Liposomes as delivery system for allergen-specific immunotherapy

Leboux, R.J.T.

Chapter 7
Summary, discussion, conclusion

In this thesis, we set out to explore the use of liposomes as adjuvants in subcutaneous allergen specific immunotherapy (SCIT). We evaluated different methods for associating a recombinant birch pollen allergen, Bet v 1, with liposomes. This led us to develop a new association method, based on the strong interaction of two complementary peptides, which resulted in a formulation that is able to induce a strong immune response upon subcutaneous administration in mice.

To evaluate how different antigen association methods affect physico-chemical properties and immune response of cationic liposomes, Bet v 1 was associated with cationic liposomes in various ways in **chapter 2**. Bet v 1 was adsorbed onto the lipid bilayer surface, encapsulated in the aqueous core of the liposomes, or both adsorbed and encapsulated. With increasing amount of Bet v 1, the size and polydispersity of the liposomes increased slightly for the adsorption method, but not for the encapsulation method or the combination. Above a protein/lipid ratio of 0.15 (w/w), aggregation was observed for both the association method and the combination method. For both methods, the association efficiency decreased with increased amounts of Bet v 1. Balb/c mice immunized with Bet v 1 either adsorbed to or encapsulated in cationic liposomes produced more antigen-specific IgG1 than Bet v 1 adsorbed to colloidal aluminum hydroxide. A combination of encapsulation and adsorption resulted in an even stronger IgG1 response as well as more cytokine (IL-4, IL-5, IL-10 and IL-13) production in lung draining lymph nodes. None of the formulations induced measurable antigen-specific IgG2a levels. While Bet v 1 in combination with cationic liposomes induced a stronger response than colloidal aluminum hydroxide-adjuvanted Bet v 1, these results reflect a Th2 skewed immune response. For allergen specific immunotherapy a Th1 or Treg type is more desirable, the latter has reportedly been induced by using anionic liposomes. Therefore in **chapter 3**, anionic liposomes were used. The effect of liposome rigidity on antigen-specific T-cell responses was evaluated. The rigidity of nanoparticles, such as liposomes, is an often overlooked property, for which no standard measurement method is available. Atomic force microscopy (AFM) was used to measure the rigidity of anionic liposomes containing OVA323, a MHC-II restricted epitope. A series of liposome formulations was prepared, in which surface charge, surface chemistry (*i.e.*, phospholipid head group), antigen content and particle size as measured by dynamic light scattering (DLS) were kept the same, in order to focus on the effect liposome rigidity. The incorporation of cholesterol in the lipid bilayer increased the rigidity of fluid-state liposomes and decreased that of gel-state liposomes. With the exception of liposomes consisting solely of dioleoylphosphatidyl phospholipids, all formulations showed a direct correlation between formulation rigidity and uptake by antigen presenting cells (APCs). Moreover, a significant correlation was observed between rigidity of liposomes and the regulatory

T-cell responses that were induced *in vitro* and *in vivo*, *i.e.*, the more rigid the liposomes, the stronger the responses. An adjuvant that induces a stronger regulatory T-cell response is in potential a better adjuvant for immunotherapy.
In **chapter 4**, the behavior after intradermal injection of both cationic and anionic liposomes was compared. Ovalbumin (OVA) and Bet v 1 were encapsulated in both types of liposomes, after which the uptake by skin resident dendritic cells of these formulations after intradermal injection in *ex vivo* human skin was assessed in a DC crawl-out model. There was a major difference in uptake between antigens: OVA was taken up very efficiently by skin resident dendritic cells, while Bet v 1 uptake was minimal. Encapsulation of Bet v 1 in liposomes, both anionic and cationic, increased its uptake drastically (>10-fold), whereas OVA uptake was decreased by encapsulation. While antigen uptake did not vary significantly between cationic or anionic liposomes, uptake of anionic liposomes was more efficient as compared to cationic ones. This was an unexpected finding, as it is generally accepted that cationic liposomes are taken up more efficiently by dendritic cells.

As observed in the previous chapters, in general the encapsulation of (usually precious) antigen is not efficiently achieved. For cationic liposomes, 60-80% of Bet v 1 was lost during preparation and purification and this process was even less efficient for anionic liposomes. Therefore, in **Chapter 5** a new association method was developed and evaluated with model antigens and Bet v 1. Antigen association to liposomal membranes was based on the interaction of two complementary alpha-helical peptides, pepE and pepK, to form a heterodimeric coiled coil (CC) complex. For this, pepK was conjugated to cholesterol, resulting in CPK, and incorporated in the lipid bilayer of cationic liposomes, while the antigenic peptides OVA323 (= pepE-OVA323, MHCII restricted epitope) and OVA257 (= pepE-OVA257, MHC-I restricted epitope) were extended at the N-terminus with pepE. Antigen affinity for liposomes via CC-formation was very high (Kd ≈ 100-400 nM range), as compared to traditional association methods based on electrostatic interactions (Kd > 10^5 nM). Moreover, CC-associated antigens remained associated with the liposomes after insertion in serum-containing culture medium or after intravenous injection in zebrafish larvae, whereas the electrostatically adsorbed antigens were mostly dissociated from the liposomes under these conditions. PepE-OVA323 associated to CPK-functionalized liposomes resulted in a 4-fold stronger $CD4^+$ T-cell proliferation *in vitro* as compared to non-functionalized liposomes. Surprisingly this was not the case for pepE-OVA257 and $CD8^+$ T-cells, which showed a dose-dependent $CD8^+$ T-cell proliferation regardless of the formulation (free antigen, non-functionalized liposomes, CPK-functionalized liposomes). Finally, antigen associated via CC formation resulted in stronger $CD4^+$ T-cell responses after subcutaneous immunization in mice. PepE-OVA323 associated with either non-

