Liposomes as delivery system for allergen-specific immunotherapy

Leboux, R.J.T.

Chapter 1
General introduction

Allergy

We are constantly exposed to airborne allergens. Not everyone is allergic or develops an allergy over time, but the incidence of allergic rhinitis, conjunctivitis and asthma is increasing rapidly. Estimates indicate that allergies affect approximately 30% of the population in the developed countries [1-5]. Sensitization to airborne allergens, such as pollen, is an overreaction to an otherwise harmless substance. Pollen from birch and other Fagales family members are the most dominant tree pollen in Northern and Central Europe. Birch pollen allergy often comes with allergies to other types of pollen (e.g. hazelnut, oak, chestnut) [6] and even food allergies [7, 8] as a result of protein homology. Based on samples in populations throughout Europe, it is estimated that between 10 and 20% of all citizens are sensitized to the main allergen of birch pollen: Bet v 1 [2-4, 9]. Among pollen-allergic patients, between 50 and 99% have Bet v 1-specific immunoglobulin (Ig) E.

An allergy is developed in two phases: the sensitization phase (Figure 1) and the effector phase (Figure 2). During sensitization, the immune response to an allergen is skewed towards a T-helper (Th) 2-type response. Allergen-specific Th2 cells secrete cytokines, including interleukin (IL)-4, IL-5 and IL-13, which ultimately results in antibody isotype switching towards IgE. In the effector phase, mast cells (MCs) and basophils are loaded with allergen-specific IgE. Upon binding of allergen to these specific IgE molecules, the MCs and basophils degranulate and thereby release a storm of molecules, such as histamines, prostaglandins and leukotrienes [10-15]. These are responsible for the most common symptoms of allergy, including sneezing, rhinitis and conjunctivitis.

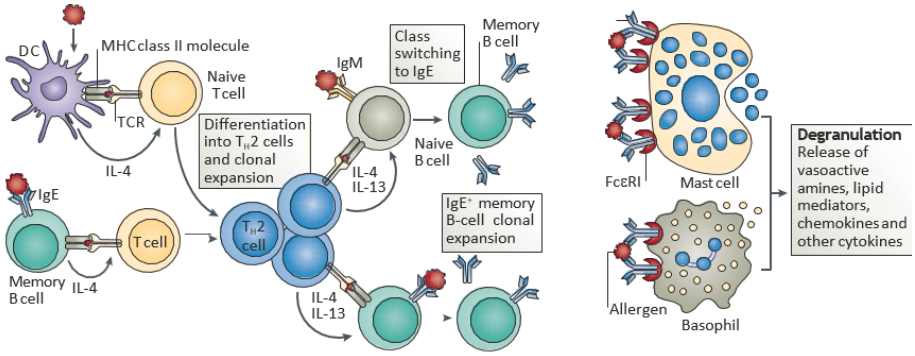

Figure 1. Proposed mechanism of the sensitization phase of developing an allergy. Dendritic cells are exposed to allergens and, in the presence of IL-4, induce the differentiation of naive CD4+ T-cells into Th2 CD4+ T-cells. These cells produce IL-4, IL-5 and IL-13 and subsequently induce antibody isotype switching towards IgE on B-cells. This allergen-specific IgE can then bind FcεRI receptors on basophils and mast cells. Image was adapted from Larché et al. [15].

Even though allergic reactions most often are not lethal, the economic burden of allergies is severe. It was estimated to be 2-5 billion dollar in the United States in 2003 (approximately 20 dollar per citizen) [16], and more than 1 billion euro in Sweden in 2015 (approximately 960 euro per citizen) [17].

