Anti -IL-4, -IL-13 and -IgE vaccination for the treatment of allergic diseases

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For my family

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"Lo importante es no tener arrugas en el cerebro"

"The important thing is not to have wrinkles in the brain"

Margarita Salas (1938-2019)

First scientific woman in the Royal Spanish Academy

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Abstract

Allergies represent major public health problems of increasing prevalence and for which there is still no efficient long-term therapy. IL-4 and IL-13 cytokines, and IgE antibodies play key roles in allergic reactions, and therefore represent good therapeutic targets. Moreover, these targets have been clinically validated with approved monoclonal antibodies (mAb) (Dupilumab to target IL-4 and IL-13 signaling and Omalizumab to target IgE). However, use of mAb in chronic diseases is limited by high cost and the need to perform injections over years to lifelong. Therefore, there is a clear need to improve current strategies in order to reach long term effects. The objective of this thesis was to develop anti-IL-4, anti-IL-13 and anti-IgE vaccines called kinoids, and provide a proof-of-concept of their safety and efficacy of these vaccines in models of asthma and allergic shock (anaphylaxis). We developed conjugate vaccines against IL-4 and IL-13, and demonstrated their prophylactic and therapeutic efficacy in reducing IgE levels, airway hyperresponsiveness, eosinophilia and mucus production in a house dust mite (HDM)-induced mouse model of asthma without any detectable adverse effect. The human version of the IL-4/IL-13 kinoid was also efficient at neutralizing human IL-4 and IL-13, and reducing IgE levels in mice humanized for IL-4, IL-13 and their common receptor subunit IL-4Rα. In addition, we also developed a conjugate vaccine against human IgE. We showed that this anti-IgE vaccine induces long-term production of anti-human IgE neutralizing antibodies in a novel mouse strain we characterized and which is humanized for both IgE and its high-affinity receptor FcεRI. Anti-human IgE vaccination reduced both circulating and FcεRI-bound hIgE, and fully protected against IgE-mediated anaphylaxis in IgE/FcεRI humanized mice. Altogether, our results showed that vaccination against IL-4, IL-13 and IgE could be a valuable strategy to target allergic disorders.

Résumé

Les allergies représentent un problème de santé majeur avec une prévalence en nette augmentation et pour lesquelles il n'existe pas de thérapie à longue durée. L'interleukine-4 (IL-4), l'IL-13 et l'immunoglobuline E (IgE) jouent un rôle clé dans les réactions allergiques. De plus, ces cibles thérapeutiques ont été validées en clinique, grâce aux anticorps monoclonaux Dupilumab et Omalizumab (ciblant l'IL-4 et IL-13 ou l'IgE, respectivement). Néanmoins, l'utilisation d'anticorps monoclonaux dans des maladies chroniques reste contraignante de par leur coût excessif et la nécessité de réinjections fréquentes. L'objectif de cette thèse a été de développer des vaccins contre l'IL-4, l'IL-13 et l'IgE, appelés kinoïdes, et d'apporter la preuve de concept de l'efficacité de ces vaccins dans des modèles d'asthme et de choc allergique (anaphylaxie). Nous avons démontré qu'une vaccination combinée contre l'IL-4 et l'IL-13 permet de réduire les taux d'IgE, l'hyperréactivité bronchique, l'éosinophilie et la production de mucus dans un modèle murin d'asthme chronique. De plus, nous avons montré qu'une vaccination avec des kinoïdes IL-4/IL-13 humains induit des anticorps neutralisants anti-IL-4 et IL-13 humaines et et réduit les niveaux d'IgE dans des souris humanisées pour l'IL-4, l'IL-13 et la sous-unité α de leur récepteur commun IL-4R. Nous avons également développé un vaccin conjugué contre l'IgE humaine. Nous avons montré que ce vaccin induit une forte production d'anticorps neutralisant anti-IgE humains, dans une nouvelle souche de souris humanisée pour l'IgE et son récepteur de haute affinité FcεRI. Une vaccination des souris humanisées IgE/FcεRI avec le kinoïde IgE humain réduit fortement les taux d'IgE et protège contre un choc anaphylactique induit par les IgE. L'ensemble de ces études démontre qu'une vaccination contre l'IL-4, l'IL-13 ou l'IgE pourrait représenter une solution thérapeutique contre les maladies allergiques.

1. Introduction

From the greek allos meaning “other or different” and “andergia” meaning “change in reactivity or capacity to react”, Von Pirquet was the first scientist to propose the terminology allergy, in 1906. He noted that the exposure of the body to an external substance resulted in antibody production and was responsible for a change that he called “subject-specific reactivity” to the substance. In his book, he insisted that responses to an antigenic stimulus or allergen could be divided into two categories: one that was immunity or the classical protection against infectious diseases, and a second that he called allergy or altered reactivity, in which immune response mediates clinical illness. The latter category included serum sickness, hay fever, anaphylaxis, Arthus reaction and asthma. He showed that antigen and antibody together could form “toxic bodies” (today’s immune complexes) (*1, 2*). Since then, the definition of allergy has evolved. Nowadays, allergy is considered as a hypersensitivity reaction initiated by specific immunologic mechanisms (*3*). It involves an abnormal reaction to a usually harmless substance called an allergen. Common allergens comprise pollens, dust mites, pets, some food components, insect venoms and some medications.

Allergic diseases have become some of the most common chronic diseases worldwide. Their occurrence has doubled during the past twenty years, affecting a large proportion of about 15-30% individuals all over the world (300 million people, estimated by the World Health organization) (*4*). Moreover, its incidence is continuing to increase in association with Western lifestyle. Allergies have thereby become one of the major medical challenges of the twenty-first century, requiring to improve global comprehension on pathophysiological mechanisms, underlying both allergic sensitization and allergic reactions. Although there are relatively few deaths from anaphylactic shock (estimated to cause death in 0.65 - 2% of patients (*5*)), the most severe clinical form of allergy, there are extensive burdens for sufferers, also representing an important economic loss (*6, 7*).

Allergic diseases include allergic rhinitis, food allergies, atopic dermatitis, angioedema, urticaria, allergic asthma, insect and drug allergies. The most dramatic clinical manifestation of allergy is anaphylaxis, an acute and potentially fatal systemic reaction which can occur in response to food, drugs and venoms.

For most of allergic disorders, a combination of symptom-relieving and control therapies (*i.e.* inhaled corticosteroids) forms the basis of management guidelines. Allergen-specific immunotherapy (AIT) is also recommended for the treatment of some allergies. Despite that, for some patients, the disease is refractory to the medication. In the era of monoclonal antibodies (mAbs), mAbs targeting IgE (Omalizumab) or Th2 cytokines (*e.g.* the anti-IL-5 mAb Mepolizumab or the anti-IL-4Rα [a common subunit for IL-4 and IL-13 receptors] mAb Dupilumab) are now commercialized for the treatment of some specific allergic conditions.

As an alternative of passive immunization, which is costly and required life-long injections, we want to propose a new approach to target important mediators in allergy. We make the hypothesis that vaccination against IL-4, IL-13 or IgE using conjugate vaccines called Kinoids (*8*) would promote long-term benefits in allergic diseases. The general aim of this thesis is to develop and characterize such IL-4-, IL-13- or IgE-targeting kinoids, and test their efficacy in relevant mouse models of allergy to perform a proof of concept.

1.1. Allergy: overview of the pathophysiology

The allergic response is characterized by a Th2 response, mediated by the expansion of T helper 2 cells (Th2 cells) and isotype switching of B cells generating IgE antibodies (*9*). The allergic cascade is initiated by a sensitization phase. Antigen-presenting cells (APCs) are activated after the contact with allergens. Activated epithelial cells release cytokines namely IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) favoring fully activation of APCs (*10*). In particular, TSLP is responsible for the complete dendritic cells (DC) maturation and costimulators upregulation (*i.e.* OX40L) (*11*). After allergen uptake, APCs migrate to the lymph nodes and present allergen peptides to naïve T cells (*12, 13*). The cytokine microenvironment, and in particular IL-4 drives the Th2 cells subset expansion. Th2 cells can then release an array of cytokines, including IL-3, IL-4, IL-5, IL-9, IL-13, IL-25, IL-31, IL-33, TSLP and granulocyte/macrophage colony-stimulating factor (GM-CSF) (*14, 15*). These cytokines are involved in the class-switching of B cells to IgE synthesis, and their differentiation into IgE-producing plasma cells. In addition, this complex cytokine interplay also drives recruitment of major effector cells of allergy, including mast cells, eosinophils and basophils, which will be discussed in more details below.

Upon re-exposure to the same allergen in sensitized individuals, the allergen can bind specific IgE on the surface of basophils and mast cells. This leads to aggregation and activation of the FcεRI receptor (a process called "cross-linking"), resulting in induction of a complex signaling cascade: it is the type I hypersensitivity reaction. During this early phase of the response, mast cells and basophils degranulate and release a range of preformed mediators, including histamine and proteases. This response is very fast and occurs minutes after the exposure to the allergen (between 1 and 30 minutes after). Finally, some patients also develop a late phase reaction that appears several hours after allergen exposure (within 6 to 72 hours). Chemokines and cytokines released by mast cells and other cell types direct the recruitment and activation of inflammatory cells, including Th2 cells and eosinophils (**Figure 1**).

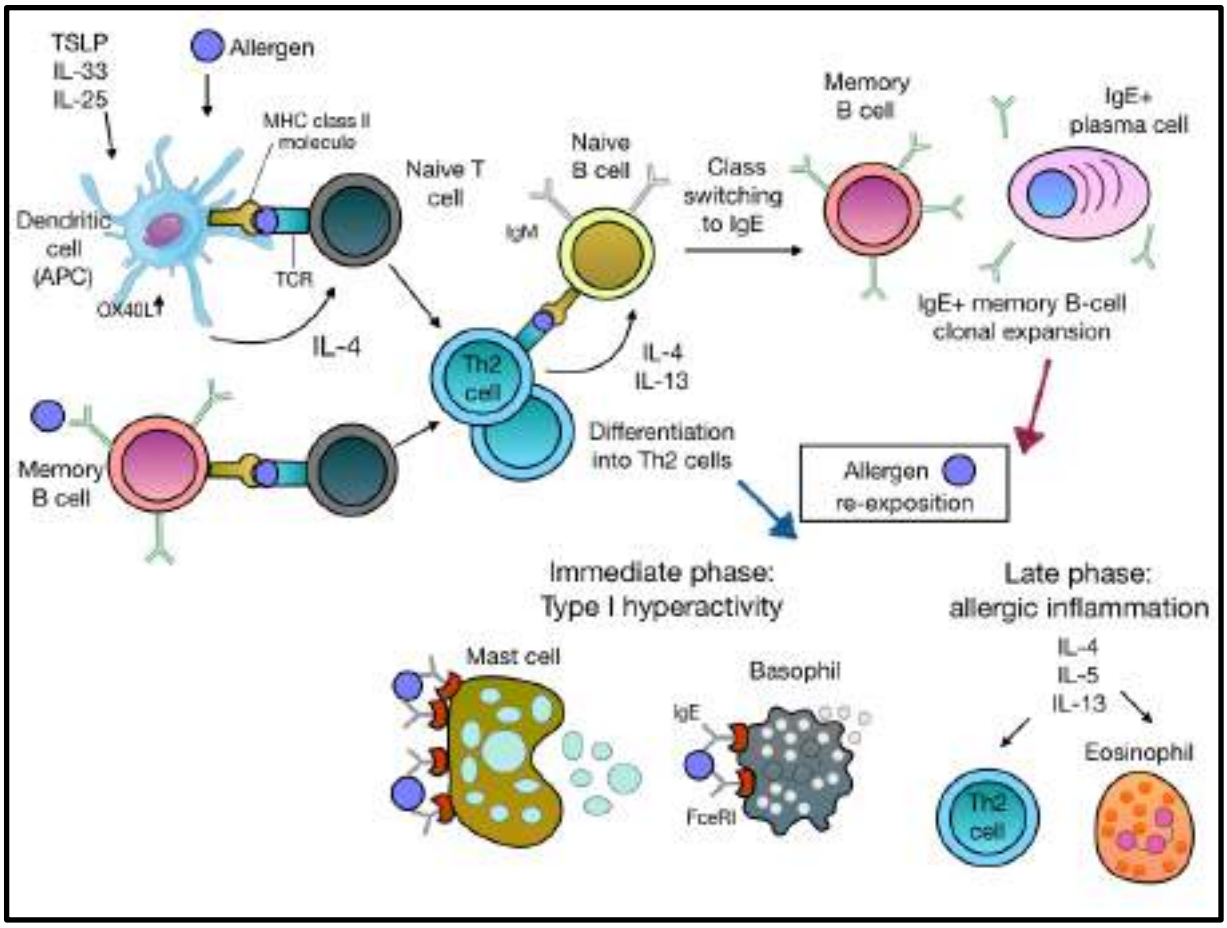

Figure 1. Mechanisms of allergic reactions. *Sensitization to allergens and development of specific B cell and T cell memory. Differentiation and clonal expansion of allergen-specific Th2 cells leads to cytokine production, such as IL-4 and IL-13, which induce immunoglobulin class switching to IgE and clonal expansion of naive and IgE+ memory B-cell populations. During second exposition to the allergen, type I hypersensitivity reaction is triggered as well as allergic inflammation (late phase of the allergic reaction). Following migration to sites of allergen exposure under the influence of chemokines and other cytokines, allergen-specific T cells are reactivated and clonally expanded. Th2 cells released cytokines, such as IL-4, IL-5 and IL-13, which are responsible for recruitment and/or activation of inflammatory cells, epithelial cells, endothelial cells and smooth muscle. Adapted from (16).*

1.2.Origins of allergy

The prevalence of allergic diseases has dramatically increased over the past years, especially in industrialized countries. More than 30% of children are allergic, among which up to 10% suffer from asthma and allergic rhinitis and 5-7% from food allergies (*17*). A complex interplay between genetic determinants and environmental stimuli is at the basis of allergies. Candidate genes of allergic asthma are the ones that regulate IgE production and the maturation and proliferation of important effector cells, such as mast cells and eosinophils. In 1989, markers on chromosome 11q were identified as linked to atopic phenotype (*18*). The β chain of the high affinity IgE receptor is encoded by a gene located on this chromosome. Some polymorphisms on this gene have been significantly associated with atopy (*19*). At least 14 genes in the 5q

region have been associated with an asthma or atopy phenotype, including some of the most replicated genes (IL4, IL13, CD14, ADRB2, SPINK5, LTC4S) (*20*). Additionally, the broad linkage region on 12q is also a strong candidate for being linked with allergy. This region encodes for several genes, including the vitamin D receptor (VDR) gene, interferon (IFN)- γ, nitric oxide synthetase, stem cell factor and signal transducer and activator of transcription 6 (STAT 6) and have been linked to asthma and total serum IgE levels (*21*). Moreover, other genes have been associated to asthma or an atopic phenotype in several studies, including a mutation in IL4RA (I75V), located in 16p12 (*22, 23*). Finally, reduced filaggrin expression is implicated as a major predisposing factor to atopy. Filaggrin is a key protein that facilitates terminal differentiation of the epidermis and formation of the skin barrier. Loss-of-function mutations (R510X and 2282del4) in the gene that encodes filaggrin (FLG) are one of the most well-known causal factor of barrier dysfunction, triggering atopic dermatitis (eczema) (*24*). Even if FLG is not expressed in the respiratory epithelium, FLG is now also considered as an asthma gene susceptibility, which risk mechanisms is not yet fully understood (*25*).

Additionally, environmental exposure has emerged as a dominant factor in the development of allergies. During the past years, several hypotheses have been proposed to explain this phenomenon.

Following birth, newborns evolve from the intrauterine milieu to the external environment. In this period of time, gut colonization is influenced by different factors and the immune system postnatal maturation is highly impacted. First, delivery mode influences the microbial colonization. Then, during the first months of life, feeding mode (breastfeeding or not), dietary fatty acids and other compounds in the dietary can impact the microbiome (*26*). Thus, appropriate intestinal microbial stimuli during early life are critical for inducing an immunoregulatory network that protects from IgE induction at mucosal sites.

