Liposome-based vaccines for immune modulation: from antigen selection to nanoparticle design

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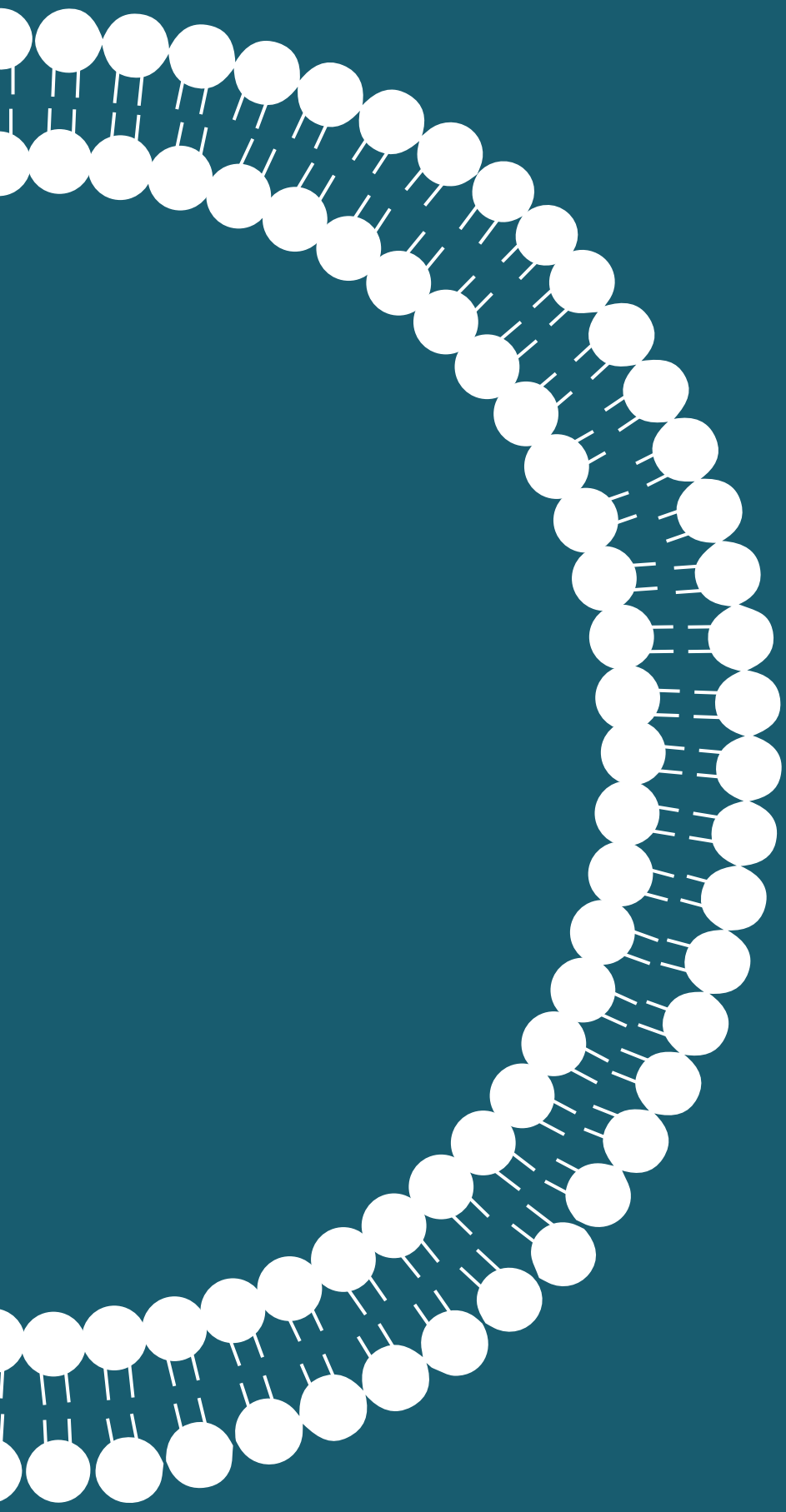

