Vaccination and targeted therapy using liposomes : opportunities for treatment of atherosclerosis and cancer

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General Summary and Perspectives

The immune system is our body's defense against infections. However, it can also be involved in diseases such as autoimmune diseases[1-3] or cancer[4,5]. T cells are vital for the homeostasis of the immune system, and therefore are a target for treating such diseases[6-8]. In this thesis, we have focused on the treatment of diseases in which T cells are too active (atherosclerosis) or not sufficiently activated (cancer) and propose new treatment strategies using liposomes, which is introduced in **chapter 1**.

Liposomes are versatile vesicle-like structures, that can be engineered into various physical forms. In **chapter 2** we reviewed how particle size, shape, and rigidity of particulate vaccines affect biodistribution, cellular uptake, antigen presentation and the resulting immune response. While there are trends that can be observed between the physicochemical parameters of such vaccines and the resulting immune responses, it is often very difficult to alter just one parameter without affecting others. However, it is clear that a rational design approach is necessary to optimize a vaccine for a specific use; the physicochemical parameters for optimal antigen-presenting cell (APC) uptake may not be ideal for eliciting the desired immune response. In later chapters, this is always considered and studied in detail when designing the liposomes.

In **chapter 3** we aimed to understand the effect of liposomal rigidity, one of the most over-looked parameters for nanoparticles, on antigen-specific T cell responses. One of the reasons that rigidity has not been studied as extensively as other physicochemical parameters is the difficulty in accurately measuring it[9-14], and a lack of standardization in the literature. We used atomic force microscopy (AFM) to accurately measure the rigidity of individual anionic liposomes containing an ovalbumin-derived $CD4^+$ T cell epitope. The surface charge, phospholipid head group composition, antigen content and size of the liposomes were controlled in order to minimize alterations in other physicochemical parameters. We found that the incorporation of cholesterol in the lipid bilayer decreases the rigidity of gel-state liposomes and increases that of fluid-state ones. Furthermore, the transition temperature of the phospholipids influenced the overall liposome rigidity. Interestingly, almost all formulations showed a positive correlation between liposomal rigidity and APC association. However, dioleoylphosphatidylcholine (DOPC):dioleoylphosphatidylglycerol (DOPG) liposomes showed the highest association to dendritic cells (DCs) of all tested formulations despite having the lowest rigidity, indicating that liposomal rigidity alone does not control APC uptake. We did observe a significant correlation between the rigidity of the liposomes and the regulatory T cell (Treg) responses they elicited *in vitro* and in *vivo*. Our findings may contribute to a better understanding of the factors driving Treg responses, and support a rational design of liposomal as well as other nanoparticulate vaccine formulations aiming to enhance antigen-specific Treg responses for the treatment of autoimmune diseases.

Building upon the information presented in **chapters 2** and **3**, **chapters 4** and **5** focus on using liposomes to treat atherosclerosis. Atherosclerosis is the predominant underlying pathology of cardiovascular disease, which affects millions of people worldwide[15]. Lipids in the form of low-density lipoprotein (LDL) accumulate in the subendothelial space in medium- and large-sized arteries, which leads to chronic inflammation[16]. LDL can modify to form oxidized LDL (oxLDL), which attracts immune cells, continuing the inflammation[16]. Some of these immune cells, such as monocytes, can differentiate into macrophages which can phagocytose oxLDL, leading to foam cell formation[17]. Manipulation of immune cells, especially T cells[2,18] or foam cells[19], can be an effective way to treat

atherosclerosis. In **chapter 4** we designed liposomes that can induce Tregs. Liposomes were composed of distearoylphosphatidylcholine (DSPC):distearoylphosphatidylglycerol (DSPG):cholesterol (CHOL) (also one of the most rigid and potent formulations in **chapter 3**) and were loaded with an ovalbumin-derived $CD4^+$ T cell epitope. These liposomes were shown to elicit high production of antigen-specific Tregs *in vitro* and *in vivo* in mice as compared to free antigen, DSPC:dipalmitoylphosphatidylserine (DPPS):CHOL, and DSPC:dipalmitoyltrimethylammoniumpropane (DPTAP):CHOL liposomes. As an atherosclerosis-specific antigen, we turned to ApoB100, the protein component of oxLDL, which was already shown to be a relevant antigen[20-31]. We identified a major-histocompatibility complex (MHC)-II-restricted ApoB100-derived peptide, referred to as p3500, using a peptidomics strategy. When encapsulating p3500 in DSPG-liposomes it successfully reduced atherosclerotic plaque formation, as measured by a reduction in total plaque size, and an increase in plaque stability.