The “hygiene hypothesis” proposed that there is an inverse relation between incidence of infectious diseases in childhood and appearance of allergic diseases afterwards (reviewed in (*17, 27, 28*)). Emerged in 1989, Strachan reported that children who grew up in large families in the United Kingdom had a lower risk of hay fever and eczema compared with children in small families, and suggested that “allergic diseases were prevented by infection in early childhood, transmitted by unhygienic contact with older siblings, or acquired prenatally from

mother infected by contact with her older children" (*29*). Studies comparing children in different environments suggested that farmer's children are more protected from allergies than their peers not living in agricultural environment (*30*). The high exposure to endotoxin levels (including lipopolysaccharides (LPS) contained in the outer structure of Gram-negative bacteria cell wall) seem to be associated with an increase of Th1 cytokines, decreasing the Th2 phenotype. However, it does not seem to be the case for respiratory pathogens (*31*). Most wheezing episodes in children younger than 3 years old are due to viral infections in the lower respiratory infections, resulting from rhinovirus and respiratory syncytial virus infections (*32*). The mechanism underlying this effect is not completely understood but can result from an important and poorly controlled inflammation in the airways.

Additionally, some evidences seem to indicate an important influence of the environment *in utero*. Placenta and its associated membranes produce a wide variety of cytokines throughout normal gestation (*33*). IL-4 and IL-13 are detectable in all phases of pregnancy at the feto-maternal interface. They are produced by the placenta immune cells and also by other cells types such as maternal and fetal endothelial cells (*34, 35*).

Epidemiological studies in humans have revealed conflicting results regarding the role of maternal allergen exposure in the development of allergies in offspring (reviewed in (*36*)). Recent findings showed that maternal diet exposure and sensitization to food allergens could protect offspring from suffering from allergic disorders, in humans and mice (*37, 38*). In addition, studies have highlighted that maternal diet could be directly associated with child predisposition to develop allergic and/or respiratory disorders. In line with this observation, a significant relation has been found between the mother's intake of omega-6 polyunsaturated fatty acid and the risk to develop asthma for the child at the age of 4-6 years old (*39*).

1.3.Main allergic diseases and clinical manifestations

1.3.1. Asthma

Asthma is a highly prevalent chronic respiratory disease affecting 300 million people worldwide (*40*). It is characterized by chronic activation of the innate and adaptive system in the airways releasing preformed or newly synthetized mediators, such as cytokines that can be

found in bronchoalveolar lavage (BAL) fluid or pulmonary secretions (*41*). Compared to the intact epithelium of a healthy subject, the bronchial epithelium from patients with mild asthma is characterized by a cellular infiltrate (mainly lymphocytes, eosinophils, neutrophils and degranulated mast cells), goblet-cell hyperplasia and collagen deposition in the submucosal area (*42*) (**Figure 2**).

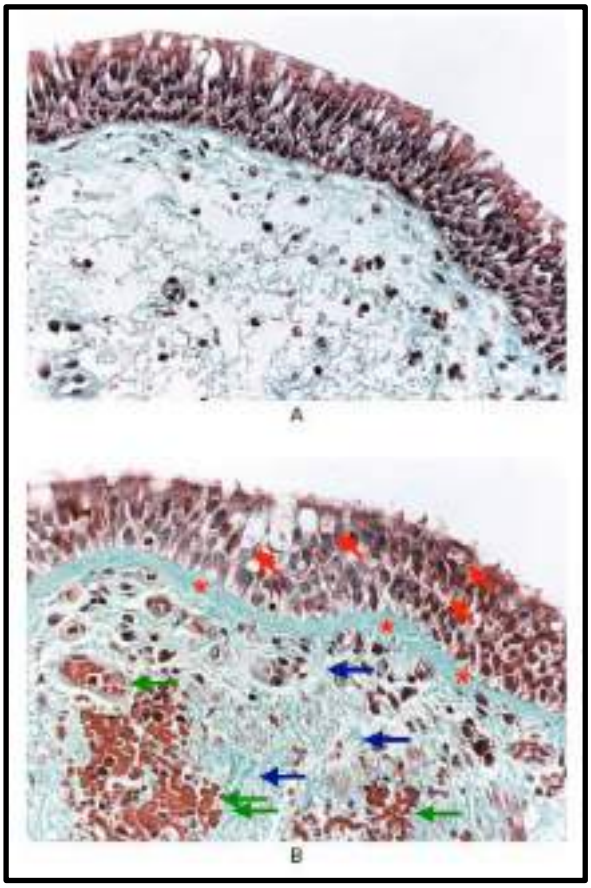

Figure 2. Specimen of bronchial mucosa from (A) a subject without asthma and (B) a patient with mild asthma (Hematoxylin and Eosin, ×40). *In the subject without asthma, the epithelium is intact; there is no thickening of the sub-basement membrane, and there is no cellular infiltrate. In contrast, in the patient with mild asthma, there is evidence of goblet-cell hyperplasia (red arrows) in the epithelial-cell lining. The sub-basement membrane is thickened (red asterisk), with collagen deposition in the submucosal area (blue arrows), and there is a cellular infiltrate (green arrows). Adapted from (42).*

Common symptoms include airway hyperreactivity (AHR) (tendency of smooth muscle cell to react to nonspecific stimuli such as cold air and exercise), mucus overproduction, airway wall remodeling and airway narrowing (causing airflow obstruction). It leads to repeated periods of shortness of breath, wheezing, cough and chest tightness (*43*). The most common hospitalization cause for asthmatics patients are exacerbations requiring an urgent treatment with systemic corticosteroids (*44*). The obstruction in asthma exacerbation occurs because of an important smooth muscle contraction, an edema in the airway wall and the bronchial lumen

obstruction with mucus. Asthma exacerbations are associated with accelerated loss of lung function triggered by activated inflammatory pathways and remodeling.

Spirometry is the main test for detecting and measuring airway obstruction in children over 5 years old and adults (*45*). Forced expiratory volume in one second (FEV1) is widely used as functional index in asthma follow up. The maximal expiratory flow-volume curves are considered as gold standard for lung function assessment. Regular assessment of FEV1 might help to identify children at risk for developing progressive decline in airflow. Among asthmatic children, a FEV1 lower than 60% is a risk factor for exacerbations and its decrease is associated with increasing asthma severity (*46*).

Asthma is an heterogenous disorder with a broad spectrum of phenotypes ranging from mild to severe forms with different degrees of response to standard treatments. It has been generally classified in two main forms according to the clinics. Most of the children and around 50% of adults suffer from allergic asthma (*43*). The disease starts with a sensitization phase, as described above. During childhood, the disease potentially evolves from eczema and rhinitis to asthma. This stepwise progression has been called "the atopic march" (*47*). The disease is characterized by elevated IgE levels against a specific allergen, such as house dust mite (HDM), pollen or peanut. Airway and blood eosinophilia also occur, as well as the presence of biomarkers that depend on the type 2 cytokines IL-13, such as periostin in serum and the exhaled fraction nitric oxide (FeNO) (*48, 49*). Both periostin and FeNO are mainly produced by the airway epithelium that express the common IL-4 and IL-13 receptor (*50*).

Non-allergic asthma is the other type of asthma, more common in adults, especially in women. This form of disease is often related to rhinosinusitis, polyps and obesity and often requires long term treatment with steroids (*51*). Although not being the most common type of asthma, some patients show a neutrophil-predominant disease. Studies in large cohorts enable the identification of neutrophilic inflammation through induced sputum as an important hallmark of a distinct cluster of patients with moderate to severe asthma (*52*). It is frequently associated to a more severe form (reviewed in (*53*)), resistant to standard treatments with steroids with less reversible airway obstruction. Tumor necrosis factor (TNF)-α is thought to play an important role in the pathophysiology of this disease form, as increased TNF-α levels in blood and BAL have been reported in patients with severe refractory asthma (*54*). More recently, in mice,

neutrophilic asthma has also been associated with T helper 17 (Th17) cells and the cytokine IL-17 (*55, 56*). However, the role of this cytokine has not been fully elucidated. Some studies suggested that IL-17 has a protective role in asthma, being important in allergic asthma resolution, but also having a role in initiating allergic asthma by induction of neutrophil recruitment (*57*). Other studies suggested that IL-23 and Th17 cells not only induce Th17 cell-mediated neutrophilic airway inflammation but also up-regulate Th2 cells mediating eosinophilic airway inflammation (*58*). Discrepancies can be due to the different times of analysis, as the protective role of IL-17 has only been shown during the challenge phase of the disease. Cytokine production by Th17 cells seem to be particularly resistant to inhibition by standard treatments with steroids, which could explain why this type of asthma is more refractory to first-line treatments.

Despite this general classification, clinicians suggest it may represent an oversimplification, with asthma pathology being more heterogeneous and complex. Although these two extreme phenotypes exist, there is a continuous spectrum of distinctive disease endotypes, with a considerable overlap of the disease, related its severity (*59*). Due to this complexity, no single drug will be effective for all patients. An accurate categorization of patients, by identifying immunologic basis underlying disease, is decisive to propose the most adapted therapeutic strategy.

1.3.2. Atopic Dermatitis

Atopic dermatitis (AD), also known as atopic eczema, is the most common inflammatory skin disorder, with a prevalence of 15-20% in developed countries (*60*). The disease is associated with cutaneous hyperreactivity to environmental antigens. The condition most often develops during childhood and is characterized by recurrent eczematous lesions (erythematous (red) patches with exudation, blistering and crusting at early stages and scaling, fissuring cracking and thickening at later stages) with intense itch and discomfort. Moreover, the disease is extremely heterogenous with a wide spectrum of intensity and disease subtypes. Distinctive degrees of epidermal barrier disruption, activation of different T cell subsets and commensal skin microbiota dysbiosis interact and contribute to cause the varying clinical presentations (reviewed in (*61*). Remarkably, atopic dermatitis frequently predates the development of allergic rhinitis or asthma suggesting a strong correlation between these diseases. It has notably been described as the atopic march (*62, 63*).

Although most of AD patients show high total and allergen-specific IgE concentrations in blood and skin, others have normal total IgE levels, and negative serum allergen-specific IgE (*64*). Thus, AD has been classically subdivided in IgE-associated or extrinsic-type AD and non-atopic dermatitis, or intrinsic-type AD (*65*). In extrinsic-type AD, Th2 cytokines expression is increased and high levels of circulating IgE as well as eosinophils have been observed (*66, 67*). The majority (80%) of patients with AD are generally classified as having extrinsic-type disease, based on the detection of high serum IgE levels (*68*). However, it is important to note that while high serum IgE levels are detected in most AD patients, the utility of targeting IgE in AD is still debated (*69*).

1.3.3. Food allergies

Food allergy (FA) is an immune-mediated adverse reaction to food, affecting 5% of adults and 8% of children (*70*). Reactions are elicited rapidly (within minutes up to two hours) after ingestion of the offending food. Symptoms can affect the skin (hives), respiratory tract (wheezing, coughing) and gastrointestinal tract (nausea, vomiting, diarrhea). Important food allergens include peanut, cow's milk, tree nuts, soy, fish and egg (*70*). Despite the increasing prevalence of food allergy, there are currently no approved treatments beyond allergen avoidance and urgent treatment (*i.e.* adrenaline injection) after accidental food intake.

Tolerance perturbation promotes allergen sensitization and it is characterized by Th2 cells responsible for increasing IL-4, IL-5 and IL-13. Production of allergen-specific IgE is central to FA development. The mechanism and site of IgE induction responses in FA are poorly understood. However, a recent study indicates that local IgE production in the gastrointestinal tract might play an important role in FA (*71*). In addition, Lin *et al*. reported that duodenal IgE-positive cells are increased in adults with FA, as well as activated eosinophils and T cells (*72*).

1.3.4. Anaphylaxis

Anaphylaxis is a severe, life-threatening generalized or systemic hypersensitivity reaction. It is the most extreme manifestation of allergic reaction characterized by an acute, systemic and potentially fatal response upon contact with an allergen. While anaphylaxis symptoms can involve any organ, the most commonly affected are the cutaneous (affecting around 88% of cases), respiratory (76.1%), cardiovascular (41.9%), and gastrointestinal systems (12.8%) (*73*).

Severe reactions (associated with hypotension) are more likely to be drug induced, representing up to 58% of fatal anaphylaxis (*74*). Anaphylaxis is usually presented as an acute episode, with a peak at 30 to 60 minutes after allergen contact (*75*). This uniphasic type resolves over the next hour with no recurrence of symptoms. Biphasic anaphylaxis is defined by recurrence of symptoms hours after resolution of the initial event in the absence of re-exposure to the trigger (*76*).

Anaphylaxis can be classified as allergic or non-allergic anaphylaxis depending on the underlying mechanism (*77*). Allergic anaphylaxis is defined as reactions mediated by IgE, IgG or immune complexes, and non-allergic anaphylaxis for all other non-immunological causes. Allergic anaphylaxis can be mediated by an IgE-dependent or -independent mechanism (*78*), whereas non-allergic anaphylaxis involves direct mast cell activation (*79*), relying on distinct mast cell receptors. Of particular, cell surface receptor MRGPRb2 in mouse and MRGPX2 in human were found in connective tissue mast cells (*80*). In mice, authors demonstrated that this receptor recognizes multiple cationic proteins and many small molecule drugs (*e.g* neuromuscular-blocking agents (NMBAs)) that are known to trigger anaphylaxis in humans. Independent of the underlying mechanism, symptoms are similar and caused by the release of mediators such as histamine, tryptase, platelet activating factor (PAF), cysteinyl leukotrienes, and others, including cytokines and chemokines (reviewed in (*81*)).

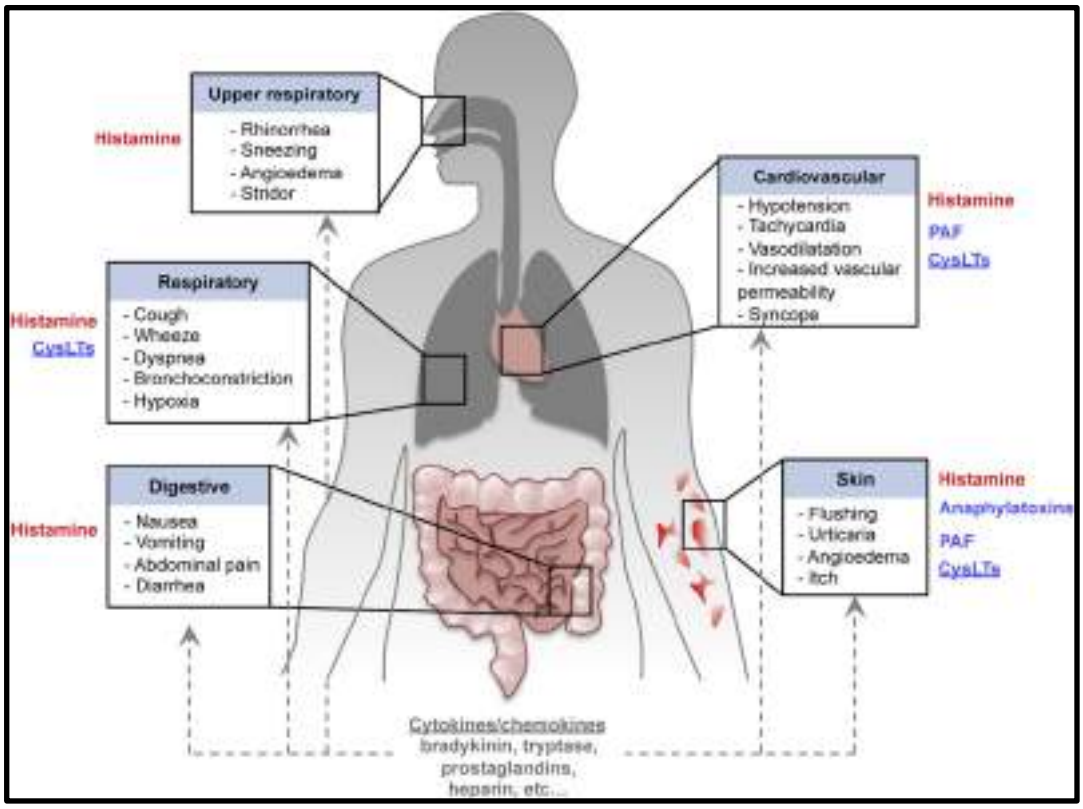

***Figure 3**. **Pathophysiologic changes in anaphylaxis and mediators that have been implicated in these processes** (from (81)).*

Anaphylaxis was classically described to rely on IgE antibodies bound on the surface of FcεRI expressing cells. Upon allergen re-exposure, IgE induced crosslinking of FcεRI, which triggers cells activation (as described in section 1.1). Highlighting the importance of IgE and mast cell-mediated reactions in mice, IgE-mediated passive anaphylaxis was abrogated in mice deficient for the high affinity receptor, FcεRI (*82*), as well as in several strains of mast cell deficient mice (*83-85*). Therefore, IgE-dependent mast cell activation can indeed produce many of the pathological changes that are associated with anaphylaxis.