Chapter 8

General summary and future perspectives

GENERAL SUMMARY

The immune system is a set of organs, cells and molecules that defend the organism against pathogens or substances recognized as foreign. It can be broadly divided in two arms, the innate and the adaptive immunity. The innate immunity is the first line of defence against an infection, it is rapidly mounted, but it is not antigen-specific and it cannot generate immunological memory. The adaptive immunity, on the other hand, needs more time to develop since it requires the clonal expansion of antigen-specific immune cells but it can generate immunological memory that will rapidly mount a secondary immune response upon reinfection with the same pathogen. Antigen-presenting cells (APCs) are the connection between innate and adaptive immunity, since they can capture antigens, process them, and present them in their cell surface to cells from the adaptive immune system such as T cells, initiating the adaptive immune response[1]. An over or under activation of the immune system is the root of many diseases. In autoimmune diseases, the natural tolerance towards self-antigens is broken, leading for example to the destruction of β cells in type 1 diabetes (T1D) or the myelin sheath of neurons in multiple sclerosis (MS) [2, 3]. Not only in autoimmune diseases, but also in other highly prevalent diseases, such as atherosclerosis, the main underlaying cause of cardiovascular diseases, the inflammatory response triggered against self-antigens seems to be involved in the aetiology of the disease[4]. In other cases, the immune system can fail to mount a sufficient immune response against a pathogen leading to widespread infection that can cause the host's death. In this thesis, we show that liposomes, a versatile type of nanoparticle, can be applied to restore immune tolerance in autoimmune diseases and to activate antigen-specific protective immune responses against infections.

Nanoparticles can be used as delivery systems for both small molecules and macromolecules such as proteins, peptides or oligonucleotides. The majority of this thesis focuses on the use of liposomes, nanometric vesicles formed by a phospholipid bilayer enclosing an aqueous core. Liposomes are highly versatile delivery systems since they can transport both hydrophobic and hydrophilic cargo loaded in the phospholipid bilayer or in the aqueous core, respectively. Furthermore, fine-tunning their physicochemical properties such as size, shape, rigidity or surface charge (ζ-potential) allows the control of the liposome's biodistribution and biological effect[5]. Among the different applications for liposomes, antigen delivery is especially interesting. Liposomes can protect antigens from proteolytic degradation, and they can direct the antigen delivery to specialized cells such as APCs[6]. Furthermore, the co-delivery of antigens and molecules with adjuvant capacity allows the modulation of immune responses. Liposomes can transport cargo to APCs, such as dendritic cells (DCs), by taking advantage of the intrinsic high endocytic capacity of these

cells[7]. The type of antigen presentation by DCs determines the type of adaptive immune response. For example, the presentation of antigens in the context of a high expression of co-stimulatory molecules such as CD86, CD80 or CD40 and pro-inflammatory cytokines will skew the immune response towards a pro-inflammatory response mediated by Th1 or Th17 lymphocytes, necessary to fight viral and bacterial infections[8]. On the other hand, the presentation of antigens by DCs in the context of low levels of co-stimulatory molecules and high levels of anti-inflammatory cytokines will induce a tolerogenic responses mostly mediated by T regulatory cells (Tregs)[9]. The use of nanoparticles for the co-delivery of antigens and molecules that modulate the expression of co-stimulatory signals is therefore a promising strategy to apply in conventional prophylactic vaccines and tolerogenic vaccines to restore immune homeostasis in autoimmune diseases.

Although lipid nanoparticles have been widely and successfully used in the SARS-CoV2 vaccines against the COVID-19 pandemic, there are still many unknowns that prevent nanoparticle-based therapies and vaccines to realise their full potential. In this thesis, we try to shed light to some of the gaps in knowledge in the field. On one hand, there is still limited knowledge on the precise contribution of the different physicochemical properties of nanoparticles to the elicited immune response. This is particularly challenging to study in the case of liposomes where altering the lipid composition will likely lead to changes in several physicochemical and biological properties of the nanoparticle such as ζ-potential, rigidity and protein corona. Furthermore, although there have been significant advances in the last few years in the mass production of liposomes and lipid nanoparticles, certain formulations are still challenging to produce in a high-throughput manner. For instance, liposomes containing phospholipids with high transition temperatures such as DSPG. The high rigidity and negative charge of DSPG-containing liposomes are key for the induction of Treg responses by tolerogenic vaccines as previously shown[10]. Finally, in the case of tolerogenic vaccines, a key challenge to overcome is the need to precisely characterize the antigens and epitopes driving autoimmunity. This is specially challenging in diseases that are not classical autoimmune diseases, such as atherosclerosis, where the immune response towards self-antigens is one of the factors contributing to the disease's progression, together with genetic and environmental factors[4, 11, 12].