functionalized liposomes or CPK-functionalized liposomes induced a Th1-skewed immune response, as illustrated by the expression of T-bet by $CD4^+$ T-cells and the production of IFN-γ. Coiled coil associated antigen induced more antigen specific IFN-γ and IL-10 production by $CD4^+$ T-cells, indicating both Th1 and Treg properties of these $CD4^+$ T-cells and therefore a promising immune response for immunotherapy against allergens.

Based on these findings, in **chapter 6** a genetic fusion antigen of pepE and Bet v 1 (= pepE-Bet v 1) was produced in *E. coli* to further study the effect of CC-based antigen binding to cationic liposomes. A rat basophil leukemia assay revealed that CC-associated Bet v 1 to liposomes was approximately 10-fold less allergenic than wild type Bet v 1, in terms of mediator release in IgE-loaded basophils. Alum adsorbed Bet v 1 was compared to cationic liposomes with Bet v 1 adsorbed in various ways. CC adsorbed pepE-Bet v 1 induced a strong antigen specific IgG1 and IgG2a response after 3 immunizations, which was significantly higher than the responses induced by alum adsorbed Bet v 1 or the antigens without adjuvant. Moreover, neither cationic liposomes with Bet v 1 adsorbed nor non-functionalized liposomes with pepE-Bet v 1 induced as strong responses as CC adsorbed pepE-Bet v 1. These results clearly show that CC-mediated Bet v 1 association with cationic liposomes is an effective way to elicit a strong antigen-specific immune response *in vivo*.

General discussion

While allergen specific immunotherapy already exists for over 100 years, the formulations for SCIT have not substantially changed in decades [1-3]. Typically, an allergen extract is mixed with an adjuvant and administered subcutaneously, requiring up to 54 injections over the course of 3-5 years before the therapy is completed [4-6]. The most commonly used adjuvant is colloidal aluminum hydroxide, while microcrystalline tyrosine, monophosphoryl lipid A (MPLA) and calcium phosphate are also available. Aluminum hydroxide initially boosts the ongoing Th2 skewed immune response, before inducing tolerance [7-9]. Despite SCIT being effective, there is room for improvement.

This can be achieved by strategies leading to 1) reduction of the number of injections, 2) reduction of the time period before symptom relief occurs and 3) fewer side effects. Therefore, safer formulations with increased immunogenicity are urgently needed and various innovations are studied in this thesis: recombinant proteins to replace allergen extracts, new administration routes and new adjuvants to replace colloidal aluminum hydroxide [2, 5]. Rather than using a birch pollen extract, recombinant Bet v 1 and pepE-Bet v 1 were used. In chapter 4 we explored a model for intradermal administration and evaluated the uptake of antigen by antigen presenting cells (APCs). And finally, the major

thread throughout this thesis was the use of liposomes as adjuvant for SCIT.

Liposomes as adjuvant for SCIT

Currently the most used adjuvant for SCIT is colloidal aluminum hydroxide. It has high adsorption capacity and upon injection forms a depot from which the antigen is slowly released. The former means it can be used for a large variety of antigens while the latter leads to prolonged antigen exposure [1]. Moreover, aluminum hydroxide has been used for decades and is considered safe.

The desired immune response for SCIT is one that induces antigen-specific IgG4 and IL-10, which is associated with a tolerogenic response (characterized by regulatory T- and B-cells) towards the allergen. It is argued that induction of a Th1-type immune response could also be beneficial [4, 5]. Aluminum hydroxide however has been shown to enhance allergy-associated IgE prior to the induction of IgG4 in patients receiving SCIT [10, 11]. If the phase of SCIT where levels of allergy-associated biomarkers (*e.g.*, IgE) are increased, can be avoided, the therapy may become more efficient.