In addition, the fact that some patients experience anaphylaxis despite having undetectable levels of circulating allergen-specific IgE suggests the existence of IgE-independent pathways of anaphylaxis (*86*). Upon allergen recognition, IgG binds to IgG activator receptors (FcγRs) expressed on myeloid cell surface, leading to mediators release, such as histamine and PAF. Such mediators release can cause physiologic manifestations similar to those seen in patients with IgE-dependent anaphylaxis (mainly hypothermia, vasodilatation, and cardiopulmonary changes). A very recent study conducted in suspected anaphylaxis to NMBAs patients found

that concentrations of anti-NMBA IgG and markers of FcγR activation, PAF release, and neutrophil activation correlated with anaphylaxis severity (*87*).

Evidences for IgE-independent, IgG-dependent anaphylaxis was provided by studies in mice either by passive (PSA) or active systemic anaphylaxis (ASA). PSA can be elicited by systemic injection of antigen-specific IgG followed by challenge of the corresponding antigen. Passive sensitization with mouse IgG1 specific antibodies conferred mice the ability to develop systemic anaphylaxis upon allergen exposure (44,213). By using either knock-out mice for the different low-affinity FcγRs or by selectively blocking these receptors, different studies have highlighted the importance of low-affinity IgG receptors, with a notable role for mouse FcγRIII (*88*). Other evidences came from ASA models when mice are actively immunized and then challenged with the same model antigen. In these models, mice develop a polyclonal IgE and IgG response before the challenge. ASA can still occur in the absence of IgE (*89*), FcεRI (*90*) and even mast cells or basophils (*90, 91*).

This thesis is particularly focused on allergic asthma and IgE-mediated anaphylaxis. Proof of concept of vaccine candidates have been conducted in mouse models of these allergic clinical manifestations.

1.4. Main effector cells and mediators of allergy and asthma

1.4.1. Main effector cells

Several immune cell populations contribute to allergic disorders and asthma. An overview of important cellular mediators is described in this section. When developing new therapeutic strategies, the decrease of this cells is desired.

Mast cells

Mast cells (MCs) are derived from hematopoietic stem cells and they are generated in the bone marrow. These granular cells then migrate through the blood to the tissues where they mature, residing in vascularized tissues near surfaces exposed to external environment like skin, gut

and lung. MCs maturation and proliferation is stimulated by stem cell factor (the ligand of the KIT receptor or CD117) and by cytokines, like IL-3 or IL-9 (*92*). MCs have diverse functions in physiological and pathophysiological processes and they are one of the first immune cells, with DCs, to interact with environmental antigens. MCs are usually found close to blood and lymphatic vessels, epithelial surfaces and smooth muscle, influencing their flow, secretion and contraction.

MCs are involved in Th2 responses against helminth infections (*93*) but also in allergic disorders. In allergy, MCs are notably involved in the IgE hypersensitivity reaction. MCs constitutively express on their surface the FcεRI, the high affinity receptor for IgE (*94*). FcεRI expression is positively regulated by IgE increase (*95*). After allergen exposure and IgE-dependent activation, FcεRI aggregates and activates MCs. This FcεRI-dependent MC activation response includes a rapid release of preformed mediators stocked in cytoplasmatic granules. Histamine, heparin and other proteoglycans, several proteases (*i.e.* tryptase, chymases and carboxypeptidase A3) and some cytoplasmatic-granule-associated cytokines are rapidly secreted. When activated, MCs also produce newly formed lipid mediators, including cysteinyl leukotriens (LTB4, LTC4, LTD4 and LTE4), prostaglandins (PGD2), platelet-activating factor (PAF) as well as many cytokines, chemokines and growth factors (*i.e.* TNF-α, VEGF, IL-3, IL-4, IL-5, IL-13, GM-CSF, MCP1) (*96*). MCs play a fundamental role in asthma as evidenced by increased mast cells numbers in the airway smooth muscle layer in mild asthmatics, associated with bronchial hyperreactivity, and a positive correlation observed between the mast cells numbers and disease severity (*97*). Recently, a study showed that increased tryptase levels in BAL fluid correlated to asthma severity, with severe asthma patients exhibiting the highest levels. In addition, elevated mast cell tryptase levels in severe asthma patients, independent of their type 2 biomarker status (blood eosinophils and periostin) (*98*). Finally, MCs are able to produce and respond to IL-4 and IL-13 (expressing both IL-4R1 and IL-4R2 receptors) (*99*).

Studies in mice demonstrated that mast cells can amplify AHR, mucus production and eosinophilia in chronic asthma models developed in the absence of artificial adjuvant (*98*). However, these asthma features are still developed in mast cell deficient mice in asthma models using adjuvants such as aluminum salts for the sensitization phase (*100*), indicating mast cell contribution can be bypassed in these conditions. Mast cell significance has also been studied in other mouse models of allergic diseases, such as in AD and severe contact hypersensitivity

(CHS). Results obtained differed, suggesting that mast cell contribution might be different depending on the use of different MC-deficient animals in several experimental conditions (*101, 102*). It has been shown that MCs indeed represent a significant source of IL-10 in their mouse model of severe CHS responses, significantly limiting both the inflammation and the tissue pathology observed in severe CHS reactions (*102, 103*). Finally, the role of mast cells was also evaluated in a peanut-induced anaphylaxis in mice. Authors showed that inducible and selective ablation of MCs can significantly reduce peanut-induced anaphylaxis suggesting a role of these cells in the pathology (*104*).

Eosinophils

Eosinophils are one of the main effector cells in type 2 inflammation. In a non-inflammatory state, eosinophils are circulating granulocytes and represent about 1 to 3% of total pool of white blood cells. Normal eosinophil count in the peripheral blood ranges between 50 and 500 x 10^9 cells/L (*105*). They are characterized by a bi-lobed nucleus with highly condensed chromatin. The cytoplasm contains two major granule types. Primary granules contain Charcot-Leyden crystal protein and eosinophil peroxidase (EPO), and the secondary granules contain various cationic proteins, including eosinophil major basic protein (MBP), which is an endogenous antagonist for M2 muscarinic receptors. Eosinophils are produced in the bone marrow, from hematopoietic stem cells. The precursors undergo maturation in the bone barrow upon exposure to IL-3, IL-5 and GM-CSF (*106*). IL-5 was demonstrated as crucial for eosinophils development, participating in their expansion, differentiation and activation (*107, 108*).

During inflammation, eosinophils respond to inflammatory signals and are recruited from bloodstream into tissues including the lungs. Eosinophils recruitment is possible thanks to a network of cytokines and chemokines. CCR3 is a chemokine receptor expressed on eosinophils that signals eotaxin subfamily: CCL1, CCL24 and CCL26 (or eotaxin-1, eotaxin-2 and eotaxin 3, respectively) (*109, 110*). Eotaxins are produced by activated epithelial cells under IL-4 and IL-13 influence. Then, IL-5 takes advantage over IL-4 for eosinophil production whereas IL-4 and IL-13 are critical for their recruitment to the inflamed tissues (*111*). In the tissue, IL-5 is also responsible for half-life increase of eosinophils. Eosinophils express the type 2 IL-4 receptor (IL-4Rα+ IL-13Rα1) and respond to both IL-4 and IL-13 (*112*). Moreover, eosinophils constitutively express both IL-4 and IL-13 mRNA transcripts (*113*), contain pre-formed IL-4 (*114*) and IL-13 (*115*) and might represent important early sources of these type 2 cytokine

during allergic immunity (*116*). Recently, Charcot-Leyden crystals have been shown to be more than just markers of eosinophil activation. Authors showed that galectin-10 (one of the most abundant proteins in human eosinophils) is released and undergoes a phase transition to a crystalline state that actively promotes key features of asthma in mice (*117*).

Beneficial eosinophil functions have been attributed to their ability to defend the host against parasitic helminths (*118*). In addition, eosinophils may also play a protective role against specific RNA viruses, as respiratory syncytial virus (RSV) (*119*). However, eosinophilia in the lungs is one of the hallmarks in asthmatic patients. Eosinophils increase in the airways is observed in the lungs, as well as in the blood, due to an increased production and recruitment to the inflammation sites. Even if eosinophils are not the unique cell type participating in airway pathophysiology, they contribute to asthmatic inflammation by various preformed mediators release. In particular, MBP secretion has been described to contribute to tissue damage, to indirectly increase smooth muscle reactivity and to trigger other important effector cells degranulation: mast cells and basophils (*120*). In addition, eosinophils generate eosinophil extracellular traps (EETs), which are composed by mitochondrial DNA and granule proteins, such as MBP (*121, 122*). EETs were detected in skin biopsies from patients with different eosinophil skin diseases, including infectious diseases and allergic diseases (*123*). In addition, the number of eosinophils secreting EETs is increased in patients with severe asthma (*124*). Another study showed that the percentage of EET-forming peripheral blood stimulated eosinophils was significantly higher in patients with severe asthma that in those with non-severe asthma and that this percentage was negatively correlated with baseline FEV1 and associated with reactive oxygen species production (*125*).

Basophils

Basophils are the rarest type of the three granulocytes subsets and constitute less than 1% of peripheral blood leucocytes in humans and mice (*126*). Originated in the bone marrow, basophils enter the circulation as mature cells and have a short life span (around 60h) under physiological conditions (*127*).

During allergic reactions and helminth infections, basophils are recruited into peripheral tissues where they rapidly degranulate (*127*). Basophils also express FcεRI in their surface and are also activated upon allergen exposure and crosslinking of FcεRI-bound IgE, releasing preformed

mediators stored in cytoplasmatic granules including histamine (*128*), proteases (MCPT8 and MCPT11 in mice) (*129, 130*), Th2 cytokines (including IL-4 and IL-13) (*131*), chemokines and lipid mediators (leukotrienes LTC4, LTD4 and LTE4) (*132*). Of note, basophils can also be activated upon an allergen-unspecific mechanism. Some cytokines have been described to contribute to basophil activation. For instance, in mouse and human, IL-33 and IL-3 are able to activate basophils in an IgE-independent manner leading IL-4 and IL-13 release (*133, 134*). Moreover, some protease allergens can also promote basophil activation, inducing IL-4, IL-5 and IL-13 production. This was shown in a study using basophils purified from human peripheral blood where authors observed that Der p 1, the major allergen from house dust mite (HDM) leads to cytokine expression by basophils (*135*). Increased basophils numbers are found during helminth infections as well as in the lung of asthmatic patients probably contributing to pathogenesis of asthma, but this remains to be demonstrated (*136*). In mouse and human, basophils have also been associated to the Th2 polarization, releasing IL-4 within minutes of activation and making these cells a possible candidate for the IL-4 innate source necessary for Th2 differentiation (*131, 137, 138*). Additionally, basophils are recruited to the lymph nodes of allergen-sensitized mice (*131*). Basophils primarily express type 1 IL-4 receptor, with a strong expression of IL-4Rα (*139*).

Even if the role of basophils still remains unclear, these cells could be important in the clinic for allergic disorders diagnostic. The basophil activation test (BAT) can be used to detect allergic reactions to drugs, food and venom in patients. This flow cytometry-based functional assay assesses cell activation degree after exposure to a stimuli, following basophil activation markers (CD63 and CD203c) (*140*).

Dendritic cells

Dendritic cells (DCs) have a crucial role in allergic asthma as antigen-presenting cells (APCs), for their signaling to CD4+ Th2 cells. DCs are present in tissues that are in contact with the external environment, such as the skin, the inner lining of the nose, lungs, stomach and intestines. They can also be found as immatures in the blood. DCs originate in the bone marrow and give rise to conventional (cDCs) and plasmacytoid DCs (pDCs). It is now established that cDCs can be subdivided in cDC1 responsible for cross presentation to CD8+ T cells and cDC2, whose main function is to present antigens to CD4+ T cells (*13*). In the lung, DCs can be found throughout the conducting airways, lung interstitium, blood vessels, pleura and bronchial lymph

nodes. They recognize pathogens or allergens via toll-like receptors, NOD-like receptors and C-type lectin receptors and they are probably activated in response to epithelial cytokines (IL-33, IL-25 and TSLP). In addition, DCs produce IL-13 (*141*) and respond to both IL-4 and IL-13 (expressing both IL-4R1 and IL-4R2) (*142, 143*). DCs represent the connection between innate and adaptive immune systems as they are activated after danger signals, process the antigens and migrate to the lymph nodes. A study using an HDM-induced asthma mouse model showed that DCs and not basophils are necessary for Th2 immunity as well as some asthma features induction, proposing a model where DCs initiate and basophils amplify the inflammatory response (*144*).

Macrophages

Macrophages are abundantly present in tissues and can take several forms (Kupffer cells, alveolar macrophages, microglia, and others). These professional phagocytes arise from monocytes under GM-CSF influence (*145*). They play a significant role in pathogen recognition and elimination, as well as in innate immunity. Based on differences observed in their phenotypes, macrophages have been classically classified as either activated (M1) or alternatively activated (M2) macrophages (AAMs). M1 macrophages have been associated to Th1 inflammation, induced by IFN-γ and LPS and were considered as pro-inflammatory macrophages.

On the other hand, M2 macrophages or AAMs have been associated to Th2 inflammation, induced by IL-4 and IL-13 and considered pro-repair and regulators (reviewed in (*146*)). AAMs produced increased levels of tissue remodeling factors, producing factors such as TGF-β, suggesting that AAMs enhance fibrogenesis and increase collagen deposition (*147*). Moreover, AAMs were suggested to promote angiogenesis and wound repair (*148*) during the healing phase of acute and chronic inflammatory disease. Macrophages express both the type I and type II IL-4 (also called type I IL-13) receptors, as well as IL-13Rα2 (*149*). Heller *et al*. suggested that human AAMs, appear to respond stronger to IL-4 than IL-13, probably because of the presence of presence of the γC subunit (*150*). Increased M2 polarization was observed in bronchoalveolar lavage fluid from patients with asthma (*151*).

However, this classification is now considered an over-simplification of macrophage heterogeneity and plasticity. Indeed, scientists have revised it in favor of a model that considers

a spectrum of macrophages polarization states. Current data indicates that macrophage polarization is a multifactorial process in which a large number of factors can contribute, producing different activation scenarios. Importantly, once a macrophage adopts a phenotype, it still retains the ability to change in response to new environmental influences (*152*).

Mouse M2 macrophages have also been studied in mouse models of parasite infections (*153*). Mouse studies showed the presence of M2 macrophages markers suggesting that, early after infection, M2 macrophages are generated probably contributing to mediate protection against parasite infection (*154*). In mouse models of asthma, M2 polarization contribution to asthma pathogenesis has to be clarified since some studies have demonstrated unaltered asthma severity in the absence of M2 polarization whereas others showed clear protection from asthma (*155, 156*).

Interestingly, lung macrophages population is heterogenic. Alveolar macrophages and interstitial macrophages are two different phenotypes that differentially exert immunological function under physiological and pathological conditions (*157*). Alveolar macrophages reside in the inner surface of the lung and can differentiate to major subsets (M1, M2) in response to different stimuli. Unlike the second type of lung macrophage, interstitial macrophages, which reside in the interstitial areas of the lung, maintain homeostasis and induce tolerance for harmless antigens (*158*). Recently, in a mouse model of allergic airway inflammation study, authors found that adoptive transfer of these interstitial macrophages isolated from CpG-treated mice recapitulated CpG protective effects and showed that it was dependent on IL-10 (*159*).

Neutrophils

Neutrophils are polymorphonuclear and represent the most abundant circulating leucocytes in humans, accounting for 50% to 70% of blood leucocytes (*160*). Although eosinophilia is recognized as an important feature in allergic asthma, evidence supports an important role for neutrophils also in other types of asthma. Neutrophils play an essential role in the immune system, acting as the first line of defense against bacterial and fungal infections. Indeed, neutrophils are the first cells recruited to the inflammation site. They are essential for the pathogen recognition leading to their phagocytosis and to cytotoxic granules secretion. Neutrophils are produced in the bone marrow from stem cells that proliferate and differentiate under the control of key transcription factor such as C/EBPα or Gfi-1. Granulocyte colony-

stimulating factor (G-CSF) is also essential for neutrophil production (*161*). Neutrophils are produced in high amounts, with 1 to 2 x 10^{11} cells being produced per day in a healthy adult. They have long been considered as short live innate immune cells, with a half-life between 6 - 12 hours. However, recent studies have shown that neutrophils can exhibit an extended half-life in certain inflammatory conditions (reviewed in (*162*)).