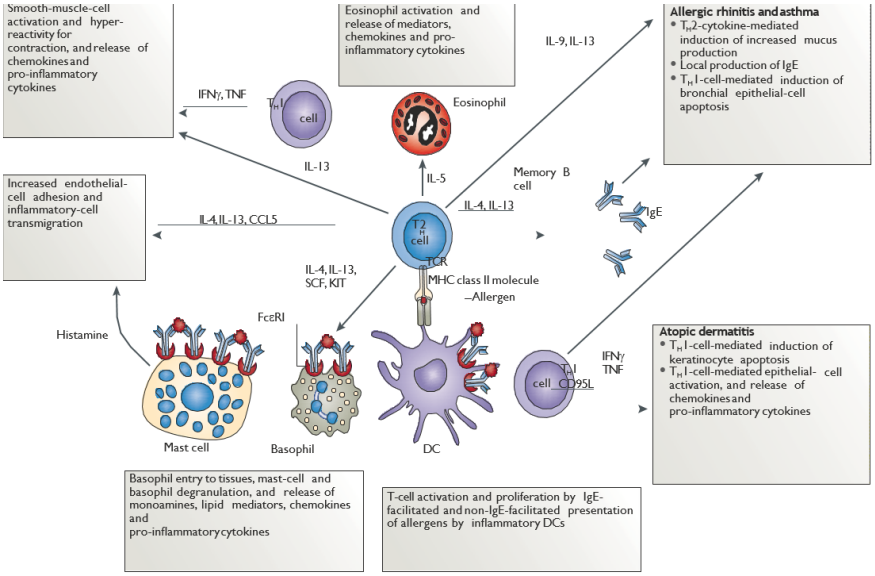

Figure 2. Proposed mechanism of the effector phase of an allergy. Upon subsequent exposure to the allergen, DCs take up allergen and IgE-bound allergen and activate Th2 CD4+ T-cells, which produce various cytokines and chemokines, resulting in activation of eosinophils, smooth muscle cells, mucus production and basophils. Image was adapted from Larché et al. [15].

Allergy therapies

The most effective allergy treatment is avoiding the allergen source. Unfortunately this is very difficult if not impossible for airborne allergens. First line therapy includes the administration of drugs, such as anti-histamines or corticosteroids [18]. These treat the symptoms, but not the underlying cause. The only curative treatment is allergen-specific immunotherapy (AIT) using allergy vaccines [18]. AIT is available as sublingual immunotherapy (SLIT) or subcutaneous immunotherapy (SCIT). In SLIT, atopic patients must administer themselves allergen sublingually daily for at least a year. SCIT consists of weekly subcutaneous injections by a general practitioner during the scale-up phase (first 3-6 months) of therapy, and monthly or bi-monthly injections during the maintenance phase (3-5 years) [19-21].

Allergy vaccines for SCIT are usually composed of an allergen extract, containing water-soluble proteins, adsorbed to aluminum hydroxide. The latter is a commonly used colloidal adjuvant in childhood vaccines, known to induce

strong Th2 mediated responses [22]. A recent advance in the field of AIT is the use of (well-defined) recombinant proteins instead of (poorly defined) allergen extracts [23-26]. As allergen extract content varies between allergen-source and even between batches of the same supplier [23, 27-29], this is the first step towards well characterized and reproducible vaccine formulations.

While proven effective, SCIT requires at least 3 years before sufficient effect is achieved. IgE levels first increase, before decreasing in the course of SCIT [12]. This may be explained by the Th2-type of immune response that aluminum hydroxide initially induces, resulting in IgE induction, before inducing IgG4 and IL-10 production. If the vaccine could directly induce either a regulatory response or a Th1 type response, the increase of allergy-related biomarkers probably would not happen. Thus, by replacing the adjuvant, the therapy might require less injections and work more quickly [10, 26, 30, 31].

Some issues about the safety of aluminum salts have been raised [21, 32-34]. Although the toxicity is debatable, development of new allergies has been reported as a result of AIT [35-37], which may be attributed to the induction of antigen-specific IgE as a result of the aluminum adjuvant. Moreover, there is a regulatory wish to replace aluminum salts in allergy vaccines [10, 26, 31]. Nanoparticles are an interesting alternative adjuvant for immunotherapy. A broad range of nanoparticles has been prepared and described as alternatives. Liposomes are among the most studied and several liposomal formulations are for purposes other than allergy vaccination are on the market [38].

Liposomes

Liposomes are nanoparticles composed of one or more (phospho-)lipid bilayers. Liposomes are a biocompatible, bio-degradable and highly versatile and tunable delivery system [39]. Molecules can be physically associated to liposomes in three ways (Figure 3): water-soluble molecules can be encapsulated in the aqueous core or adsorbed to the surface, while lipid-soluble molecules can be incorporated in the lipid bilayer [40]. Incorporation of molecules in liposomes typically alters the bio-distribution after administration. This formulation strategy has successfully been used to alter the pharmacokinetics of cytostatic drug molecules, such as doxorubicin [41]. Liposomes are also investigated and used as adjuvant for vaccination, to carry antigenic peptides or proteins as cargo.

Liposomes can serve as vaccine adjuvant in two ways. Firstly, they can act as a delivery system to enhance the delivery of antigenic cargo towards and into antigen presenting cells (APCs) [42, 43]. Secondly, they can induce, enhance or direct the subsequent immune response via various immunostimulatory mechanisms [43-45]. In order for an adjuvant to induce its effect, both antigen

and adjuvant need to be taken up by the same APC [46, 47]. The *in vivo* behavior of liposomes strongly depends on the physico-chemical properties, which in turn is determined by the content and lipid composition. The hydrodynamic diameter and zeta potential of liposomes and their impact on immunogenicity have been widely studied [42, 48-53].