In **Chapter 2**, we review tolerogenic strategies against prevalent autoimmune diseases such as multiple sclerosis (MS) and type 1 diabetes (T1D). We summarize the lessons that can be learned from the efforts to bring these therapeutic approaches to the clinic and the challenges to apply tolerogenic therapies to atherosclerosis, one of the most prevalent chronic diseases in the western world[13]. Atherosclerosis is an inflammatory disease of the arteries, characterized by the

infiltration and accumulation of low-density lipoproteins (LDL) in the subendothelial space of the arteries, forming atherosclerosis plaques. The accumulation of LDL particles also triggers the recruitment of immune cells to the incipient lesion, initiating an inflammatory response that leads to further grow of the plaque[14]. These plaques can restrict blood flow to certain parts of the body or rupture generating a thrombus, which leads to the most common clinical manifestation of atherosclerosis in the form of myocardial infarction or stroke. Several lines of evidence highlight the importance of autoimmunity in the development of atherosclerosis. On one hand, the presence of auto-reactive B and T cells and circulating antibodies against LDL demonstrates that the immune response in atherosclerosis is, at least in part, directed towards self-antigens[15, 16]. Furthermore, there is a clear correlation between atherosclerosis and classical autoimmune diseases, such as rheumatoid arthritis (RA), with RA patients having a significantly higher risk of suffering a cardiovascular event[4, 17]. Finally, the opportunity to target the immune component of atherosclerosis as a therapeutic strategy is evidenced by the significant residual cardiovascular risk of patients after intensive statin therapy, mostly associated with higher levels of inflammatory markers[18]. Clinical trials, such as the CANTOS or COLCOT trials, have shown that systemic immune suppression can significantly reduce the incidence of cardiovascular events, but at the expense of higher risk of fatal infections[19, 20]. Therefore, the side effects of chronic immune suppression would hardly be acceptable for the prevention of cardiovascular events. The induction of antigen-specific immune tolerance, mediated by Tregs, would be a better approach and it has been studied in clinical trials against RA, T1D and MS. In these diseases, both cell-based and peptide-based strategies have been used in clinical trials and have proven to be generally safe and well-tolerated. The use of cell-based approaches involves the *ex vivo* differentiation of DCs into tolerogenic DCs and the coating to the cells with the target antigens. The costs associated with the manufacture of these tolerogenic DCs has often limited the number of patients enrolled in the trials and therefore the power of the safety and efficacy conclusions[21]. The peptide-based approaches have shown promising results in RA, T1D and MS[22-24], however in some cases the efficacy was limited to subgroup of patients with specific HLA types, as seen in a phase II trial with MS patients[22]. The use of cocktails of peptides instead of single peptides has shown better results[25]. The careful selection of the peptide dose is important since too high doses can lead to unwanted pro-inflammatory T cell activation, therefore these clinical trials often include a dose escalation period[25]. Finally, the administration route also plays a key role for peptide-based tolerance induction, with mucosal and intradermal routes showing the best results[24, 26]. In summary, antigen-specific

tolerogenic therapies have shown to be safe but their effectiveness depends on the careful choice of the target antigen(s), dose and administration route.