Neutrophil numbers are not increased in airway secretion of patients with mild asthma, however patients with severe asthma have shown an increased neutrophil number in airway lavage (*163*). Neutrophils are one of the first cells to be recruited into the lungs during specific asthma-related events, as shown after bronchial allergen challenge in asthmatic patients, comprising several steps initiated by adhesion molecules endothelial expression (*164, 165*). In addition, neutrophils increase has been found in airway secretions from individuals with asthma experiencing an exacerbation (*166*). Among all the neutrophil products, some of them have been found to be more concentrated in neutrophils from asthmatic patients compared with control neutrophils. Matrix metalloproteinase 9 (MMP-9) and reactive oxygen species (ROS) produced by neutrophils were detected in bronchoalveolar lavage fluids and bronchial biopsies from patients with allergic asthma after allergen challenge (*167*). Finally, IL-4 may also be related to neutrophil recruitment during allergic asthma. Human neutrophils are targets of IL-4 and IL-13 expressing both type I and II IL-4 receptor (*168, 169*). Recent studies demonstrated that IL-4 and IL-13 adversely affect several functional properties of neutrophils, inhibiting its expansion, migration and NET formation (*169, 170*).

Although a vast majority of studies performed in animal models seek to model eosinophilic airway inflammation, some studies showed that airway neutrophilia and AHR can occur together (*171*). These models are often triggered by a viral infection and AHR can persist after the acute viral infection resolution. A recent study in mice has found that rhinovirus-induced exacerbation of asthma is in part mediated by neutrophil extracellular traps (NETs) and double-stranded DNA neutrophil release in the airway (*172*). In addition, data in mice using neutrophil-depleting antibodies have suggested that neutrophils could be implicated in IgG-mediated anaphylaxis (*173*). Human and mouse neutrophils express several activating receptors for the Fc portion of IgG (FcγR) (*174*).

Effector T-cell subsets

T helper 2 (Th2) cells and cytokines that they secrete are a distinct feature of allergic response. Th2 cells are characterized by the specific transcription factor expression of GATA 3 and are considered central cells in the development of allergic diseases and asthma. CD4+ lymphocytes in peripheral blood and BAL fluid from patients with severe acute asthma demonstrate increased expression of activation markers (CD25) as compared with those from patients with chronic airflow limitation or normal controls (*175*). Moreover, activated T cell number correlates with the degree of peripheral-blood eosinophilia (*176*), as well as eosinophil number in BAL fluid, symptoms severity and bronchial hyperresponsiveness degree (*177*). They are considered as important Th2 inflammation drivers being crucial sources of IL-4, IL-13 and IL-5, illustrated by an increase expression of mRNA and protein level in BAL from asthmatic patients (*178*). In peanut allergic patients, specific T cell clones have been found in peripheral blood and characterized *in vitro*. Compared with healthy donors, an increase of T cells was shown with a specific Th2 profile (*179, 180*). Different cohorts of children with AD have also found an increase in Th2 cells in peripheral blood (*181, 182*). Additional studies suggested that while acute AD lesions are characterized by a marked Th2 (but also Th17) activation, chronic lesions are characterized by mixed Th profiles (*183*).

T helper 17 (Th17) cells are a pro-inflammatory T helper subset defined by IL-17A production characterized by the specific expression of the transcription factor retinoic acid–related orphan receptor-γt (RORγt). As introduced, although asthma is classically associated with eosinophilia and Th2 cells and cytokines, some patients show a neutrophil-predominant disease with absence of Th2 cytokines and a mixed Th1 and Th17 cytokine milieu. A large number of studies suggest that IL-17 presence in asthmatic airways is correlated with asthma severity. Elevated IL-17A levels have been found in sputum from asthmatic patients and IL-17A concentration found at these sites positively correlates with asthma severity (*184*). The same observation was found in lung biopsies (*185*) as well as in the BAL fluid (*186*). In skin biopsies from AD patients, marked infiltrates of T cells were found with a marked Th2 and Th17 activation profile (*68, 187*).

IL-17A contribution in neutrophilic asthma has also been confirmed in several mouse studies (*56, 188*). However, as discussed previously (see section 1.3.1.), IL-17A exact contribution in asthma is still unclear, particularly because some reported have demonstrated that IL-17A or Th17 cells alone are not sufficient to trigger the disease and interestingly another study in mice

has shown that IL-17A can mediate anti-inflammatory mechanisms during the challenge phase (*57*). In AD, Th17 have also been found after epicutaneous OVA exposure in mice (*189*).

Regulatory T cells (Treg cells) are T cells functionally distinguished by their capacity to limit T cell proliferation and effector functions. They are characterized by the transcription factor expression of forkhead box protein 3 (Foxp3). Allergic symptoms reduction after successful allergen immunotherapy has been associated with IL-10 and Treg cells increase (*190*). In asthmatic patients, Treg cells are functionally impaired, affecting Th2 regulation (*191*). Finally, studies in mouse showed that after adoptive transfer, Treg cells suppress asthma features through IL-10 and TGF-β production, two cytokines implicated in the suppression of DC activation (*192*).

Type 2 innate lymphoid cells (ILC2s)

ILC2s are effector lymphocytes that are embedded at mucosal sites and are involved in tissue homeostasis and response to diverse environmental insults. ILC2s lack antigen-specific receptor, but like Th2 cells react to epithelial cytokines (IL-25, IL-33 and TSLP). ILC2s produce IL-4, IL-13 and IL-5 (*193, 194*). However, IL-4 expression appears to be tightly regulated and it is probably produced by ILC2s under very defined conditions (*195-197*). For example, in mice, CysLT1 receptor expression was found on lung ILC2s and LTD4 potently induced CysLT1R-dependent ILC2 production of IL-4 (*198*). ILC2s were first described in the gut, associated with adipose tissues in the peritoneal cavity from mice infected with helminths (*195*). ILC2s are produced in response to transcription factor GATA-3 and RORα from a common lymphoid progenitor that also give rise to T and B cell lymphoid progenitor.

ILC2s do not express antigen receptors but react promptly to "danger signals" from inflamed tissue and produce an array of cytokines that direct subsequent immune response. Allergen proteases (Derp 1 from HDM or papain) might indirectly activate ILC2s through an effect on lung epithelial cells (by IL-25, IL-33 and TSLP production). In addition, LTD4, a major proinflammatory mediator released from activated mast cells, leads to ILCs activation through the cysteine leukotriene receptor (CysLT1R) (*198*). Following IL-25 and IL-33 administration, ILC2s accumulate in the lung and are a major source of IL-13 and IL-5, which are essential for many critical aspects in allergic asthma (described in section IL-13 and IL-5) (*199*). In ILC2s, IL-4 and IL-13 receptors expression is still unclear. ILC2s also contribute to airway

hyperreactivity development in response to respiratory virus, such as rhinovirus or influenza virus. A role for ILC2s cells in viral infections might help to explain how viral exposure can cause asthma symptoms in some people, promoting a nonallergic eosinophilic airway inflammation (*200*).

1.4.2. Main mediators

Cytokines IL-4 and IL-13, and IgE antibodies play key roles in allergic reactions, and therefore represent good therapeutic targets.

IL-4

IL-4 was first cloned in 1986 from a mouse T cell line and was described as an IgG1 induction factor (*201*). The same year, Yokota and colleagues isolated, from a concanavalin A-activated human T-cell cDNA library, the human homologous (*202*). IL-4 is a pleiotropic cytokine implicated in allergic disorders but also represents a multifunctional regulatory agent of the immune system. It is produced by CD4 T cells but also by basophils, eosinophils, mast cells and natural killer T (NKT) cells (*131, 203, 204*). ILCs can probably also produce small amounts of IL-4 (*198, 205*).

IL-4 is implicated in growth control, survival and gene expression. This interleukin has been involved in allergy development, as increased levels have been observed in serum of allergic patients and in bronchoalveolar lavage (BAL) of asthmatic patients (*206*). It regulates a wide spectrum of cellular functions such as T lymphocytes proliferation and Th2 phenotype generation (*207*). IL-4 is also the major cytokine driving IgG1 and IgE production by B cells (*208*). It is also implicated in expression regulation of the high affinity IgE receptor (FcεRI) (*209*) and the low affinity IgE receptor (CD23) (*210*), in the alternative activation of macrophages (M2 macrophages) (*211*) and adhesion molecules expression such as the vascular cell adhesion molecule-1 (VCAM-1) important for eosinophil and T cell infiltration (*212*). IL-4 plays a predominant role in controlling parasite infection, limiting adult worm survival. Treatment with anti-IL-4 antibody substantially abrogated protective immunity to *Heligmosomoides polygyru* infection (*213*). In addition, IL-4 treatment was able to cure gastrointestinal nematode infections in mice (*214*). In contrast with these initial results, IL-4-deficient mice retained the capacity to expel N. *Brasiliensis* whereas IL-4Rα deficient mice failed probably suggesting overlapping functions with IL-13 (*215*).

IL-4 gene is located in chromosome 5, in 5q31 (*204*) and occurs naturally in two splice isoforms (*216*). The conventional IL-4 is encoded by four exons and has been well described. The second isoform produced by alternative splicing is called IL-4δ2 and it is encoded by exons 1, 3 and 4, omitting exon 2. Human IL-4 protein is a 15 kDa globular protein with 129 residues, 4 helices and 3 disulfide bonds formed between 6 cysteines present in the sequence. In 1987, J. Ohara and W.E. Paul reported that a saturable high affinity chain receptor existed on the surface of T cells (*217*). IL-4 signaling is regulated via two receptors, both signaling via the Janus Kinase (JAK) / signal transducer and activator of transcription (STAT) pathway (*218*). Type 1 IL-4 receptor, comprises the high affinity IL-4 receptor alpha chain (IL-4Rα) and the common gamma chain (γc) and exclusively binds IL-4 (**Figure 4**). IL-4Rα chain is widely expressed whereas γc subunit is more restricted to specific hematopoietic cell type, including lymphocytes and myeloid cells (*219*). Type 2 receptor binds both IL-4 and IL-13 and is composed by IL-4Rα and IL-13Rα1 subunits. Engagement of both receptors initiates recruitment of signal transducer and activator of transcription factor 6 (STAT6) by the consensus sequence GYKxF and its consequent phosphorylation. The STAT 6 phosphorylation results in its dimerization and translocation to the nucleus to finally induct transcription of IL-4 responsive genes involved in Th2 response. Type 1 receptor can also activate the insulin receptor substrate (IRS)-2 pathway (*150*).

IL-13

Discovered in 1993 by A. Minty *et al.*, IL-13 is also a pleiotropic Th2-type cytokine implicated in allergic disorders (*220*). The cytokine is produced by activated Th2 cells, NKT cells, mast cells, basophils, eosinophils, B cells, macrophages and activated type 2 innate lymphoid cells (ILC2) (*221-224*). Studies have revealed that IL-13 plays a pivotal role in host defense against parasite infection (*215*) as revealed using IL-13 deficient mice but also in the pathogenesis of allergic diseases being one of the most important cytokines in asthma pathology (*225, 226*).

IL-13 drives many relevant cellular responses. Some of the functions are similar to IL-4, including B cell proliferation, promoting class switching to IgE and inducing CD23 expression on B cells (*227*). On macrophages, IL-13 is also able to promote M2 polarization which is a specific functional phenotype, characterized by anti-inflammatory and tissue regeneration functions (see section 1.4.1. Macrophages) (*228*). On eosinophils, IL-13 promotes survival, activation (*229*) and recruitment to the site of inflammation by inducing expression of IL-5 and

eosinophil chemokines such as eotaxins, as well as expression of VCAM-1 on endothelial cells (*230, 231*).

In addition, IL-13 has important functions on non-hematopoietic cells. It is a potent inducer of VCAM-1 expression on human lung fibroblasts, boosting muscular contraction and enhancing smooth muscle cell proliferation (*232*). IL-13 also induces collagen production by fibroblasts forcing airway remodeling in the context of asthma. IL-13 has also been described as one of the major cytokines implicated in mucus hypersecretion and goblet cell metaplasia (*233*), in airway hyperresponsiveness and in airway narrowing observed in patients with asthma (*234*). Finally, IL-13 induces vascular endothelial factor (VEGF) expression in the lungs, leading to angiogenesis (*235*). Recently, it has been demonstrated that IL-13 and IL-4 directly activate sensory neurons that express IL-4Rα and IL-13Rα1 chain (*236*).
IL-4 and IL-13 share one receptor: Type II IL-4 receptor also called Type I IL-13 receptor (expressed in non-hematopoietic cells). The different IL-4 and IL-13 functions can be explained first because IL-13 is more expressed in the lung effector sites, whereas IL-4 is mainly produced in the lymph nodes (*196*). It can also be explained by the fact that IL-4 signaling occurs via Type I and Type II IL-4 receptor, as detailed above, whereas IL-13 activates the Type II IL-4 receptor or Type I IL-13 receptor. IL-13 does not activate Type I IL-4 receptor but can also bind Type II IL-13 receptor which exclusively recognizes IL-13 (**Figure 4**).

Heterodimerization of subunits forming Type 1 IL-13 receptor also causes activation of Janus kinases, Tyk2 and Jak1, followed by STAT 6 activation (similar activation pathway by IL-4). Interestingly, IL-13 also binds Type II IL-13 receptor composed of IL-13Rα2. Traditionally, Type 2 receptor was always considered as a decoy receptor as the cytoplasmic tail is only 7 amino-acid. However, recent studies have shown that the receptor exists in both soluble and membrane forms encoded by distinct transcripts generated by alternative splicing (*237*). Although mechanisms by which this receptor mediates IL-13 functions are still unclear, one study proposed that IL-13 promotes TGF-β1 synthesis by macrophages, inducing fibrosis and participating in the allergic responses (*238*) .

Finally, it has been shown that binding to the Type II IL-4 receptor varies in signaling potencies and kinetics depending on the cytokine. IL-13 first binds IL-13Rα1 and then this binary complex recruits IL-4Rα. On the other hand, IL-4 first interacts with IL-4Rα and then recruits

IL-13Rα1 to form a ternary complex (*239*). IL-4 and IL-13 are then required for receptor heterodimerization. IL-4 binds with higher affinity and the cytokine is able to signal more effectively at lower concentrations than IL-13. However, the higher concentration of IL-13Rα1 than IL-4Rα appears to allow high concentration of IL-13 to signal more strongly that IL-4 (*239*).