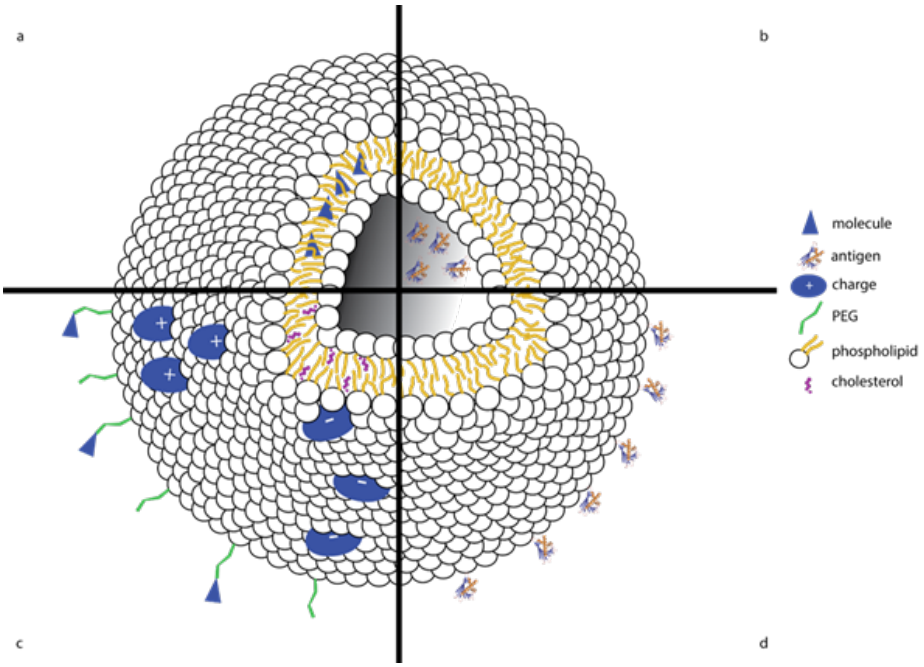

Figure 3. Schematic overview of structure and composition of a unilamellar liposome. Liposomes consist of one or more lipid bilayers, in which hydrophobic molecules can be incorporated (A). The aqueous core can be used to encapsulate hydrophilic molecules (B). The surface of liposomes can contain charged lipids, PEG-conjugated lipids and lipids conjugated to targeting molecules (C). Molecules can also be adsorbed to the surface via e.g. electrostatic interactions (D).

Small particles (<500 nm) are thought to migrate towards the lymph node and then be taken up by lymph node resident APCs, whereas larger particles (>500 nm) are primarily taken up at the injection site [54, 55]. Consequently, small liposomes were shown to disappear more quickly from the injection site than large ones [56]. While smaller solid nanoparticles are often associated with a Th1-skewed immune response and stronger CD8 T-cell responses, this does not seem to be the case for liposomes [48]. Large neutral liposomes induced a strong Th1 response, whereas small liposomes induced a Th2 response [53, 57]. Small cationic liposomes containing pDNA were more effective at inducing both a humoral [56] and especially CD8+ T-cell response [51, 56]. This illustrates that not only size, but also other parameters, such as zeta potential and surface chemistry, are to be considered.

Liposomes can be either neutral, anionic or cationic. Neutral liposomes are typically composed of phosphatidylcholines (PC), phosphoethanolamines (PE) and cholesterol. Neutral liposomes are not stabilized by electrostatic repulsion, which can result in rapid aggregation or sedimentation [58]. Despite this challenge, neutral liposomes are the basis of AS01 (in combination with MPL-A and Saponin), GSK's adjuvant used in malaria and recombinant Zoster vaccine [46]. Cationic liposomes generally form a depot at the site of injection, where they accumulate and need to be taken up by APCs to remove them from the injection site. Cationic liposomes are efficiently taken up by APCs and generally induce a strong T-helper cell type (Th) 1 response and are able to induce antigen specific CD8+ T-cell responses [44, 59-62]. The uptake mechanism for cationic liposomes seems to be based on electrostatic interaction between liposomes and cell surface [45, 68]. Anionic liposomes are taken up via scavenger receptors present on macrophages and liver sinusoidal endothelial cells [66, 67]. Studies with anionic liposomes have shown that liposomes containing lipids with either a phospho-glycerol or a phospho-serine headgroup can induce a regulatory immune response rather than an inflammatory Th1 or Th2 response [45, 63-65].

Besides improving the delivery of the antigen, another advantage of using liposomes, or nanoparticles in general, is their ability to co-encapsulate molecular immune modulators [66-72]. This will ensure the delivery of both antigen and immune modulator into the same APC. This co-delivery is necessary for an optimal effect of the immune modulator on antigen specific immune responses. Additionally, targeting moieties could be coupled to the surface to enhance uptake by specific subsets of cells (e.g. dendritic cells, Langerhans Cells or foam cells) [73-77]. In this thesis we use liposomes as antigen delivery systems for two different antigens: model antigen ovalbumin (OVA) and Bet v 1, the major allergen in birch pollen allergy.