A key knowledge gap that differentiates autoimmune diseases like MS and T1D from atherosclerosis is that while autoimmune responses against myelin and β cells proteins are clearly characterized, the antigens driving pro-atherogenic immune responses are far less established. The study of antigen-specific immune responses in atherosclerosis is essential for the development of tolerogenic vaccines against the disease. In **chapter 3**, we aim to shed light on this using an immunopeptidomics approach. Previous studies have made use of *in silico* analysis of candidate proteins such as ApoB100 to scan the amino acid sequence of the protein and determine potential good binders to HLA molecules[27, 28]. In this chapter, we use immunopeptidomics to isolate and identify peptides presented by HLA class II molecules directly from atherosclerosis plaques of patients undergoing endarterectomy surgery. We identified 20 epitopes derived from ApoB100, the main protein in LDL particles. Using the expression of the T cell activation marker CD40L as a proxy, we show that a subset of 22% of atherosclerosis patients have detectable levels of $CD4^+$ T cells that respond to these epitopes. Interestingly, the level of $CD4^+$ T cell response in this subset of patients correlated positively with histologically determined plaque vulnerability. Future studies should investigate the use of these ApoB100-specific $CD4^+$ T cell responses as biomarkers of atherosclerosis progression. Further characterization of this $CD4^+$ T cell response showed that upon peptide stimulation, these cells produce IL-17 and IL-10, but also other cytokines such as IL-5, IL-9 or IL-6, suggesting that the ApoB100-specific T cell population does not present a unique phenotype. These findings are in line with previous studies showing that the ApoB100-specific T cell response in atherosclerosis evolves from a Treg mediated response towards a pathogenic Th1/Th17 phenotype in advanced stages of the disease[29]. Restoring the immunological balance by inducing ApoB100-specific Tregs or preventing Th1/Th17 polarization using tolerogenic nanoparticles loaded with the epitopes identified here is a promising therapeutic strategy that should be explored further.

Besides the definition of the target antigens, the delivery system needs to be optimized for tolerance induction in human. Previous studies have shown that liposomes composed of DSPC:DSPG:Cholesterol are good candidates for this task and have shown promising results in animal models[10]. However, the first studies with this formulation in an *in vitro* human system did not recapitulate the tolerogenic properties seen in mice[30]. In **chapter 4**, we show that the translation from pre-clinical models to patients might require the presence of a tolerogenic molecule such as 1α,25-dihydroxyvitaminD3 (vitaminD3). Liposomes loaded with vitaminD3 were able to induce a tolerogenic phenotype *in vitro* in human monocyte-derived DCs. This

tolerogenic phenotype was characterized by the expression of ILT3 and a lower expression of the co-stimulatory molecule CD83. Furthermore, these tolerogenic DCs were able to induce FoxP3$^+$ CD25$^+$ Tregs that also expressed high levels of CTLA-4 and TIGIT. The anionic DSPG liposomes loaded with vitaminD3 were also able to induce IL10-producing Tregs, an anti-inflammatory cytokine essential for the immunomodulatory function of Tregs. This is in line with mouse studies showing that anionic liposomes are better internalized by APCs and have better tolerogenic capacity than cationic liposomes[30]. Most importantly, these tolerogenic DCs inhibited the polarization of T cells towards the pro-inflammatory subsets Th1 and Th17. In a further step towards the translation of these tolerogenic formulations to human, we studied the intradermal administration of vitaminD3-loaded liposomes in *ex vivo* human skin. We observed a selective migration of CD14$^+$ dermal DCs, that have previously shown to be able to induce Tregs[31]. All in all, in this chapter we show that upon inclusion of vitaminD3, anionic liposomes can induce tolerogenic immune responses not only in animal models but also in human *in vitro* and *ex vivo* setups. The next steps in the clinical translation of peptide-based liposome vaccines will require the evaluation of antigen-specific T cell responses in *ex vivo* human models such as the intradermal skin injections shown in this chapter.