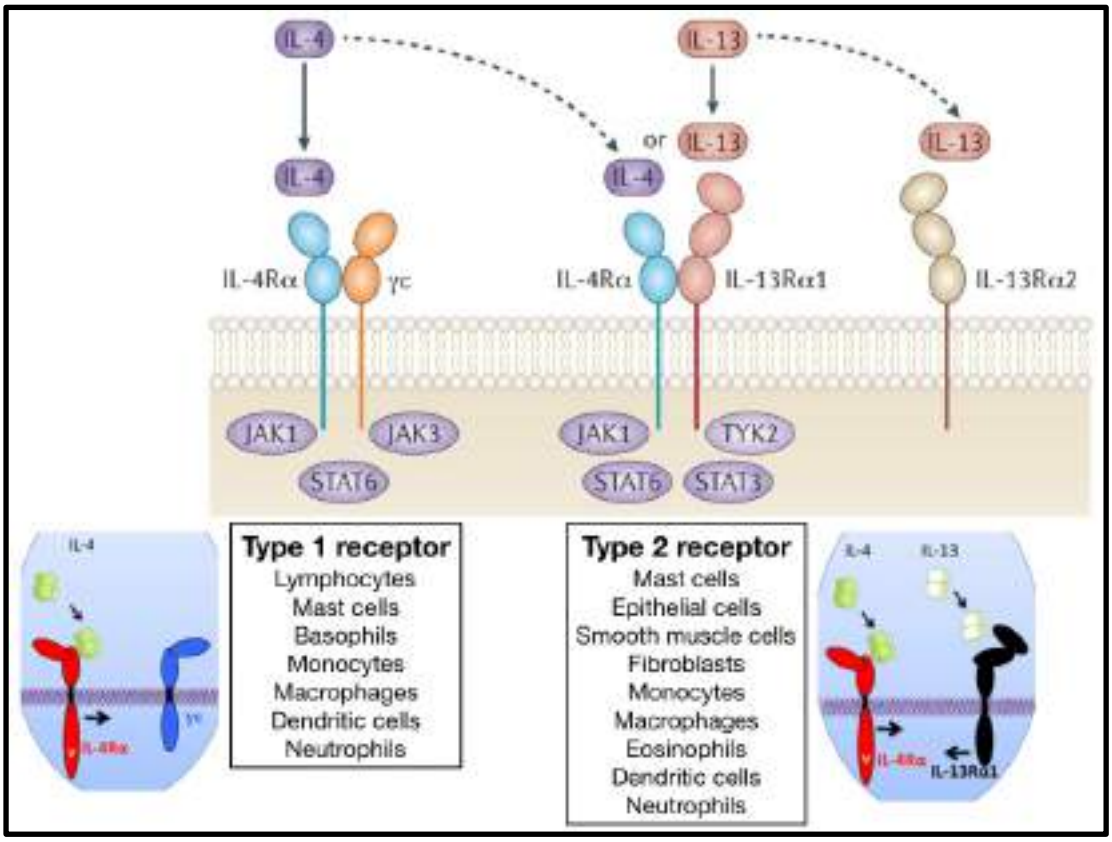

Figure 4. Receptor systems for IL-4 and IL-13 and cellular distribution*. The distinct and overlapping functions of IL-4 and IL-13 can be explained because both cytokines shared a receptor chain: IL-4Rα. IL-4Rα forms two distinct heterodimeric receptor complexes. Type 1 receptor comprising IL-4Rα and γc chain only binds IL-4. Type 2 receptor formed by IL-4Rα and IL-13Rα1is the primary receptor of IL-13 but also binds IL-4. However, binding of both cytokines occurs in a different manner. In addition, IL-13 binds to a second receptor, IL-13Rα2, the function of which is unclear but is postulated to be a decoy receptor. Adapted from (219, 240).*

IgE antibodies and their receptors

Discovered about 50 years ago, IgE represents the isotype found at the lowest concentration in the circulation (50-200 ng/mL IgE in healthy individuals vs. around 10 mg/mL for IgG). Molar concentration is no more than 5 x 10^{-10} M, which is 100 000 times lower than IgG. IgE is very short-lived in plasma (around 1 day) but when bound to its receptor, it can remain fixed to cells in tissues for weeks and even months (*241*).

Antibodies are composed by two functional different parts: the antigen-binding fragment (Fab) and the fragment crystallizable (Fc) region. Two antigen binding sites are contained within the Fab region of antibodies, and they are covalently linked through the antibody heavy chain to Fc effector domains. The Fc domains provide specificity for the activation of downstream effector functions and are exclusively derived from antibody heavy chain constant domains (**Figure 5A**). Isotype switching after B cell activation produces immunoglobulins with identical antigenic specificity connected to different heavy chain constant regions: IgA, IgD, IgE, IgG and IgM. These different isotypes are important since they interact with distinct cellular receptors and thus initiate different defense mechanisms.

Secreted IgE can bind the high affinity receptor for IgE (FcεRI) constitutively expressed at high levels on the surface of mast cells and basophils but also (in humans) in other cell types including neutrophils, eosinophils, platelets, monocytes, Langerhans cells, dendritic cells and airway muscle cells (*242*). Due to the low circulating concentration of IgE, interaction with FcεRI has a high affinity degree (Kd 10^{-9} to 10^{-10} M). The high affinity receptor FcεRI is composed by 4 subunits. The α-chain contains an extracellular portion composed by two Ig-like domains (D1 and D2) responsible for binding with IgE and a short cytoplasmic domain which is thought to have no signaling function. The γ-chains homodimer is responsible for the cell signaling and contains two immunoreceptor tyrosine-based activation motif (ITAM) domains. Finally the β-chain includes an extra ITAM domain and promotes signal amplification (**Figure 5B**) (*210*). Apart from expression on mast cells and basophils, in humans, FcεRI is also expressed on other cell types, as mentioned above, and the receptor is reported to be an αγ2 trimer. The FcεRI αγ2 trimer biological function is less well understood. Recently, Greer and collaborators showed that human FcεRI internalization by conventional DCs and monocytes (which express the αγ2 trimer) contributes to serum IgE clearance (*243*). Since FcεRI has no intrinsic enzymatic or channel activity, its ability to signal involves FcεRI-associated enzymes activation (tyrosine kinase of the Src family Lyn and Fyn) (*244*). Antigen binding to receptor-bound IgE causes receptor aggregation, triggers cellular activation and mediates IgE biological functions. A circulating soluble FcεRI form (sFcεRI) of about 4 kDa has been described in human serum. However, cell types releasing the protein as well as the physiological role remains to be identified (*245*).

The low affinity receptor FcεRII (or CD23) is a Ca2+ dependent type C-lectin composed by a unique polypeptide and exists in different forms. Its affinity for IgE is lower than FcεRI affinity (Kd 10^{-5} M) (*246*). CD23 was initially considered as the low affinity receptor for IgE but an increased affinity was later evidenced, by oligomers formation (*247*). While FcεRI is homologous to a family of antibodies receptors for IgE, IgG and IgA, CD23 belongs to a different protein structural class and is uniquely associated with the IgE system (**Figure 5C**). CD23 also differs from FcεRI in terms of function. CD23 is involved in immune responses regulation and responsible for IgE negative regulation (*248*). In humans, the CD23 gene has two transcription initiation sites and differential usage of these sites and alternate splicing of RNA transcripts gives rise to two distinct proteins isoforms. Both forms differ in their N-terminal intracellular domain in 6/7 amino acid. CD23a is constitutively expressed on B cells and mediates IgE endocytosis. CD23b is induced in particular by IL-4 and can be expressed on the surface of T cells, Langerhans cells, monocytes, macrophages, platelets and eosinophils. The extensive CD23 distribution on human cells is due to the use of the CD23b promoter. In mouse, only one isoform is found that most closely resembles human CD23a. Therefore, CD23 expression is relatively limited in the mouse and is found only on B cells and follicular dendritic cells (*249*). Membrane CD23 is responsible for multiple functions, including IgE antibody-dependent presentation in B cells, feedback inhibition of IgE synthesis in murine B cells and IgE antibody-dependent killing of tumor cells by human monocytes (*250*). In addition to its role as a low-affinity receptor for IgE, CD23 can be cleaved from cell surfaces to yield a range of soluble CD23 (sCD23) proteins that have pleiotropic cytokine-like activities. The metalloprotease responsible for CD23 release is ADAM 10, which cleaves CD23 to generate a 37 kDA or 33 kDA molecule (*251*). The major allergen found in HDM, Der p 1, is also able to cleave CD23 into a smaller 16 kDA fragment (*252*). The oligomerization state of sCD23 influences its function on B cells. Monomeric sCD23 species inhibit IgE synthesis in activated B cells and trimeric sCD23 interacts with IgE with a higher affinity and enhances IgE synthesis by activated B cells (*253*).

The extremely slow dissociation rate of IgE from its FcεRI receptor represents a distinctive feature in the human immune system and has left many questions open until now. IgE share the same basic structure with IgG with two identical heavy chains and light chains, but the heavy ε chain contains one more domain than the heavy γ chain of IgG (**Figure 5A**). The Cε3 and Cε4 domains are homologous with the Cγ2 and Cγ3 IgG domains. Cε2, located in the position

equivalent to flexible hinge of IgG is the most different part of IgE. This "extra" domain was thought to be a simple spacer between the antigen-binding site (variable chain) and the Cε3 - Cε4 domains. Interestingly, Zheng and collaborators showed that the distance between the N-terminal and the C-terminal was less than half that expected for a planar IgE molecule, suggesting that the variable and the constant fragments of IgE were very close (*254*). When the IgE crystal structure was determined, this data was validated showing that IgE is in a bent conformation, with the Cε2 folded back onto the rest of the structure, making extensive contact with the Cε3 domains and even touching the Cε4 domain of the other chain. The IgE structure is asymmetric with respect to the Cε3 domains (*255*). While Cε2 from chain B makes extensive contact with Cε3 from chain A and touches Cε4 from chain A, Cε2 from chain A makes very few contacts with Cε3 and Cε4 from chain B. The two Cε3 chains make no contact with each other whereas the two Cε4 domains are more related (*256*). IgE Cε2 domain do no play a role in receptor engagement but interact with Cε3-Cε4 domains to influence receptor binding kinetics and complex stability, contributing to the slow dissociation rate of IgE to FcεRI (*257*). Several intra- and inter-domain disulphide bridges control IgE structure and activity, which is also regulated by glycosylation at various sites. In particular, disruption of the glycosylation site found in the Cε3 domain at asparagine-394 (N394) in humans, and N384 in mouse abrogates IgE binding to FcεRI, highlighting glycosylation importance in IgE biology (*258*).

Comparison of Cε3-Cε4 crystal structure alone in solution or bound to FcεRI suggested that Cε3 domains can undergo large conformation rearrangement, adopting either a "closed" or an "opened" conformation by rotation relative to Cε4 (**Figure 5D**). Cε3 from chain A adopts a closed position preventing interaction with its receptor FcεRI, whereas Cε3 from chain B adopts a more open structure enabling to interact with FcεRI (*259*) . A number of intermediate states are also observed in different IgE-Fc crystal forms. Conformational flexibility in the IgE-Fc affects the interaction between IgE and its receptors and might be important for unique aspects of IgE biological function (*259-261*). The existence of a closed conformation and demonstration that this closed conformation is incapable to bind FcεRI opens the door to new strategies for the designing inhibitors of this interaction. Stabilization of the closed confirmation by molecules could block the interaction with FcεRI, leading to a novel class of therapeutics.

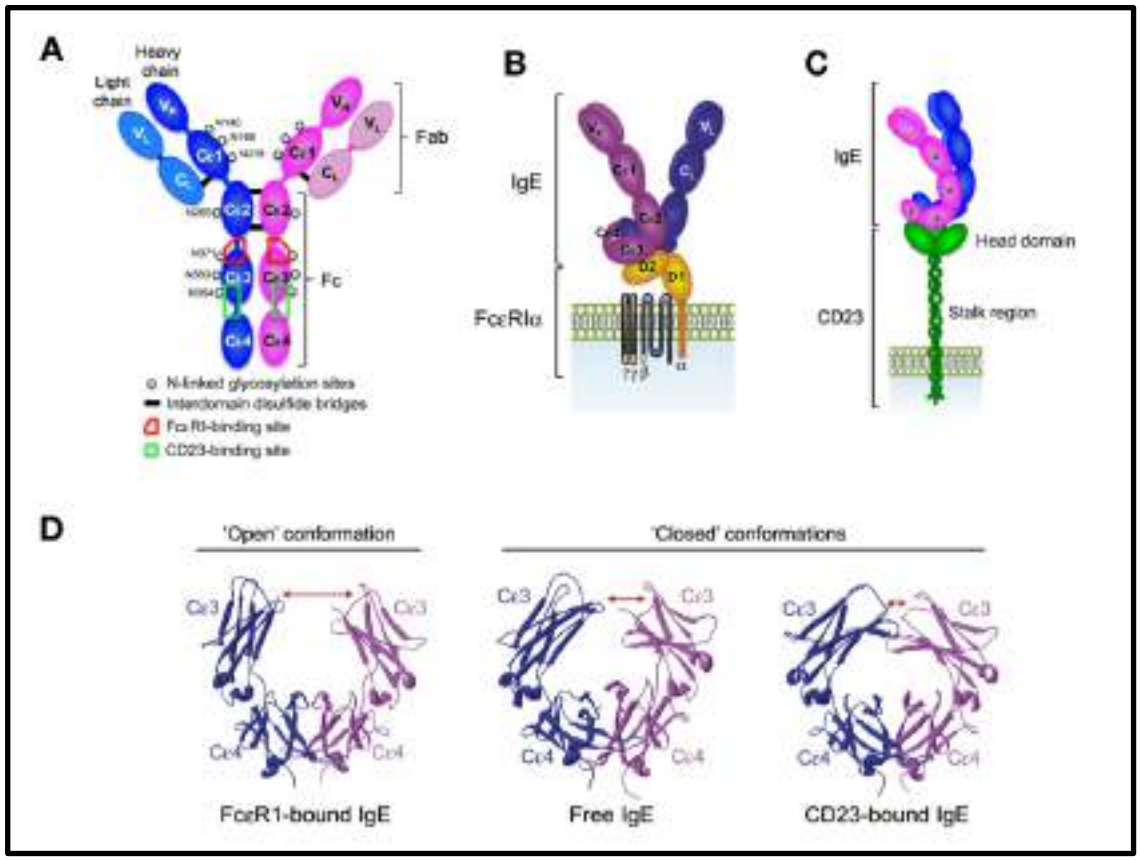

Figure 5. IgE. *A) IgE and its interaction with B) FcεRI and C) CD23 structure. C) Conformational changes in IgE Fc portion upon binding to FcεRI or CD23.*

To further explore this possibility and to develop new reagents for the identification of IgE-Fc conformation modulators, Wurzburg and co-workers produced a disulfide bond mutant (Cys-335) of the IgE-Fc that is "trapped" in the closed conformation state. They reported that the fragment, produced in insect cells, does not bind FcεRI unless the disulfide bond is reduced (*262*).

Monoclonal antibodies that bind IgE in the closed conformation have demonstrated to be safe, avoiding mast cell degranulation and are a suitable strategy for allergic diseases treatment. Omalizumab structural basis has been recently clarified (*263*). The structure reveals the mechanism of omalizumab-mediated inhibition of IgE interactions with FcεRI and CD23 and explains why the antibody selectively binds free IgE. Omalizumab does not appear to inhibit IgE-FcεRI interaction by disorting FcεRI binding site. Instead, the Omalizumab-Fab is positioned between the binding sites of both FcεRI and CD23, thus locking interactions with both receptors, with the heavy chain proximal to the CD23 binding site and the light chain proximal to the FcεRIα-binding site.

IgE antibodies play an important role in mediating allergic reactions. Both IgE and mast cells are concentrated in mucosal tissues, thus IgE are among the first defense molecules that the pathogen encounters. When an allergen crosslinks IgE-FcεRI complex on mast cell surface, it rapidly triggers the so-called early phase of the allergic reaction, releasing preformed molecules contained in mast cell granules. The late phase of the reaction is initiated hours after (see section Mast cells). Mast cell activation is a central event and leads to characteristic allergy signs. As FcεRI is also expressed in other cell types in humans, allergen also activates IgE on the surface of sensitized APCs (dendritic cells), promoting IgE production by B cells. Hence, consumed IgE can be replaced to maintain mast cell and APC sensitization.

Additionally, it has been shown that IgE can also facilitate antigen presentation. The first evidence came from patients with atopic dermatitis (AD). Bruijnzeel-Koomen and co-workers detected IgE on epidermal Langerhans' cells surface (*264*). This observation was then extended to other skin diseases (*265*). Following allergen capture, Langerhans' cells migrate to lymphoid nods or stay in the tissue where they present the processed antigen to naïve T cells. It has been suggested that human peripheral blood DCs use the αγ2 trimer for allergen uptake and presentation to naïve T cells (*266*). Similarly, allergen-IgE complexes bound to CD23 on activated B cells surface may also mediate antigen presentation to T cells. *In vitro*, experiments showed that mouse and human B cells incubated with antigen-specific IgE were up to 100-fold more efficient than untreated B cells at presenting low concentration of the respective antigen (*267*). Mice pretreatment with anti-CD23 monoclonal antibody completely inhibited the IgE-mediated effect (*268*).

1.4.3. Other mediators

Depending on inflammation pattern (Type 2 or Type 1/Type 17), cytokines pattern differs. Cytokines produced during the induction and function of Th2 responses include IL-4 and IL-13 but also other important cytokines including IL-5, IL-25, IL-33 and TSLP. Neutrophilic asthma is more characterized by the presence of IL-17 or TNF-α (*50*).

IL-5

IL-5 was initially described as an eosinophil and B cell growth factor. The primary IL-5 physiological sources are activated Th2 cells and ILC2. However, other cell types contribute to IL-5 production including mast cells, NK cells and eosinophils themselves (reviewed in (*269*)).