Aim and outline of this thesis

In this thesis we investigate liposomes as a delivery system for SCIT. We set out to gain fundamental knowledge on the effect of antigen association method on the subsequent induced immune responses *in vitro* and *in vivo*. Moreover, we developed a new method of antigen association that can both reduce antigen loss during preparation and improve the immunogenicity of the antigen. We have prepared cationic and anionic liposomes and focused on the delivery of antigens in different animal models and in *ex vivo* human skin biopsies.

Chapter 2 compares different methods of Bet v 1 association to cationic liposomes. Bet v 1 was adsorbed to the surface, encapsulated in the aqueous core and resulting association efficiencies were determined. Aggregation was observed after exceeding a 0.15 protein/lipid ratio (w/w) in all cases. Liposomes with Bet v 1 encapsulated, adsorbed, and unbound induced the strongest IgG1 response.

In **chapter 3** atomic force microscopy was used to accurately measure the rigidity (Young's modulus, Ym) of anionic liposomes as function of lipid composition. The Ym was correlated to liposome uptake *in vitro* and induction of regulatory T-cells *in vivo*. A linear correlation was observed between liposome rigidity and the uptake of liposomes by cultured dendritic cells. Moreover, the linear correlation was also observed between liposome rigidity and induction of antigen-specific regulatory T-cells (Tregs) *in vivo*: the more rigid the liposome membrane, the more Tregs.

We evaluated the fate of two liposome formulations (cationic and anionic) after intradermal injection in human skin biopsies in **chapter 4**. Fluorescent OVA or Bet v 1 was encapsulated in fluorescent anionic and cationic liposomes. Subsequently, we analyzed which subsets of dendritic cells had taken up antigen and liposomes. Antigen uptake in different subsets was not affected by injection depth. We found that most CD14+ dDCs take up antigen, while Langerhans cells showed the smallest fraction of antigen-positive cells. Moreover, encapsulation of Bet v 1 in liposomes greatly enhanced uptake by APCs.

In order to reduce the loss of precious antigen during the preparation of liposomal antigen formulations, we developed a novel antigen association platform, which is described in **chapter 5**. This association method is based on the interaction between two complementary peptides (pepE and pepK, figure 4) that form a coiled coil. PepE was covalently linked to an antigenic peptide sequence (yielding a pepE-antigen conjugate), while pepK was covalently linked to cholesterol (yielding CPK). CPK was subsequently incorporated in the lipid

bilayers of cationic liposomes. This coiled coil-based association of pepE-antigen conjugates to liposomes was compared to association to non-functionalized cationic liposomes and showed superior *in vivo* co-localization, as well as *in vitro* and *in vivo* antigen specific CD4+ T-cell responses.

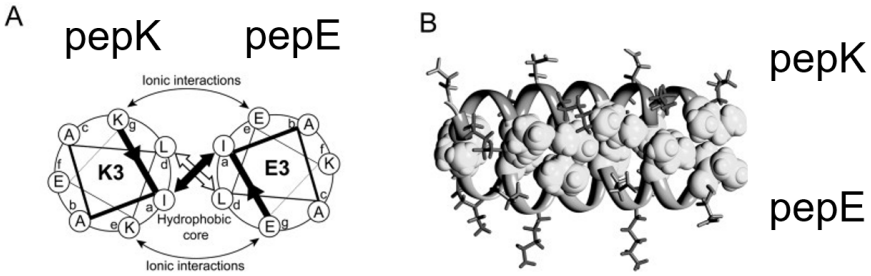

Figure 4. Schematic overview of the intermolecular interactions that happen when pepE and pepK form a coiled coil (A) and a representative image of what the coiled coil looks like on a molecular scale (B). Image was adapted from Fernandez-Rodriguez et al. [78]

Mice were immunized with Bet v 1 associated to liposomes or aluminum hydroxide in **chapter 6**. We compared the coiled coil-based association to the gold standard in SCIT: adsorption to aluminum hydroxide. Coiled coil associated Bet v 1 resulted in a superior immune response compared to plain adsorbed Bet v 1 to either cationic liposomes or aluminum hydroxide, with high levels of Bet v 1-specific IgG1 and IgG2a. Moreover, cells derived from lung draining lymph nodes produced high levels of IL-4, IL-5, IL-10 and IL-13.

Finally, in **chapter 7** the results of this thesis are summarized and the possible application of liposomes as adjuvant in (allergen specific) immunotherapy is discussed.

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