After showing in chapter 4 that DSPC:DSPG:Cholesterol liposomes incorporating vitaminD3 have potential applicability in humans, in **chapter 5** we aim to tackle the problem of the manufacturing of these formulations. Traditional lab-scale methods for the preparation of liposomes, such as the lipid film hydration method, have little upscale potential. In short, this method starts with the creation of a dry lipid film by evaporating the organic solvent in a rotary evaporator followed by hydration of the dry lipid film with an aqueous solvent that contains the antigen to be encapsulated[32]. The process often involves a freeze-drying step after the hydration of the lipid film to increase the loading efficiency of the antigen. The hydration step generates a suspension of large multilamellar vesicles that needs to be extruded through multiple filters at high pressure until the desired particle size is achieved. Furthermore, the extrusion process needs to occur at a temperature above the transition temperature of the phospholipids, which in the case of DSPC:DSPG:Cholesterol liposomes is 55°C. This process is labour intensive, involves multiple steps and it has high energy requirements, making very difficult the production of large batches of formulations necessary for the clinical development of these nanoparticles. The production of liposomes using microfluidics is a one-step process that does not require solvent evaporation or extrusion. There are commercially available systems such as the NanoAssemblr® platform from Precision Nanosystems, but this equipment can be expensive, microfluidics cartridges are not reusable, and the temperature control is not optimal. Therefore, in chapter 5 we propose the use of a reusable and off-the-

shelf glass herringbone micromixer for the preparation of DSPC:DSPG:Cholesterol liposomes. The formation of liposomes in this system is dominated by the controlled mix of an organic solvent containing phospholipids and an aqueous solvent containing the antigen to load[33]. The micromixer can be fully submerged in a temperature-controlled water bath, ensuing the desired temperature in the mixing channel. Using this system, we show that the average particle size of the liposomes can be fine-tuned by changing the flow rate ratio (FRR) between organic and aqueous solvents and that peptides with a wide range of charge and hydrophobicity can be encapsulated. Furthermore, we show that the encapsulation efficiency of the tolerogenic adjuvant vitaminD3 in DSPC:DSPG:Cholesterol liposomes was substantially increased in the formulations prepared with microfluidics compared to the conventional lipid film hydration method. The biologically active form of vitaminD3 is costly therefore the increase in encapsulation efficiency presents a significant advantage. The manufacture of DSPC:DSPG:Cholesterol liposomes using microfluidics facilitates the production of these formulations under Good Manufacturing Practice (GMP) conditions[34], a pre-requirement for moving these tolerogenic nanoparticles closer to the clinic. Future development of the technology should focus on the use of scalable and in-line methods for the down-stream processing of formulations, for example using tangential flow filtration to separate the liposomes from the non-encapsulated peptides and adjuvants.

Besides the inclusion of tolerogenic molecules, such as vitaminD3, previous research has shown that nanoparticle rigidity is a key physicochemical parameter that determines the tolerogenic capacity of liposomes. For instance, highly rigid liposomes composed of DSPC:DSPG:Cholesterol have shown to have better capacity to induce Tregs compared to liposomes with a more fluid membrane like DOPC:DOPG[35]. The level of unsaturation of the phospholipids´ acyl chains and the presence of cholesterol in the bilayer are the main determinants of liposome rigidity[36]. However, the lipid composition of the bilayer can also affect other properties of the nanoparticle such as the protein corona, the set of proteins that interact with the liposome surface in a biological fluid[37]. In **chapter 6**, we use a hybrid nanoparticle consisting of a PLGA particle covered with a DOPC:DOPG lipid bilayer (DOPG/PLGA hybrids) in order to obtain a highly rigid nanoparticle but with fluid lipid bilayer. We show that while these particles can deliver the antigen to APCs and induce Treg responses *in vitro*, they fail to replicate the same effect *in vivo*. The DOPG/PLGA hybrid nanoparticles were not able to induce antigen-specific T cell proliferation *in vivo* while the DSPC:DSPG:Cholesterol liposomes induced significant T cell expansion. Furthermore, although previous studies have shown the capacity of rigid anionic liposomes to arrest the development of atherosclerosis in mice[10], these hybrid particles did not have any effect on atherosclerosis progression