Th2 cells produce and secrete IL-5 upon a complex activation process triggered by inhaled allergens and driven by dendritic cells (*50*). In this regard, the presence of IL-4 is essential, because of its requirement for Th2 cell activation. IL-5 release from ILC2 is induced by epithelial innate cytokines including IL-25, IL-33, and especially TSLP. IL-5 promotes proliferation, activation, differentiation, survival and degranulation of eosinophils, contributing to airway hyperreactivity in patients (*107*). Increased IL-5 levels are detected in bronchoalveolar lavage from patients with asthma (*270*). Levels of IL-5 were also elevated in patients' skin with AD and further increased in patients with elevated IgE levels (*271*). IL-5 biological effects are mediated by the IL-5 receptor (IL-5R), composed by the alpha subunit (IL-5Rα) and a non-specific βc heterodimer (which also binds IL-3 and GM-CSF). When IL-5 is present, it binds the IL-5Rα chain, allowing then the recruitment of the dimer βc and signaling pathway activation (*272*).

Epithelial Type 2 cytokines: IL-33, TSLP and IL-25

The epithelium is the interface with the environment and forms a physical barrier providing a first line of defense. Epithelial cells can rapidly detect and respond to pathogen-associated molecular patterns as well as cellular stress or tissue damage. Activation of epithelial cells from the skin, lung and intestine leads to cytokines, chemokines and antimicrobial peptides release, responsible for immune cells recruitment and activation (presented below). In the lung, epithelial cells have been shown to play a central role in controlling DCs activation (*13*).

IL-33 is expressed by epithelial cells but also by fibroblasts and endothelial cells and acts as an alarmin after allergen exposure through its receptor ST2. This receptor is highly expressed on ILC2s and Th2 cells (*273*). In physiological conditions, IL-33 resides in the nuclei of resting epithelial cells and it is released after allergen exposition (*274*).

TSLP was initially described as a CD11c+ DCs activator to promote CD4+ naïve T cells differentiation to Th2 cells (*275*). TSLP is highly expressed by epithelial cells and has been found to be overexpressed in the airways from patients with severe asthma (*276*) as well as in the skin from AD patients (*275*). Finally, in mouse models, studies have shown that TSLP acts on DCs promoting OX40L expression, monocytes, CD4+ T cells, mast cells and basophils also promoting the development of Th2 inflammatory response in cooperation with IL-33 (*277, 278*).

IL-25 is also released by activated epithelial cells and might play a role in enhancing Th2 phenotype in the lung. IL-25 receptor is expressed on Th2 cells surface. When activated, IL-25 promotes Th2 expansion and Th2 cytokines release. Elevated expression of IL-25 and IL-25R transcripts was observed in lung tissues from asthmatic patients, relating IL-25 with the exacerbated allergic disorder (*279*). Moreover, in mice, transgenic IL-25 overexpression in the intestine enhanced experimental food allergy, while mice lacking IL-25 receptor were resistant to food allergy (*277, 280*).

1.5.Approaches to target allergic diseases

This section will provide an overview of current treatments and innovative therapeutics for allergy and asthma.

1.5.1. Established treatments

For most allergic disorders, a combination of symptom-relieving and control therapies forms the basis of management guidelines. Inhaled corticosteroids (ICs) and short- and long-acting β2 adrenoceptors agonists (SABAs and LABAs) represent first line of asthma treatment according to guidelines. In the case of rhinitis, α-adrenoceptor agonists are used to relieve nasal congestion, and non-sedating H1-antihistamines (such as cetirizine) and topical corticosteroids are well established control therapies. Antihistamines act upon histamine receptors (H1 to H4), extensively expressed by many cell types. H1 receptor has been associated with many actions in relation to allergic inflammation, such as rhinorrhea, smooth muscle contraction, and many forms of itching (pruritus) (*281*).

Corticosteroids reduces Th2 mediated inflammation in asthmatic airways by inhibiting cytokines and chemokines expression. Free corticosteroids diffuse across the cell membrane, activating cytoplasmatic glucocorticoid receptors and modulating transcription of important genes implicated in the inflammation (*282*). ICs are effective at suppressing airway inflammation but they do not modify disease natural history. In addition, there is a concern about repetitive use of inhaled corticosteroids because of possible side effects that appear after long term use of treatment, such as eye cataract, weight gain, increased blood pressure, impaired growth in children or decreased bone mineral density (causing osteoporosis) (*283, 284*).

β2-adrenoceptor agonists are important bronchodilators for preventing and relieving bronchoconstriction. Inhaled SABAs includes salbutamol and turbutaline and are used for rapid relief of asthma symptoms. These molecules are agonists of β2-adrenoceptor activating cyclic adenosine 3'5'-monophosphate (cAMP) production, which is then responsible for protein kinase A activation mediating smooth-muscle relaxation. A major advance in this area of treatment has been the introduction of LABAs, salmeterol and formoterol. They induce bronchodilatation for at least 12 hours (*285*). They are used as a supplementary therapy for asthma not controlled by ICs. The use of an inhaler that combines ICs with LABAs is now the most-effective available therapy for asthma (*286*).

1.5.2. Allergen immunotherapy and allergen vaccines

Allergen immunotherapy (AIT) is an immune modifying therapy that has been recommended for the treatment of allergic rhinitis, venom hypersensitivity, some drug allergies, mild bronchial asthma and food allergies. Compared with ICs and LABAs, AIT is disease modifying, rather than palliative (*16*). The aim of this type of treatment is to induce immunological tolerance through repeated exposure to allergens. AIT modifies the response of APCs, T cells and B cells as well as number and function of effector cells mediating the allergic response (*16*). Effective immunotherapy modifies the allergen-specific response from a Th2 response to a nonallergic response characterized by an increase of the Th1 profile. AIT is also responsible for the induction of T-cell tolerance increasing Treg cells number (*287*). This response is characterized by anti-inflammatory cytokines production, such as IL-10 and TFG- β (*288*). Induced by IL-10, the humoral response is also modified with AIT (*289*). Protection is mediated through the induction of allergen specific IgG4 instead of IgE response (*290*). The protective role for IgG4 might result from its unique structural features of the hinge region resulting in a lower affinity for certain Fcγ receptors (*291*). IgG4 does not fix complement and can inhibit immune-complex formation by other isotypes, giving IgG4 anti-inflammatory characteristics. In addition, IgG4 is unique among IgG subclasses as its heavy chains can spontaneously dissociate and re-associate *in vivo* (a process called "Fab arm exchange"). Thus, most of the IgG4 pool is bispecific, making it unable to cross-link antigens to form immune complexes (*292*).

Historically, variability in safety and clinical efficacy has limited the application of AIT as well as high costs and long duration (3-5 years) of treatment. Increasing safety while maintaining or

even improving efficiency are the main research subjects for AIT (*293*). To improve AIT safety, the use of hypoallergenic recombinant allergens or chemically modified allergens (allergoids) can reduce reactivity with IgE (*294*).

Hypoallergenic peptides derived from Der p 2 (one of the most representative allergens of HDM) were described to lack IgE reactivity and reduced ability to induce allergic inflammation while preserving immunogenicity (*295, 296*). In mice and rabbit, the injection of these peptides (coupled to a carrier protein) induced specific IgG antibodies which were able to block specific IgE recognition. Further studies in mice evaluated the use of this peptides as a prophylactic strategy to prevent HDM-induced asthma. Authors showed that the recombinant hypoallergenic peptide derived from Der p 2 decreased airway hyperreactivity in response to methacholine and airway inflammation (*297*). This approach has also been evaluated in peanut allergy. Authors removed conformational and linear IgE epitopes in Ara h 2 (one of the most representative allergens in peanut), and showed that such hypoallergenic Ara h 2 has reduced binding to IgE and reduced capacity to activate basophils from patient's blood samples, as well as reduced anaphylactogenic potency in sensitized mice as compared to native Ara h 2 (*298*). Finally, this strategy has also been used in birch and grass allergy using continuous overlapping Bet v 1-derived peptides (*299*) or recombinant hypoallergenic derivatives of the major timothy grass pollen allergens Phl p 1- 6 coupled to a carrier protein (*300, 301*) respectively, with encouraging results in the clinics. Thus, by manipulating allergen structure and its IgE binding, allergenicity can be reduced, thereby reducing systemic reactions, making recombinant and peptide vaccines a safe and effective form of immunotherapy. This strategy can prevent some severe AIT complications including anaphylactic shock, which occurs in 0.1-5% of AIT treated patients (*302*).

Improvement of adjuvants or implementation of new administration routes are also strategies that can be adopted to develop a safer and more efficient vaccine for AIT. Subcutaneous immunotherapy (SCIT) has been first adopted. Intralymphatic and epicutaneous vaccination have recently been studied and showed similar efficacy to SCIT in grass pollen allergy reached with fewer injections and lower total allergen dose (*303*). IgE blockage during AIT has also been reported to increase efficiency and reduce side effects in allergic patients, including flood allergic patients. (*304, 305*).

1.5.3. IgE as therapeutic target

Because of its key role in type I hypersensitivity reactions, IgE has been historically a prime target for intervention in allergic diseases. Different strategies have been developed in order to target IgE.

Omalizumab is a recombinant humanized IgG1 monoclonal antibody directed against human IgE marketed by Novartis and Genentech under the trade name of Xolair® (*306*). It was FDA-approved for treatment of mild to severe allergic asthma and chronic spontaneous urticaria (*307, 308*). Omalizumab binds circulating free IgE, reducing potentially accessible IgE amount for binding to FcεRI or CD23 (*309*). Precisely, Omalizumab binds free IgE Cε3 domain. Importantly, Omalizumab does not recognize IgE that is already bound to FcεRI or CD23, avoiding receptor activation. As already mentioned, the IgE binding site of Omalizumab has been determined by molecular modeling and crystallography (*263, 310, 311*). Moreover, data obtained *in vitro* suggested that Omalizumab could facilitate FcεRI-bound IgE dissociation (*312*). In 1996, the two first randomized, double blind placebo-controlled trials were conducted (*313, 314*). These studies showed that the antibody was well tolerated and induced a reduction in serum IgE levels, an increased allergen dose needed to provoke an early asthmatic response and a reduced mean maximal fall in FEV1 during the early response. In addition to free serum IgE reduction, Omalizumab induced other indirect effects, as illustrated by a downregulation of FcεRI expression on the surface of basophils, DCs and mast cells, leading to a decrease of the inflammatory response mediated by T helper cells and preventing IgE-dependent allergen presentation (*315*). After 25 years of clinical research, more than 150 clinical trials have been completed, in different allergic conditions including food and venom allergies or mastocytosis. Although being generally well tolerated, Omalizumab administration can induce side effects ranging from skin inflammation (at the site of subcutaneous injection) to systemic anaphylaxis (0.2% of patients) (*316*). Very recently, it has been suggested that adverse reactions to Omalizumab are triggered by IgG receptors engagement due to immune complexes formed between Omalizumab and IgE. An Fc-engineered mutant version (unable to bind IgG Fcγ receptors) of Omalizumab was equally potent at neutralizing IgE as the original version but safer, as it did not induce IgG receptor-dependent adverse reactions in a humanized mouse model (*317*).

More recently, new anti-IgE therapies have been developed. Ligelizumab (QGE031), manufactured by Novartis, is also a humanized anti-IgE antibody directed against Cε3 but with an increased IgE affinity, as evidenced by increased equilibrium dissociation constant (Kd). It binds IgE Cε3 domain with 50-fold higher *in vitro* affinity and 6 to 9-fold greater potency *in vivo* compared to Omalizumab (*318*). In 2016, Ligelizumab was tested in patients with mild allergic asthma and was shown to have greater efficacy than Omalizumab on inhaled and skin allergen responses (*319*). However, in a more recent phase II clinical trial, Ligelizumab effect did not surpass Omalizumab and further development for asthma was discontinued (NCT01716754). Nevertheless, a recent phase IIb study indicates that Ligelizumab outperforms Omalizumab for the treatment of chronic spontaneous urticaria (CSU), an autoimmune disease thought to largely rely on anti-IgE or anti-FcεRI autoantibodies (*320*). Quilizumab (MEMP1972A), developed by Genentech, is also an anti-IgE antibody but targets IgE-expressing B cells by binding to the membrane bound IgE (the M1' epitope). It lyses IgE-expressing B lymphocytes, thus preventing allergen-induced generation of IgE producing plasma cells and indirectly reducing serum IgE levels (*321*). However, results obtained in a clinical trial with chronic spontaneous urticaria (CSU) were not convincing and its clinical development was stopped (*322*). In addition, XmAb7195 is a humanized monoclonal anti-IgE antibody developed by Xencor. The antibody was obtained after affinity maturation and Fc engineering of Omalizumab (introducing two-point mutation) to increase IgE binding and FcγRIIB binding (which is an inhibitory IgG receptor) (*323*). Finally, a different strategy has also been described to target IgE using small engineered proteins that can recognize targets with high specificity and affinity (*324*). Designed ankyrin repeat proteins (DARPins) are extremely stable and some molecules have been reported to bind human IgE with affinities in the low nanomolar range (*325*).

1.5.4. Monoclonal antibodies targeting cytokines

The good results in terms of efficacy and safety obtained with the first monoclonal antibodies have significantly increased their popularity. Nowadays, they represent an important impetus in academic research and industry, as highlighted by their large production for a large variety of applications ranging from research to therapeutic and diagnostic purposes. Because of the important role of cytokines in the allergic cascade, cytokines and their receptors represent promising therapeutic targets. Antibodies already on the market or under development are listed in **Table 1**.

Anti-IL-4 and anti-IL-13 monoclonal antibodies

A variety of monoclonal antibodies and soluble receptors blocking IL-4 and/or IL-13 have been developed for the treatment of different types of allergies, and some of them are currently evaluated in clinical development. The first antibody to be tested in humans was Pascolizumab, developed by GlaxoSmithKline (GSK). This humanized monoclonal antibody neutralizes both human and cynomolgus monkey IL-4 bioactivity and was well tolerated in monkeys (*326*). However, even if it was well tolerated in adults with asthma, results obtained in phase I/II clinical trial showed that Pascolizumab was not efficient in asthmatic patients and its development was discontinued (NCT00024544).

As IL-13 constitutes one of the most important cytokines in asthma, the clinical efficacy of a number of antibodies directed against IL-13 has been evaluated in asthma, with mixed results. Lebrikizumab, developed by Roche (and licensed to Dermira in 2017), is a humanized IgG4 monoclonal antibody. Results from a first clinical study showed an improvement in lung function in asthmatic patients with poorly controlled disease when treated by ICs and high pretreatment levels of serum periostin (*327*). However, no consistent results were obtained in a phase III clinical trial (LAVOTA study), in patients with uncontrolled asthma because the antibody did not show significant reduction in asthma exacerbations in biomarker-high patients (high levels of periostin) (*328*). The antibody is now being evaluated in a phase III clinical trial for the treatment of atopic dermatitis (AD) (NCT04178967).

Another human IgG4 monoclonal antibody against IL-13, Tralokinumab developed by AstraZeneca (and licensed to LEO Pharma in 2017 for skin diseases), has been evaluated in two phase II studies, also leading to controversial results (*329, 330*). Tralokinumab inhibits downstream IL-13-mediated effects by preventing cytokine binding to both IL-13Rα1 and IL-13Rα2. Treatment was associated with an improved lung function but no improvement was obtained on primary endpoint which was Asthma Control Questionnaire (ACQ-6). In a subsequent clinical trial in severe asthma patients, Tralokinumab did not significantly reduce asthma exacerbations but had a positive impact on FEV1 in patients receiving the antibody every two weeks. Moreover, results suggested a possible treatment effect in a defined population with severe uncontrolled asthma. Tralokinumab was then further investigated in two phase III trial (STRATOS) but results showed inconsistent effects on exacerbation rates (*331*). Data suggested that a group of patients selected using biomarkers would allow to benefit from

therapy against IL-13. As of today, FeNO and periostin only were identified as biomarkers potentially predictive of treatment effect (*332*). In December 2019, LEO Pharma announced positive results in 3 Phase III (ECZTRA 1-3) for the treatment of AD but detailed results are still unpublished.

