive responses when administered with split or subunit H5N1 vaccines than non-adjuvanted or aluminum-adjuvanted vaccines [1] [2]. Similarly, investigation of cross-reactive responses after immunization with our nanoparticulate influenza vaccines would be of high interest.

Also, the preliminary DC maturation studies conducted on human monocyte-derived DC suggest an immunostimulatory adjuvant effect of our formulations. Future experiments should be done to investigate whether the influence of these formulations on the immune response is the same in murine DC and in other animal models, along with toxicity studies (local and systemic). Additionally, more effort should be put to investigate the persistence of the immune response and to study the immunogenicity induced by other routes of immunization, especially the intradermal route. Intradermal vaccination, e.g. by using microneedle-mediated delivery, could also be an attractive alternative to intranasal immunization in the context of the potential induction of protective mucosal IgA. Nasal administration has been associated with adverse effects, such as the occurrence of Bell’s palsy syndrome (facial nerve paralysis) induced by an adjuvanted virosomal vaccine after intranasal immunization in humans [3].

7.2.2 – Specific interaction between delivery systems with the influenza antigen

The core of each vaccine formulation is the antigen. It is therefore not surprising that the rational design of a vaccine should be based on the characteristics of the antigen. The model antigen in this thesis is (HA) a viral membrane protein, which is water soluble and negatively charged at physiological pH. As noticed in the different in vivo studies conducted in this thesis, HA is immunogenic when injected alone, but it induces mainly IgG1 antibodies and moderate HI titers. As demonstrated in chapter 4 & 5, HA interaction with cationic liposomes and peptide polymer NP is driven by electrostatic forces. Moreover, the antigen’s lipophilic domains may profit from formulation in liposomes as these membrane proteins can be incorporated in
the liposomal bilayer, thereby mimicking more closely the natural way these antigens are presented to the immune system. Cryo-TEM characterization of the liposomes produced could help understand if the positioning of HA could contribute to the differences observed between our formulations. Interestingly, in Chapter 3 the interaction of HA with the peptide polymer NPs showed that HA/polymersome association was presumably a combination of both electrostatic and hydrophobic interactions, arising from the hydrophobic membrane-anchoring domain of the HA, the localized charge on the HA, and the charged corona of the polymersomes.

Moreover, the action of HA is not limited to its immunogenic properties. Following endosomal uptake, acidification within the endosomes induces HA-mediated fusion (resulting from a conformational change in HA), which likely leads to release of the liposomes or peptide polymer NP into the cytoplasm and a potential MHC class I presentation. Therefore, even though this was not investigated in this thesis, it may be that HA itself enhances its own delivery into the cells, leading a potent immune response.

7.2.3 – Need for a better understanding of the NP’s adjuvant mechanisms

The immunogenicity of HA was notably improved by the use of DC-Chol liposomes. Although the presence of cholesterol does induce specific interaction of the liposome with the biological systems, it remains unknown whether the presence of the cholesterol backbone was also the reason for the superior immunogenicity of HA/DC-Chol:DPPC liposomes and, if so, by which mechanism. In Chapter 5, the use of DC-Chol liposomes loaded with HA in a mouse model increased the antibody responses, both the IgG1 and IgG2 a/c antibody responses, which is consistent with the results obtained in previous studies where DC-Chol lipids administered with hepatitis B surface antigen [6] or monovalent split inactivated influenza vaccine (H1N1) resulted in improved immune responses in animal models [7].

Studies to elucidate the mechanisms by which DC-Chol liposomes act as adjuvants have suggested a role for the chemokine CCL2, secreted by epithelial cells and involved in Langerhans cell recruitment [8] and complement activation [9]. Another mechanism may be the ability of DC-Chol liposomes to associate with antigen and initiate a depot-effect [10]. The raise of chemokine secretion probably leads to DC migration and local inflammation. It would be interesting to study the role of plain DC-Chol liposomes, by injecting separately the liposomes and the antigen at the same site of injection, which should preclude the antigen depot effect and enable the monitoring of the potential recruitment of the APCs and macrophages, and the activation of the NLRP3 inflammasome pathway. Finally, despite the concern about eventual cytotoxicity of cationic liposomes, DC-cholesterol has already been tested in the clinic with an HIV recombinant gp160 antigen [11]. The results showed a good tolerability of the vaccine after nasal or vaginal administration.
Following the work achieved in this thesis, the future of the peptide polymer NP as adjuvant system shou

ld be addressed. Our studies showed some efficacy for their use in vaccine formulations with strong adjuvant and vaccine carrier abilities, allowing co-delivery of the antigen with a TLR-9 ligand (CpG). Insights into the liposome adjuvant mechanism could partially be extended to these new systems, which could notably be explained by a depot effect. However, more investigations should be carried out in order to get a better understanding of their adjuvant mechanism, and the influence of the formulation. Different immune stimulators could be either added or incorporated to further enhance the immunogenicity. Since vesicles composed of polypeptide-b-designed peptides can be easily functionalized, it is expected that these peptide-based NP will be able to act as delivery vehicles to specific targets in the body. Furthermore, their peptide sequence could be designed to modulate the immune response. For instance, the IC31® adjuvant [12], currently in phase II clinical trials, is based on the cationic peptide KLKL5KLK with successful results with the tuberculosis antigen Ag85B [13] [14].

Finally, an ideal vaccine should contain a sufficient amount of immune modulators for the activation of the innate immune response and alert the immune system, but at the same time without causing hyper-immunostimulation (which may result in anaphylactic shock or local tissue damage by excess of inflammatory mediators). The immune modulator(s) should also stay associated with the antigen until uptaken by APCs. The NPs seem to be the ideal system to ensure such co-delivery.
References


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