Liposomes can replace role of aluminum hydroxide as adjuvant for SCIT. They are a versatile adjuvant that consists of (phospho)lipids with proven clinical safety [12]. The composition can be altered, which will alter the properties and consequently the behavior after administration [13]. Cationic liposomes can form a depot after injection, can adsorb a wide variety of antigens (in particular those that are negatively charged), and are considered to be taken up most efficiently and induce a Th1-skewed immune response [14-17]. Anionic liposomes have shown, in mice, to be able to induce a specific tolerogenic response to encapsulated antigens [18, 19]. Moreover, in chapter 4, we have shown that encapsulation of antigens in liposomes of either charge can increase the antigen uptake by dendritic cells after injection in human skin. The lipid composition and consequential charge will likely determine the type of immune response that is induced by liposomes. Both cationic and anionic liposomes could be able to improve SCIT as adjuvant.

To ensure that liposomes and antigen are taken up by the same antigen presenting cell (APC), antigens can be encapsulated in the core of liposomes. This process is both inefficient and expensive. Encapsulation efficiency is typically <50%, as shown in this thesis (Chapter 2-4) and the literature [20-24]. Moreover, for oppositely charged antigen it is difficult to distinguish between surface adsorbed antigen and encapsulated antigen. The difference between encapsulated and surface adsorbed antigen could have consequences for the induced immune response, adverse events and the stability of the liposomal formulation. For the induction of a strong humoral response, intact antigen is

required. If intact antigen is freely diffusing, it could bind to IgE molecules on mast cells or basophils, resulting in an allergic reaction [25-27].

An ideal liposomal adjuvant for SCIT should have the following characteristics 1) high antigen association, 2) intact antigen is available on the surface to induce a strong humoral response, 3) no antigen is released from the adjuvant to potentially induce adverse events and 4) be applicable to a range of antigens. On top of the above-listed characteristics, a SCIT formulation should foremost also be stable and induce a strong immune response, which comprises the production of antigen-specific IL-10 and high-affinity neutralizing antibodies (IgG4 in humans).

Antigen association to liposomes via coiled coil-forming peptides

The newly developed association method using CC-forming peptides (chapter 5 and 6) leads to antigen-containing liposomes matching the desired characteristics mentioned in the previous paragraph. High antigen association efficiency for several tested antigens (pepE-OVA323, pepE-OVA257 and pepE-Bet v 1) was obtained, which remained intact in the presence of serum or in circulation in live zebrafish larvae. Moreover, in chapter 6 we showed that in mice that CC-associated liposomal Bet v 1 induced a superior immune response compared to otherwise associated Bet v 1. In addition to the strong immunogenicity of the formulation, it was also hypo-allergenic in a rat basophil leukemia assay, which is a good indication of an improved safety profile.

A liposome formulation based on CC-forming peptides for antigen association will likely offer enhanced stability compared to encapsulated antigen, because the antigen and the liposomes can be stored separately and mixed prior to administration. This stability could be further increased by improving the formulation buffer, which currently only consists of an aqueous solution of sucrose and HEPES. Liposomes and antigen can be mixed shortly before injection, with high association efficiency. Charged, small liposomes are generally very stable at 2-8°C, as electrostatic repulsion avoids aggregation, while Brownian motion prevents the suspension from collapsing. This stability based on repulsion could be compromised when antigen and liposomes have opposite charges.

In summary, allergen association to liposomes via CC formation shows great promise as allergy vaccine carrier platform. The formulation could be further optimized. For an approach based on cationic liposomes, the induced immune response could be enhanced by incorporation of immunomodulatory molecules in the liposome, such as MPLA or dimethyldioctadecylammonium (DDA). These may skew towards a Th-1 biased response [28, 29]. Owing to their amphiphilic nature, incorporation in lipid bilayers without compromising liposome stability

is feasible.

Anionic liposome-based vaccines on the other hand require more formulation optimization as CPK incorporation into anionic liposomes resulted in almost instantaneous aggregation. Thus, the CC-forming peptides should probably be reversed: pepE (anionic) should be linked to cholesterol, while pepK (cationic) should be coupled with the antigen. Moreover, anionic liposomes for SCIT could benefit from incorporation of molecules such as vitamin D, to induce a stronger tolerogenic response [30].

Another interesting possibility that could be explored is the association of multiple antigens via CC formation. This could induce tolerance to multiple allergens (and consequently multiple allergies) with only one formulation. Finally, other applications than allergy could be explored, such as rheumatoid arthritis which is also associated with $CD4^+$ T-cell responses. The ability to associate antigens in a highly efficient and reproducible manner could result in a platform technology. This would improve the development of new formulations, which would require only minimal optimization for new antigens.

Concluding remarks

While aluminum has been used successfully as adjuvant to treat allergies, SCIT could probably be improved drastically by improving the formulation. In this thesis we investigated liposomes as adjuvant for SCIT. We studied several methods of antigen association with cationic and anionic liposomes, and the effect thereof on the loading efficiency and immunogenicity of the antigen. We have developed a novel association method based on a complementary peptide pair. This association method reduced antigen loss during formulation and resulted in superior immune responses compared to colloidal aluminum hydroxide-adsorbed antigen and otherwise associated antigen. The overall results provide a solid basis for further improvements with respect to the design of these liposomes, which potentially may replace colloidal aluminum hydroxide as adjuvant in SCIT.

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