The fact that IL-4 and IL-13 share a receptor explains their large functional overlap (see section 1.4.2). Targeting both IL-4 and IL-13 or their common receptor appears to be more effective than addressing a sole cytokine. Pitrakinra, marketed by Aerovance (under license from Bayer) and under the trade name of Aerovant®, is a biologic drug but not an antibody. It is a recombinant IL-4 containing two targeted point mutations and acting as an IL-4Rα antagonist by preventing IL-4Rα assembly with IL-13Rα. However, inhalation treatment did not show an overall significant improvement of asthma exacerbations (*333*). Interestingly, at the dose of 10 mg, a subgroup of patients carrying specific single nucleotide polymorphisms in the gene encoding IL-4Rα, located within the 3' untranslated region, 10 mg dosage Pitrakinra treatment significantly lowered asthma exacerbations frequency (*334*).

Dupilumab (REG668:SAR231893) is a recombinant human IgG4 antibody developed by Regeneron Pharmaceuticals and Sanofi and marketed under the trade name of Dupixent®. It targets IL-4Rα chain, the common chain shared by IL-4 and IL-13 receptors. Dupilumab then blocks transduction signal activated by IL-4 and IL-13. Two phase I studies were concluded in 2010 (NCT01015027 and NCT01484600) and two more in 2012 (NCT01537653 and NCT01537640) in healthy volunteers to evaluate tolerability, pharmacokinetics and safety of subcutaneous and intravenous Dupilumab administrations (*335*).

The first trial on Dupilumab safety and efficacy was performed in moderate to severe atopic dermatitis (AD) patients and results were published in 2014 (*336*). This study showed that Dupilumab, in a week-monotherapy, improved in a rapid and dose-dependent manner all clinical indexes reflecting AD disease activity, biomarker levels and transcriptome analysis. Finally, SOLO-1 and SOLO-2 were large international phase III clinical studies enrolling patients with moderate-to-severe atopic AD. Dupilumab improved AD signs and symptoms, including pruritus, symptoms of anxiety and depression and quality of life as compared with placebo (*337*). Dupilumab has been also clinically evaluated for asthma. Results of the first phase II were published in 2013 and showed that in patients with persistent, moderate to severe

asthma and blood eosinophil count of at least 300 cells/microliter or sputum eosinophil level of at least 3% using ICs and LABAs, Dupilumab treatment (subcutaneous injection with 300 mg once a week) improved lung function and reduced levels of Th2 associated inflammatory markers (*338*). The second pivotal phase IIb clinical trial included similar patients but a much larger group (n= 769) and also showed improvement in lung function as well as a reduction in severe exacerbations number (*339*). With this positive results, two large phase III studies were completed in 2017. The QUEST study included a broad population of patients with uncontrolled, persistent asthma treated with either 200 or 300 mg every two weeks with Dupilumab or placebo (*340*). The VENTURE study studied the effect of 300 mg Dupilumab administered every two weeks to patients with severe asthma, regularly using oral corticosteroids (OCs) (*341*). Both studies showed positive results with a decrease in exacerbations number as well as a better lung function (increase in FEV1) and asthma control associated with OCs uptake reduction. Dupilumab is now FDA-approved for the treatment of uncontrolled moderate-to-severe AD, for uncontrolled moderate to severe eosinophilic or oral steroid dependent asthma (for patients older than 12 years old). It has also been recently approved for the treatment of chronic rhinosinusitis with nasal polyposis as a consequence of the good results obtained in different clinical trials (*342*). Finally, in a recently phase IIb trial, Dupilumab administration has shown efficacy in eosinophilic esophagitis (*343*). Finally, Dupilumab is now under evaluation in a panoply of allergic and/or type 2-mediated diseases, including peanut allergy (NCT03793608), atopic keratoconjunctivitis (NCT04296864) or chronic obstructive pulmonary disease (COPD) with type 2 inflammation (NCT03930732). Good clinical results obtained with Dupilumab compared with monoclonal antibodies targeting only IL-4 or IL-13 suggest that blocking both cytokines is necessary to control pathology. Patients with the highest Th2 biomarkers would benefit the most from the treatment. A potentially useful biomarker might be FeNO, as the enzymatic activity of NOS is directly controlled by both IL-4 and IL-13. A sustained reduction of 40% in FeNO levels during treatment with Dupilumab was found.

Despite good results obtained in clinics with Dupilumab, other monoclonal antibodies targeting IL-4Rα have been developed, such as AMG317 (Amgen), but with no significant efficacy. In a phase IIb clinical trial, AMG317 did not demonstrate clinical efficacy across the overall group of patients and its development was discontinued (*344*). Several hypotheses could explain early failures of these antibodies. The antibody design (epitope choice) and administration route can

influence antibody efficacy, affecting its neutralizing activity and/or bioavaibility. Finally, study patient population definition (in this case with a marked Th2 profile) is also crucial to demonstrate product efficacy (*240*).

Anti-IL-5 monoclonal antibodies

Several biologics directly targeting IL-5 itself or its receptor IL-5Rα have been developed. As IL-5 represents a crucial cytokine in eosinophil function, targeting IL-5 pathway seems to be a suitable strategy to control eosinophilic asthma. Mepolizumab, marketed by GlaxoSmithKline under the trade name of Nucala®, is a humanized IgG1 antibody specifically binding IL-5 with high affinity. The first clinical studies showed that patients with refractory asthma with recurrent severe exacerbation treated with Mepolizumab had a reduction in exacerbations as well as an improvement of their quality of life and a reduction in blood and sputum eosinophilia (*345, 346*). Later, the efficacy of Mepolizumab was confirmed in a much larger study population with severe eosinophilic asthma, in phase IIb/III DREAM study (*347*), as well as in MENSA (*348*) and SIRIUS studies (*349*). In the context of real-life studies, preliminary data suggest that Mepolizumab can result in being even more effective in patients with higher blood eosinophil count (*272*). Reslizumab, developed by Teva and marketed under Cinqair® trade name, is also a monoclonal antibody specifically targeting IL-5 in its IL-5Rα binding site. Positive results have also been obtained in clinical trials in patients with severe eosinophilic asthma (*350*). Finally, Benralizumab, developed by AstraZeneca and marketed under Fasenra® trade name, is a monoclonal IgG1 antibody against IL-5Rα, thereby also having an impact on IL-5 pathway. It selectively recognizes the isoleucine-61 residue of human IL-5Ra, located near IL-5 binding site. Results obtained in different clinical trial are also very promising decreasing the annual rate of asthma severe eosinophilic exacerbations, improving asthma symptom control and enhancing FEV1 (*351*). All three drugs are now FDA- approved for treatment of severe asthma with an eosinophilic phenotype.

Anti-epithelial cytokines antibodies

Secreted by epithelial cells, IL-25, IL-33 and TSLP act as alarmins and therefore represent important factors in the early phase of the disease. Consequently, decreased production of these cytokines constitutes an important target for type 2 immune responses priming, such as in allergic asthma. Currently, while IL-25-related studies have not reached clinical evaluation, IL-33 and TSLP represent promising targets.

In an acute OVA-induced murine model of allergic airway inflammation, IL-25 neutralization during the sensitization phase significantly reduced (but did not abolish) Th2 cytokines production (including IL-5 and IL-13) as well as eosinophil infiltration, IgE production and AHR (*352*).

The TSLP-targeting therapeutic antibody Tezepelumab is under clinical development by AstraZeneca and Amgen. A first phase I has been realized with mild allergic patients and showed that three monthly Tezepelumab doses reduced allergen-induced bronchoconstriction and airway inflammation indexes before and after allergen challenge (*353*). Then and consistent with these results, a phase II clinical trial was conducted, during one year and including monthly antibody treatment in patients with uncontrolled asthma. The results showed a significant reduction in asthma exacerbation as well as improved lung function (*354*). Currently, a phase III in patients with severe and uncontrolled asthma is ongoing and results are expected for 2023 (NCT03927157). Tezepelumab has also been studied for AD treatment in a phase II study with no statistically significant improvement over placebo (*355*), and in combination with AIT with the aim of enhancing immunotherapy efficacy in cat allergic patients (NCT02237196).

Finally, therapeutic strategies aimed at targeting IL-33 are being explored for their potential in allergies treatment. Etokimab, developed by AnaptysBio, is currently under development. A recent phase IIa clinical trial with patients with moderate-to-severe AD demonstrated that a single Etokimab dose led to decreased clinical severity (*356*). The antibody is also being investigated in phase II clinical trial for it effect on severe eosinophilic asthma (NCT03469934). Another strategy to block pathways are antibodies developed against the IL-33 receptor ST2. Developed by Genentech under a license from Amgen, RG 6149 /AMG 282 is being tested in different clinical trials including patients suffering from with chronic rhinosinusitis with nasal polyps (NCT02170337) or from COPD (NCTT03615040).

Table 1. *Biological drug candidates targeting cytokines under clinical development*

Cytokine target	Biologic	Type of drug	Effect on disease	Stage of development
IL-4	Pascolizumab (GSK)	mAb (IgG1)	Partial efficacy in asthma.	Development discontinued

IL-13	Lebrikizumab (Roche / 2017: Dermira)	mAb (IgG4)	No significant improvement in asthma exacerbation. In phase II, efficacious in a subgroup of patients with high serum periostin levels ('Th2' high subgroup). No consistent results in phase III.	Phase III completed in asthma Ongoing Phase III in AD
IL-13	Trolokinumab (AZ / 2016: LEO Pharma)	mAb (IgG4)	Inconsistent results of efficacy in asthma. Effective in AD.	Phase III completed in asthma Positive results in 3 Phase III in AD
IL-4R	Dupilumab (Regeneron/Sanofi)	mAb (IgG4)	Effective in asthma, AD and CSwNP. Ongoing trials in eosinophilic esophagitis, food allergy and COPD with type 2 inflammation.	Distributed
IL-4R	Pitrakinra (Aerovance)	Mutated IL-4 molecule	No significant improvement of asthma exacerbations but efficacious in a subgroup of asthma patients with a specific SNP in the IL4RA gene.	Discontinued
IL-4R	AMG317 (Amgen)	mAb (IgG2)	No significant clinical efficacy in asthma patients.	Discontinued
IL-5	Mepolizumab (GSK)	mAb (IgG1 isotype)	Effective in asthma (in subgroup with high eosinophils), effective in CSwNP. Not effective in AD.	Distributed
IL-5	Reslizumab (Teva)	mAb (IgG4)	Effective in asthma (in subgroup with high eosinophils).	Distributed
IL-5R	Benralizumab (AZ)	mAb (IgG1)	Effective in asthma (in subgroup with high eosinophils).	Distributed
TSLP	Tezepelumab (AZ / Amgen)	mAb (IgG2)	Efficacious for treatment of allergic asthma, no significant improvement in AD.	Ongoing Phase III in severe asthma

IL-33	Etokimab (AnaptysBio)	mAb (IgG1)	Ongoing trials for treatment of peanut allergy, AD, allergic asthma, CSwNP.	Ongoing Phase II
IL-33R (ST2)	RG6149/AMG 282 (Genentech under a license from Amgen)	mAb	Ongoing trials for treatment of CSwNP, and COPD.	Ongoing phase II in COPD

CSwNP: Chronic rhinosinusitis with nasal polyps; COPD: Chronic obstructive pulmonary disease

1.5.5. Vaccines targeting self-proteins

Vaccination against infectious diseases has been one of most effective medical intervention in medicine. In parallel, monoclonal antibodies targeting host proteins, such as cytokines, have proven to be efficient for acute and chronic diseases treatment. However, protein therapies cost and their inconvenience for the patients are major obstacles for their wide-spread use. A novel strategy targeting the same molecules but using an active immunization strategy could be interesting and it is starting to be analyzed by different groups.

Naturally circulating anti-cytokine natural antibodies can be detected in sera from healthy individuals. Although at very low levels, in a study including 200 healthy individuals, anti IFN-α autoantibodies were isolated from all subjects' sera (*357*). Moreover, natural antibodies against other cytokines including IL-6, IL-1 and IFN-γ were detected in healthy individuals (*358*). This phenomenon can be easily explained by mechanisms underlying immunological tolerance. Tolerance occurs at the B and T cell levels. In general, T cell tolerance is more profound, and for many antigens a largely normal B cell repertoire exists along with tolerant T cells. B and T cells undergo a negative selection mainly in the bone marrow and thymus respectively, where receptors recognizing self-molecules are eliminated or inactivated, this phenomenon being defined as the central tolerance. Interestingly, as part of the central tolerance, T cells also suffer a positive selection where T cell receptors of a certain affinity or avidity are maintained and propagated within thymus.

The presence of these naturally occurring auto antibodies in certain healthy donors have not been associated with any notable effect (*359*). During a regular immune response against foreign antigens, B cells and T helper (Th) cells collaborate in the efficient production of

antibodies. However, B cells responsible for auto antibodies production do not expand in healthy subjects due to the lack of T-cell help. Thus, the primary objective to mount an antibody response against a self-protein is equivalent to circumvent Th cell tolerance, which is usually difficult to break. Of note, it might not be interesting to activate specific T cells against self-proteins by vaccination since the response could be harmful, and might be difficult to control leading to side effects (*360*). A carrier protein can be used to increase self-molecule immunogenicity. Interestingly, APCs are activated and peptides from the self-antigen but also from the linked carrier protein are presented on their MHC class II molecules, leading to carrier-specific T helper (Th) cell activation. These activated Th cells recognize antigen-derived peptides presented on B cells, helping self-antigen-specific B cells to produce antibodies (**Figure 6**). This approach is similar to classical carbohydrates conjugate vaccines where B cells recognize carbohydrates and Th cells recognize the linked carrier protein. Using this approach, it has been possible to induce high levels of specific antibodies against cytokines for treatment of allergies, as described in consecutive chapters. In addition, adjuvant use contributes to increase immunogenicity of the conjugate vaccine, contributing to DCs recruitment and the activation, or inducing a local deposition of antigen (*361*).

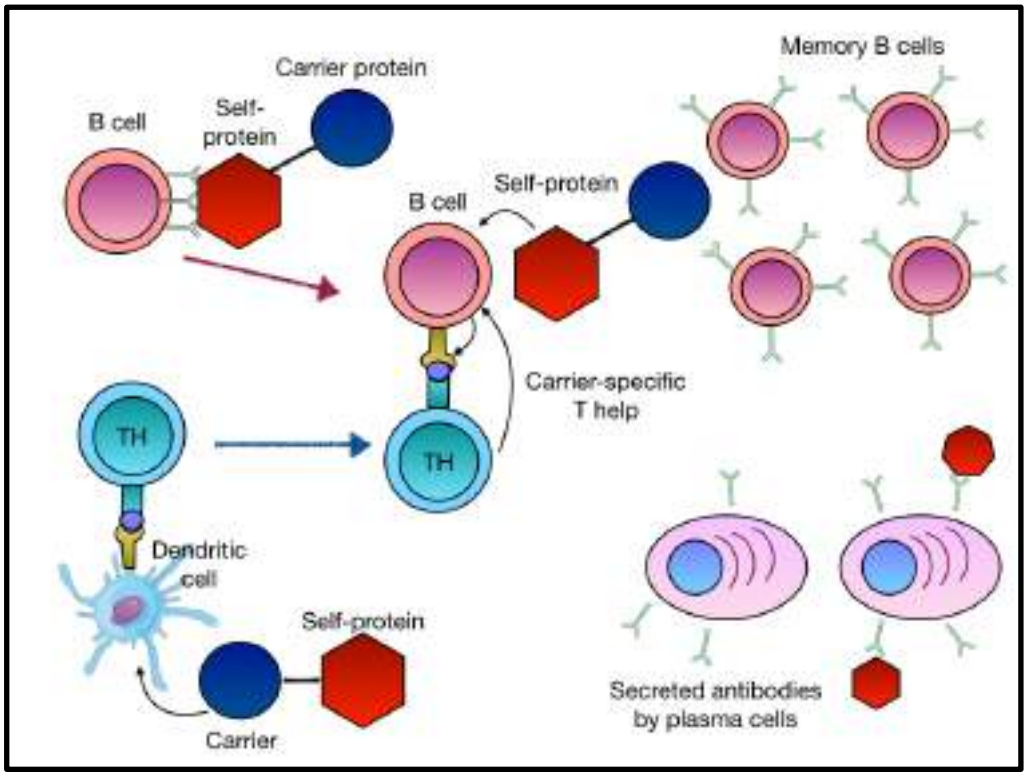

Figure 6. Vaccine strategy against a self-protein: How to circumvent Th cell tolerance? *(adapted from (362))*

Different vaccines strategies have been proposed to target IL-5. Zou and coworkers first suggested to couple IL-5 to Virus Like Particles (VLPs), derived from bacteriophage Qβ, inducing neutralizing antibodies in mice, in the absence of adjuvant (*363*). They showed that the vaccine was able to reduce eosinophilia in lungs and blood. More recently, the same group has demonstrated this vaccine efficacy in insect bite hypersensitivity (IBH) disease in horses, characterized by eosinophils in the skin (*364, 365*). Using the same strategy, *i.e.* coupling a cytokine with VLPs, a vaccine targeting IL-31 has also been described for AD treatment in dogs (*366*).