We also described the mechanism behind the potency of the DSPG-liposomes. Interestingly, it has been shown that anionic liposomes composed of PS resemble apoptotic cells, since PS becomes exposed on the surface of apoptotic cells[32]. Through scavenger receptor (SR)-mediated uptake by APCs, PS-containing liposomes can mediate an anti-inflammatory effect[33,34]. We show that DSPG-liposomes are more effectively taken up by DCs *in vitro* than DPPS-liposomes. We also demonstrate a role for the protein corona in Treg induction via SRs. *In vitro* and *in vivo*, liposomes interact with proteins in the physiological medium, resulting in the formation of a protein corona around the liposomes[35]. We found a significant reduction of uptake for both PG- and PS- liposomes in the presence of serum when SR-mediated uptake by DCs was blocked, which was not observed in serum-free conditions, suggesting that formation of a protein corona is required for liposome-SR interactions. We concentrated on the serum protein complement component 1 Q (C1q), since it was shown to bind to PS on apoptotic cells and lead to clearance via SRs[36-39]. Interestingly, C1q is also involved in the pathology of atherosclerosis[40]. We showed that C1q is present in the protein corona and binds to anionic liposomes. The addition of C1q in serum-free conditions completely restored the uptake of both PG- and PS-liposomes, while specific depletion of C1q in serum significantly reduced uptake of PG-liposomes. We further observed that the addition of C1q increases Treg responses compared to serum-free conditions, but not significantly. This suggests that C1q is partially responsible for the Treg induction of both DSPG- and DPPS-liposomes, but the protein corona likely contains more components that help to induce Tregs. Furthermore, it would be very interesting to study whether there is a relationship between liposomal rigidity and the protein corona, which is something we have not studied.

C1q does not only interact with SRs, but also binds to other proteins, such as the C1q-binding protein, also known as p32. This receptor is aberrantly expressed in plaque-resident macrophages[41]. As mentioned above, macrophages play an important role in the pathogenesis of atherosclerosis, since they can phagocytose lipids and become large lipid-rich foam cells[42]. These foam cells are unable to migrate out of the vessel wall, leading to a build-up at the site of the inflammation and formation of a plaque[43]. Therefore, a suitable treatment strategy would be to prevent and/or reverse the formation of foam cells[19], which was our goal in **chapter 5**. Macrophages are able to reverse cholesterol transport across their membrane via ATP-binding cassette (ABC) transporters ABCA1 and

ABCG1[44]. The expression of both of these transporters is controlled by the liver X receptor (LXR). A class of small molecules, called LXR agonists (*e.g.*, GW3965), can activate this receptor to induce reverse cholesterol transport in foam cells, allowing for migration of these cells from the plaque[45]. Unfortunately, when administered systemically at therapeutic doses, LXR agonists have effects in other organs, such as the liver, leading to high triglyceride levels in the plasma or liver[46-48]. Encapsulation of the LXR agonist in a drug delivery vehicle such as liposomes can overcome this problem[49]. The cyclic peptide Lyp-1 (CGNKRTRGC) binds to p32 and can therefore be used to target macrophages in atherosclerotic lesions[50,51]. For this purpose, we designed a particulate formulation that combines the targeting properties of the cyclic peptide Lyp-1 with the therapeutic effect of an LXR agonist (GW3965) and promotes reverse cholesterol transport in foam cells to stabilize atherosclerotic plaques. We used the knowledge gained from **chapter 2**, **chapter 3** and **chapter 4** to formulate liposomes with long circulation time, minimal APC uptake, and minimal immune effects. We then functionalized the liposomes with Lyp-1, and this led to greatly enhanced association to foam cells *in vitro*, while having limited affinity for macrophages. *In vivo* we saw retention of liposomes in atherosclerotic plaques, and we confirmed that the plaque-resident foam cells had taken up the targeted liposomes. We showed that using a low dose of GW3965 encapsulated in Lyp-1 liposomes can reduce plaque macrophage content and increase plaque stability while avoiding an increase in lipids in the serum and liver. These findings demonstrate that it is possible to increase the therapeutic window of LXR agonists, and may contribute to the design of other atherosclerosis therapies. In particular, Lyp-1-functionalized liposomes could be used as a platform to deliver other compounds, such as anti-inflammatory drugs, to foam cells.