or plaque composition, nor did they induce antigen-specific T cell responses in this context. We hypothesized that the *in vivo* behaviour of these lipid nanoparticles may be more influenced by the protein corona than by the particle rigidity. The changes in average particle size, polydispersity index (PdI) and surface charge (ζ-potential) of the formulations after incubation with mouse serum or foetal bovine serum confirm the formation of a protein corona when in a biological medium. Furthermore, the coating of the nanoparticles with proteins that have been previously reported to play a role in nanoparticle uptake, such as ApoE[38], ApoB100[39] or C1q[10], revealed that ApoB100 might drive cell uptake in formulations with a DOPC:DOPG lipid bilayer, regardless of their rigidity, while the complement protein C1q is the main mediator of uptake for DSPC:DSPG:Cholesterol liposomes as previously reported[10]. The presence of cholesterol in the lipid bilayer is another important difference between the formulations studied here, therefore follow up studies should address the role of cholesterol in the formation and composition of the protein corona and how it influences the tolerogenic properties of lipid nanoparticles. This chapter highlights the complexity of assigning specific physicochemical properties of nanoparticles to a certain biological effect, since changes in the phospholipid composition affects nanoparticle rigidity but also determines the composition of the protein corona. Future studies should follow a comprehensive approach to determine the effect of small changes in phospholipid composition in both physicochemical properties of the nanoparticle, the protein corona composition and their biological effect. The large number of parameters to consider and the interaction between them might require the use of more sophisticated statistical methods, such as Design of Experiments (DoE), that allow the study of multiple parameters and their interactions in a time-efficient manner[40].

In **chapter 7** of this thesis, we focus on the other main application of liposome-based vaccines, the induction of protective immune responses against viruses. In the field of vaccines against respiratory viruses such as coronavirus and influenza, there is a need for subunit vaccines that can induce cellular immune responses locally in the lungs. Cationic liposomes have been studied before for their immune activating properties[41] and these delivery systems present advantages for intranasal vaccination since the electrostatic interaction with the negatively charged surface mucosa improves the absorption of the formulation[42, 43]. In this chapter, we present a subunit vaccine formulation based on cationic liposomes loaded with the adjuvant cyclic dimeric guanosine monophosphate (c-di-GMP) and different influenza and SARS-CoV2-derived antigens. A rapid prime and boost immunization regime with this formulation induces potent and long-lasting $CD8^+$ T cell responses in mice. Compared to intravenous administration, the intranasal prime with this vaccine formulation induced more balanced systemic and lung-specific immune responses,

and more importantly this administration route allowed the induction of lung memory $CD8^+$ T cell responses. Finally, we show that the vaccination with cationic liposomes loaded with c-di-GMP and the influenza epitope PA224 leads to a reduction of lung viral titers after challenge with PR8 H1N1 influenza virus compared to unvaccinated mice and that the intranasal prime immunization is essential for this protective effect of the vaccine. The induction of a protective immune response using an accelerated prime-boost regime can be key for the effective and fast immunization of the population in response to rapidly spreading respiratory viral infections such as SARS-CoV2 and influenza.

FUTURE PERSPECTIVES

In this thesis, we aim to move forward the field of immune-modulatory nanoparticles, both from the delivery system perspective and the identification of target antigens. Nowadays, the most advanced therapies used in the clinic to target excessive inflammation and autoimmunity are biologics, mainly monoclonal antibodies, such as Adalimumab, an anti-TNFα antibody, or interferons such as IFN-β[44]. Other tolerance-inducing therapies that did not reach the clinic yet but that are being tested in clinical trials are cell-based therapies. These therapies consist of the *ex vivo* modification of DCs or T cells to transform them into tolDCs or Tregs and subsequently transfer them back to the patient. A promising type of T cell therapy to treat autoimmunity are CAR-Tregs, engineered Tregs expressing a chimeric antigen receptor (CAR) to target specific antigens[45]. Monoclonal antibodies and other immune suppressors currently used in clinical practice are not antigen-specific and induce general immune suppression leaving patients more prone to infections[19]. On the other hand, cell-based therapies such as tolDCs or CAR-Tregs are or can be antigen-specific, however the complex manufacture and their often-unstable tolerogenic phenotype are major limitations[46]. Therefore, the + *in vivo* generation of tolDCs and Tregs, such as the approach proposed in this thesis, can be considered as the next major step forward in the field of antigen-specific immune modulation.