The final research chapter of this thesis (**chapter 6**) focuses on using liposomes to treat cancer, which is another major health concern[52]. Cancer encompasses a group of diseases involving abnormal sustained cell proliferation, replication, and survival[53]. T cells are also involved in cancer, since they can recognize tumor-associated antigens[4,5], however in contrast to atherosclerosis, T cell responses in cancer are not effective and suffer from exhaustion[54]. As we have shown in **chapter 4**, DSPC:DPTAP:CHOL liposomes are potent at inducing antigen-specific pro-inflammatory T cells and may provide the type of immune response required to kill a tumor. In **chapter 6** we aimed to repurpose DSPC:DPTAP:CHOL liposomes with tumor neoepitopes as a therapeutic cancer vaccine. Neoepitopes are very interesting for treatment; they result from non-synonymous somatic mutations that encode new amino acid residues, leading to novel peptides that can be presented on the cell surface of tumor cells[55]. The Hannover Medical School previously showed that inducing strong pro-inflammatory T cell responses against $Adpgk_{mut}$ (a neoepitope) could eliminate established cancers[56]. This was achieved by isolating DCs from mice, pulsing them with antigen and adjuvants *ex vivo*, and reinjecting them[57]. These DCs can then present the tumor-specific epitopes to T cells and prime them to destroy the cancer cells[58]. To boost $CD8^+$ T cell responses, mice were injected after some time with a mix of an agonistic anti-CD40 antibody (Co), antigen (A) and a toll-like receptor (TLR) ligand (T) Poly I:C[59]. Unfortunately, *ex vivo* priming of DCs is very labor-intensive and expensive, prohibiting wide-spread use[57]. We propose DSPC:DPTAP:CHOL liposomes as a delivery system to deliver the antigen to DCs *in situ* in a simpler way[60]. First, we compared non-adjuvanted $Adpgk_{mut}$ liposomes with liposomes containing the TLR3 ligand poly(I:C), the TLR4 agonist MPLA, or the

STING agonist cdiGMP. Mice were primed with liposomes and boosted with CoAT. The cdiGMP liposomes induced the strongest antigen-specific $CD8^+$ T cell response, so we continued with this formulation in further experiments. In a direct comparison, cdiGMP liposomes performed as good as DC-CoAT vaccination. We also tested the suitability of the liposomes with another neoepitope, $Alg8_{mut}$. Similar to $Adpgk_{mut}$, encapsulation of the antigen in cdiGMP liposomes greatly boosted the immune response and led to very long-lasting responses which were similar to those achieved with DC-CoAT[56]. Next, we aimed to improve our vaccination protocol by eliciting $CD4^+$ T cell help with the $CD4^+$ epitope MTAG85B[61] and selecting a long version of $Adpgk_{mut}$ peptide[62]. The long peptide did not enhance the $CD8^+$ T cell response against the minimal epitope. We also saw no evidence of the MTAG85B-induced $CD4^+$ T cell activation boosting the $CD8^+$ responses. Due to the lack of improvement of the $Adpgk_{mut}$ responses with the addition of a $CD4^+$ T cell epitope or a long peptide, we decided to perform a tumor study using only the minimal $Adpgk_{mut}$ peptide in cdiGMP liposomes. Here, we observed extremely high antigen-specific responses to the antigen (up to 60% of all $CD8^+$ T cells responded to the antigen by producing IFNγ). We observed tumor regression within 30 days and 100% survival in mice receiving the LS-CoAT vaccination. Overall, this work shows that cdiGMP liposomes are a very powerful platform for encapsulating tumor neoepitopes to induce strong antigen-specific $CD8^+$ T cells and eliminate established tumors.

Perspectives

While we presented several different treatment strategies for different diseases, liposomes were used in all cases. We used liposomes to deliver a drug to plaque-resident macrophages by using a targeted approach. We also manipulated the physicochemical properties of liposomes to induce anti-inflammatory or pro-inflammatory T cell responses. These studies further add to the evidence that liposomes are extremely versatile drug delivery particles[63].

In **chapter 5** we explored a method for treating atherosclerosis by using targeted liposomes to deliver a drug. Aside from targeting to atherosclerotic plaques, targeted liposomes have been applied in the treatment of cancer[64], and to cross the blood-brain barrier to treat neurological diseases such as Alzheimer's[65]. Several liposomal formulations, including PEGylated liposomes, have already been shown to be safe and are FDA and EMA approved[66]. Furthermore, PEGylated liposomes have been used to passively deliver prednisolone (an anti-inflammatory drug) to atherosclerotic plaques in humans, indicating that there is some translation from animals to humans[67].