Several recommendations can be derived from this thesis as the field advances towards the clinical application of these therapeutic strategies. On one hand, the design of clinical trials to test efficacy of tolerogenic therapies against atherosclerosis represents a significant challenge due to the large and heterogeneous target population. Furthermore, the slow and progressive development of the disease makes necessary the follow up of patients in clinical trials over several years or even decades. Therefore, the initial clinical trials to put to test tolerogenic therapeutic strategies against atherosclerosis should target the subpopulation of patients that can potentially benefit the most from these therapies. In this context,

immunopeptidomics can be applied to identify common antigenic targets in cohorts of patients such as those with more vulnerable plaque characteristics or with high residual inflammatory risk.

Future clinical trials of tolerogenic nanoparticles might also benefit from research into less invasive and potentially more tolerogenic administration routes, such as intranasal and intradermal administration. The promising results from the intradermal administration of anionic liposomes in *ex vivo* human skin shown in chapter 4 of this thesis prompts further research into other administration routes, such as intranasal administration. Intranasal antigen delivery using anionic liposomes could harness the natural capacity of the airways mucosa to induce tolerogenic responses[47].

Although this thesis and previous research have shown the potential of anionic liposome to induce tolerance, the field would benefit from further optimisation of tolerogenic formulations. We observed that liposome formulations that have previously shown to have intrinsic tolerogenic capacity in mouse models, need to include a tolerogenic adjuvant such as vitaminD3 to induce tolerogenic responses in human *in vitro* and *ex vivo* models. This should be considered for the future optimisation of key parameters such as size, ζ-potential, rigidity and/or protein corona and these experiments should be carried out using human *in vitro* or *ex vivo* models as far as possible.

Apart from the liposomal formulations proposed in this thesis for the induction of immune tolerance, other type of nanoparticles have significant tolerogenic potential. One of these formulations are mRNA lipid nanoparticles (LNPs), which have demonstrated their efficacy as prophylactic vaccines during the SARS-CoV2 pandemic. Tolerogenic mRNA-LNP vaccines are being actively investigated by several industrial and academic research groups. These formulations present advantages over liposomes such as a higher and less variable loading efficiency of antigens due to the more consistent physicochemical properties of mRNA compared to peptides. Furthermore, these formulations can be used to deliver mRNA encoding for large proteins such as ApoB100, which might overcome the challenge of identifying minimal epitopes and tailoring the target antigens to the specific HLA types. This approach however also has limitations that must be overcome in the near future, such as the instability of mRNA compared to peptides/proteins. Furthermore, it is not yet clear if LNPs with anionic surface charge also have tolerogenic properties similar to the liposome counterpart and if including tolerogenic molecules like vitaminD3 will affect the loading of mRNA into the nanoparticle. As these gaps in knowledge are being actively investigated, tolerogenic mRNA-LNPs are becoming promising new tools for antigen-specific immune modulation.

CONCLUSIONS

In conclusion, this thesis shows the versatility of liposomes to induce both tolerogenic immune responses in the context of inflammatory or autoimmune diseases and protective immune responses against infections. We address some of the challenges in liposome-based therapies and seek to bridge critical knowledge gaps in the field. The identification of target antigens in the context of atherosclerosis, optimization of the delivery system and the development of scalable manufacturing methods are some of the issues covered in the chapters of this thesis. The research presented here reveals the applicability of immunopeptidomics to study the precise antigens and epitopes driving immune responses in atherosclerosis but could also be useful in the context of classical autoimmune diseases. Furthermore, we lay the foundation for the clinical translation of tolerogenic anionic liposomes by performing human *in vitro* and *ex vivo* studies with these formulations. Finally, we also explore the use of cationic liposomes as prophylactic vaccines against viral infections and show their potential to generate protective T cell responses against influenza rapidly and efficiently. Overall, this thesis highlights the potential of liposome-based immunotherapies against autoimmune diseases and in the field of prophylactic vaccines and prompts further research to advance in the clinical translation of these formulations.

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