We have emphasized the importance of designing liposomes with specific physicochemical parameters, and summarized the significance of size, shape and rigidity in **chapter 2**. Liposomal rigidity was intriguing since we could not find a substantial amount of literature concerning this parameter. Therefore, we aimed to enhance our understanding of this parameter and performed a detailed study examining the relationship between liposomal rigidity and Treg responses in **chapter 3**. For this, we presented an optimized protocol for measuring the rigidity of liposomes using AFM, which can also be applied to other particulate vaccines and drug delivery systems. Both of these chapters contribute to the understanding of the effect of physicochemical parameters of liposomes on the immune responses, and could help others with the design of liposomal vaccines. It should also be noted that liposomes are by nature relatively soft particles, so it is unknown

whether the immune effects we observed apply to a wider range of rigidities. The exact mechanism of why rigidity affects the immune response is still not fully understood, and future studies examining this in more detail would be very valuable.

The induction of antigen-specific Tregs are a focus of **chapters 3** and **4**. We identified a peptide derived from murine ApoB100 for vaccination, but human ApoB100 epitopes have been identified which were successful at reducing atherosclerosis in humanized mice[68]. These peptides can be encapsulated in liposomes to enhance their effects and safety. Unfortunately, so far, no clinical studies have been performed using ApoB100-derived epitopes, so there is still a question about the translation of these studies to a clinical setting[69]. Since many people suffering from atherosclerosis use statins to control their disease, the interaction between statins and peptides and/or liposomes needs to be studied as well. For instance, combined use of statins and LDL-mimicking liposomes led to the liposomes crossing the blood-brain barrier in an *in vitro* culture model[70], which could be dangerous. Furthermore, using fluorescence anisotropy, it has been shown that some statins interact with the bilayer of liposomes and can alter their fluidity[71], and thereby their immunogenicity. As opposed to our study with Lyp-1 liposomes, the tolerogenic liposomes in **chapter 4** were administered at the same time as starting the western-type diet; it would be more clinically relevant to administer the liposomes in mice with established atherosclerosis.

Tregs are not only important in the treatment of atherosclerosis, but are involved in many other diseases, grouped under the term autoimmune diseases[72]. We found a role for serum-derived C1q in the interaction of liposomes and SRs, and it would be interesting to study this mechanism in more detail. Autoimmune diseases include diseases such as Crohn's diseases, diabetes, multiple sclerosis and rheumatoid arthritis, and affect an estimated 7.6–9.4% of the population, with women being at a higher risk[73]. There is currently no cure for any of the autoimmune diseases, and treatment focuses on systemic suppression of inflammation with anti-inflammatory drugs. However, these therapies can result in severe side effects and susceptibility to infection, especially upon long-term treatment[74-76], emphasizing the need for more specific therapies. While we have only studied the effects of Treg-inducing liposomes on atherosclerosis, other studies have been performed with tolerogenic liposomes treating diabetes[33] and rheumatoid arthritis[77]. All of this work is vital for developing a possible treatment for autoimmune diseases.

Immunomodulation was also the focus of **chapter 6**, but instead of suppressing the immune response to an antigen, we aimed to enhance it by the use of cationic liposomes. Cancer immunotherapy via DCs[78] or T cells[79], and immunotherapy against neoantigens[80] has been studied for several years. We argue that immunotherapy via liposomes is a faster, more cost-effective treatment strategy. Indeed, nanoparticles, including liposomes, have been shown to be effective in cancer therapy[81], and our work contributes to this evidence. Tools to identify antigens specific to autoimmune diseases[82,83], including the atherosclerosis antigen p3500 identified in this thesis, and cancer[84] are improving. Combined with the ability of liposomes to encapsulate a wide range of epitopes, liposomes could be an important player in the future of personalized therapies[85]. It should be noted that, while we observed no toxicity in our animal studies, at high doses cationic phospholipids induce toxicity in *in vivo* animal models[86] and *in vitro* human cells[87]. Therefore, further development of these liposomes should always

strive for minimizing the dose of the cationic phospholipids in the liposomes. In order to progress to clinical trials, more toxicity studies need to be performed in animal models to test whether the liposomes are safe to use, either in the short-term or long-term.

Aside from the need to test the safety of the liposomes for clinical use, testing in humans requires large amounts of liposomes and the encapsulated contents (drug, peptide, adjuvant). Scale-up is not only potentially very costly (although encapsulation of a compound in a (targeted) nanoparticle greatly decreases the required dose, and therefore cost[88]), but it can alter the liposomes' physicochemical properties. This is also true for the GMP production of liposomes. Methods such as micro-fluidics[89] and ethanol injection[90,91] are proven to be able to reproducibly manufacture large amounts of liposomes under GMP conditions. However, a detailed evaluation of the effect of production method on liposomal properties should be performed. Despite this, there are a substantial number of liposomes that are already approved for humans, or in clinical trials[66], so the potential for further developing the treatments presented here is promising.

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