Some different strategies were also set up trying to target IL-13. Ma *et al.* developed an IL-13 peptide-based vaccine prepared with peptides derived from murine IL-13 receptor binding site (*367*). Reduction in eosinophilia and mucus production was reported, but with no effect on IgE levels (*368*). An IL-4 vaccine prepared with the entire cytokine coupled to KLH-carrier protein was also developed for allergic asthma treatment. In an ovalbumin (OVA)-induced asthma model, the vaccine partially reduced IgE levels and eosinophilia with minor effect on mucus hypersecretion (*369*). Recently, an anti-IL-33 vaccine has been developed and showed a reduction in AHR and eosinophilia from HDM-challenged mice. However, the vaccine had no impact on mucus production and was no able to reduce IL-5 and IL-13 production (*370*).

Finally, Hellman's group has been working on the development of a peptide-based vaccine directed against an immunoglobulin: IgE. Concretely, a chimeric Cε2-Cε3-Cε4 region was therefore used as target molecule. The IgE Cε3 domain was taken from the species to be vaccinated and Cε2 and Cε4 from a different species (nonplacental mammal) were used as carrier domains (*371*). In mice, the vaccine was well tolerated and showed high anti-IgE titers as well as positive effect on skin reactivity in response to an allergen challenge. Results from these experiments also showed that vaccination effect was reversible with only a few anti-IgE antibodies percent remaining 11 months after discontinuing the treatment. The vaccine was tested in humans and adjuvanted with aluminum hydroxide for injection, but results were not published (NCT00439621). This research group also tested the vaccine in dogs, but due to high circulating IgE levels observed in this species, obtained results were disappointing (*372*).

After structural analysis, another study focused on an IgE vaccine obtained by coupling two IgE peptides selected from different loops of the Cε3 that binds to the high affinity FcεRI receptor with VLP (*373*). The first peptide, labeled Y corresponded to the epitope recognized

by omalizumab whereas the second one, labeled P, targeted a different loop on IgE Cε3. This vaccine was tested in non-human primates an elicited an anti-IgE response lowering IgE levels. However, results in clinics were not convincing with no significant reduction of IgE levels and the vaccine development was subsequently stopped (*374*).

Optimizing adjuvants

Adjuvants represent an important question in the area of therapeutic vaccines, in particular vaccines that are developed against self-proteins. The traditional aluminum salt-based adjuvant (ALUM) has been widely used for more than 80 years. There are different aluminum compositions but the most commonly used adjuvants are aluminum hydroxide and aluminum phosphate. Mechanism by which ALUM boosts the immune response remains not fully understood. Initially, it was reported that ALUM created a depot effect at injection site, ensuring a slow absorbed antigen release (*375*). Conversely, recent studies have shown that the excision of injection site containing ALUM did not affect the immune response, suggesting that long-lasting adjuvant effect would more likely be due to innate cells that are attracted to the site and initially take up the antigen. A contribution of tissue damage from the injection site resection could also result in a sufficient inflammatory signal leading to enhanced immune response (*376*). Interestingly, a study in mice showed that OVA absorbed on ALUM triggered local accumulation of double-stranded DNA (dsDNA), suggesting that ALUM injection causes cell death and the subsequent host cell DNA release. Moreover, authors proposed that dsDNA induced B cell responses, including IgG1 responses and also stimulated Th2 responses, associated with IgE isotype switching (*377*). Despite its wide use, this type of adjuvant has shown low adjuvant activity boosting the antibody response against a self-protein. A study comparing different adjuvants effects in a vaccine targeting IgE has shown that other adjuvants might be more interesting for this type of immunization (*378*). In the study, authors evaluated five commonly used adjuvants, including Freund's, aluminum hydroxide, immune-stimulating complexes (ISCOMs), Montanide ISA™ 720 and Montanide ISA™ 51 and found that mineral oil-based adjuvant, Montanide ISA™ 51 and Freund's induced at least 5-10-fold higher anti-self IgE titers that any other candidates.

As an alternative to ALUM, emulsions composed by two distinct phases, water (the antigenic media) and oil, have been developed. In order to stabilize the emulsion, surfactants (containing a polar group which is hydrophilic and a nonpolar group which is hydrophobic) are added.

Depending of added surfactant and its hydrophilic/lipophilic balance (HLB) value, water-in-oil or oil-in-water emulsions can be obtained. Surfactants with a low HLB value have a high affinity for oily phases and are used for water-in-oil emulsions. In this type of emulsions, the antigenic phase is made of droplets dispersed into the continuous oily phase. Surfactants with a high HLB value are used to form oil-in-water emulsions. This type of emulsion is characterized by an aqueous continuous phase and oil constitutes the dispersed phase containing the droplets (*379*).

Freund's complete and incomplete adjuvant (CFA and IFA respectively) are the most commonly used adjuvants in laboratory and are an example for water-in-oil emulsions. CFA is a water-in-mineral oil emulsion containing heat-killed *Mycobacterium tuberculosis*, while IFA only contains the mineral oil and a lipophilic surfactant (mannide mooleate), as emulsifier. These formulations are potent adjuvants able to elicit both Th1 (CFA) and Th2 (IFA) responses (*380*). However, even if raw materials quality has been improved in order to avoid secondary reactions, the risk-benefit ratio is not favorable for its use in clinics.

Highly purified mineral-oils adjuvants have also been developed representing a safer alternative to CFA/IFA. Montanide ISA™ 51 is a water-in-oil adjuvant composed by a mix of mineral oil and a surfactant from mannide monooleate family, with a 50/50 oil/water ratio. Montanide ISA™ 720 is very similar, since it is composed by a mix of vegetable originating nonmineral oil with surfactant from the mannide monooleate family, with a 70/30 oil-to-water ratio (*379*). The depot effect represents a potential mechanism explaining their efficacy, by promoting a slow antigen release but also by creation of an inflammation at injection site stimulating APCs and lymphocytes recruitment, and facilitating cell association (*361*). Compared to the plant oil in Montanide ISA™ 720, the mineral oil in Montanide ISA™ 51 has probably a longer half-life in the tissue and can thereby function more efficiently as an antigen depot (*378*).

In addition, an influenza vaccine formulated in a squalene oil-in-water emulsion, named MF59 (and commercialized by Novartis), has given very good results in clinics. It is an oil-in water emulsion containing 5% v/v squalene in citric acid buffer with stabilizing nonionic surfactants polysorbate 80 (Tween 80) and sorbitan trioleate (Span 85), and emulsified under high pressure conditions in a microfluidizer resulting in small uniform droplets (*381*). Squalene is naturally synthetized in human steroid hormones pathway and is naturally present in the skin, adipose tissue and muscles. Natural squalene is obtained from shark liver. It has also been described to

increase antibody production as well as memory T and B cell activity against influenza viruses. This squalene-based adjuvant also promotes inflammatory cells recruitment at the injection site. Adjuvant together with the antigens are taken up and drained to the lymph nodes. Recently, MF59 was shown to promote differentiation of monocytes derived DCs within the draining lymph nodes, enhancing CD4 T cell responses (*382*). In addition, MF59 enhances CD4+ T follicular helper cell activation, controlling germinal center reactions which then increases B cell responses in mice (*383*). Other squalene-based adjuvants are currently under development and will be soon tested in humans (*e.g.* co-development of a GMP squalene-based adjuvant by Seppic and the Vaccine Formulation Institute).

Finally, one additional active area of adjuvant research is the use of Immune Stimulating Complexes (ISCOMs). Saponins, which is ISCOMs adjuvant active component, are a heterogenous group of sterol and triterpenoid glycosides coming from wide range of plants (*384*). A special interest has been placed on the well-defined particles made up of cholesterol and saponins from tropical tree bark, *Quillaja Saponaria*. ISCOMs have shown to promote a broad immune response, with high antibody levels and a strong T cell response, further enhancing cytokine secretion (*385*).

Kinoid Technology

Kinoids developed by NEOVACS SA are therapeutic conjugated vaccines in which a cytokine is coupled to a carrier protein. Keyhole limpet haemocyanin (KLH) has been used as carrier protein, providing T cell epitopes necessary to activate the immune system. Kinoids are used in an active immunization strategy, to induce neutralizing antibodies against an overproduced cytokine present in certain pathological conditions. Among the different kinoids developed by NEOVACS SA, IFN-α kinoid (IFN-K) is the most advanced product, targeting systemic lupus erythematosus (SLE). In transgenic mice expressing human IFN-α2b, intramuscular IFN-K injection induced a strong polyclonal response, targeting multiple epitopes, enabling to neutralize not only IFN-α2b *in vivo* but also the 12 other human IFN-α subtypes *ex vivo* (*386*). In a mouse model of SLE, IFN-K was shown to slow disease progression (*387*). With these encouraging results obtained in mouse, IFN-K was tested in clinical trials. In a phase I/IIa study in patients with active SLE, IFN-K was well tolerated, induced high titers of neutralizing anti-IFN-α antibodies, especially in patients with type I IFN gene signature, and significantly reduced IFN-induced genes expression (*388*). Follow-up analysis on a subgroup of IFN-K-

treated patients confirmed the link between anti-IFN-α antibodies persistence and IFN signature downregulation and revealed an inhibitory effect of the IFN blockage on B cell associated transcripts (*389*). Recently, results of a phase IIb have also been published (NCT02665364) (*390*). This study confirmed IFN-K-induced anti-IFN-α neutralizing antibodies, as well as a significant IFN gene signature downregulation in IFN-K-treated patients. In terms of clinical endpoint, coprimary endpoint (BILAG-Based Composite Lupus Assessment or BICLA) was not met but interesting results have been obtained with a statistically significant and clinically relevant difference in lupus low disease activity state (LLDAS) score, and a statistically significant reduction in corticosteroids uptake in IFN-K-treated group (*390*).

1.6.Mouse models for the study of allergies

To develop new therapies, their proof of concept is often demonstrated using animal models. Thus, major research efforts have been done in experimental animal models. Animal models should reflect the human pathology as closely as possible, favoring a suitable translation of basic science observations to the bedside. Mouse is the most widely used species, mainly because mice are affordable, easy to breed, maintain and handle, because of transgenic mice availability as well as the wide array of specific reagents available for their response analysis. During my thesis, I focused my work on the development of models of allergic asthma and anaphylaxis in order to produce the proof of concept for the candidate vaccines.

1.6.1. Mouse models of allergic asthma

Experimental asthma models have represented a challenge in research, mostly because of discrepancies between humans and mice disease presentation. Compared with humans, mice do not spontaneously develop asthma. In order to investigate processes underlying this disease, an artificial asthmatic-like reaction has to be induced in the airways. Investigators tried to mimic the human allergic asthma features in mouse. Because asthma is a complex, multifactorial and with multiple phenotypes disease, it is unlikely that a single animal model of asthma would recapitulate all the morphological and functional features observed in the human pathology. Different models have been developed and they replicate different aspects of the disease.

On one hand, mouse models for the acute allergic response to inhaled allergens have been traditionally used (*391*). They reproduce many human asthma features, including an increase specific IgE production, airway inflammation, goblet cell hyperplasia and AHR (*392*). Depending on mouse strain, allergen and immunization schedule used, the inflammatory response can be different. The most commonly used mouse strain in antigen challenge is BALB/c, as this strain develops a significant Th2 response (*393*). Ovalbumin (OVA) has been frequently used as allergen, inducing a robust allergic response. However, since OVA is not a relevant allergen in clinics, recently, efforts have been made developing models using other more relevant allergens. Models using house dust mite (HDM) or cockroach extracts have shown to also develop robust responses in mouse (*394*). In general, acute models include a sensitization phase where allergen is intraperitoneally injected with an adjuvant (ALUM is frequently used in this case) followed by challenge with the allergen administered directly via the airways (aerosol, intranasal or intratraqueal administration). This type of model has been widely used, but studies in B cell deficient mice (*395*) or mast cell deficient mice (*100, 396*) have shown that mice are still able to develop AHR. These results suggested that in OVA-induced model using adjuvant, B cell, IgE or mast cell are not required.

In order to overcome this drawback, adjuvant-free protocols have also been developed in order to reflect what is happening in the clinics. In OVA-induced models without the use of ALUM, FcεRI deficient (*397, 398*) and mast cell deficient mice (*399*) exhibited significant impairment in the airway inflammation as well as in AHR. Results suggested that these models, and contrary to models using adjuvants, were fully dependent on mast cells, IgE and FcεRI. Thus, results obtained in mice suggested that the extent to which mast cells contribute to airway inflammation and AHR in mice is highly dependent on the experimental model used to generate the airway response.

Even if these models helped to elucidate multiple relevant pathology mechanisms, they also present limitations. Because of the short-term nature of acute models, many lesions observed in chronic human asthma, including chronic inflammation and airway remodeling are not recapitulated and the inflammation is rapidly resolved when allergen challenge is interrupted (*400*).

On the other hand, and to overcome issues associated with acute models, chronic models have also been developed. These models more deeply reproduce what is observed in the clinics. In such models, airway remodeling and persistent AHR is noticed. It seems more interesting then to test new treatments in this type of models. Chronic allergen challenges are characterized by repeated intranasal allergen administration for a long period of time (generally weeks or months). These prolonged challenge models generally elicit less inflammation in the lungs than acute protocols, but eosinophilia is still prominent. One possible explanation for this observation is that after repeated challenges, tolerance is developed and downregulates inflammation and AHR after repeated allergen administration. Kumar *et al*. suggested that tolerance might be associated with the high allergen mass concentration in the lung parenchyma (*401*). Moreover, in some of the more recent chronic modes, it has been shown that key features of allergic asthma persist even in the absence of further allergen challenge (*402*). However, in an HDM-induced model, authors found that neither IgE nor FcεRI contributed to the pathogenesis as depicted in IgE or FcεRI deficient mice (*403*). Despite the apparent absence of FcεRI contribution in this model, Sibilano *et al*. showed that mast cells are required for full development of multiple asthma features in mice, including plasma levels of antigen-specific IgE and IgG1 antibodies, AHR, airway inflammation and airway remodeling (*404*), Furthermore, in HDM-induced asthma models, elevated levels of IL-17, as well as IL-4 and IL-13 were detected in BAL fluid, associated with an expansion of Th2 and Th17 cells in lung tissue confirming mixed Th2-Th17–driven inflammation in their mouse model, and miming inflammatory phenotype observed in certain severe asthma cases (*56*).

Finally, some mouse models have revealed a markedly Th17 phenotype characterized by airway neutrophilia and AHR. Results obtained in CXCR2-deficient mice (with impaired neutrophil recruitment) as well as with neutrophil-depleting antibodies suggested that neutrophils can be implicated in AHR (*405*). These models have been shown to be refractory to glucocorticoid treatment, and might be an interesting approach to implement when studying neutrophilic severe asthma (*406, 407*).

1.6.2. Mouse models of anaphylaxis

Anaphylaxis can be triggered in mouse using active (active systemic anaphylaxis, ASA) or passive models (passive systemic anaphylaxis, PSA) (*408, 409*).

In PSA models, mice are passively sensitized by transfer of antigen-specific antibodies (IgE or IgG), followed by challenge with the antigen to trigger anaphylaxis. Challenge is usually done the day after (16-24h after) to ensure the presence of the injected antibodies. Passive immunization studies in which mice were sensitized by injecting an antigen-specific IgE antibody, followed by allergen challenge, support IgE and mast cell importance in antigen-induced shock (*410*). In addition, passive immunization with an IgG1, IgG2a, or IgG2b (but not IgG3) also undergo anaphylaxis, after challenge with the appropriate allergen, with very similar manifestations to IgE-dependent anaphylaxis (*88, 410*).

In addition, models of passive cutaneous anaphylaxis have been developed. While they are not very useful as a model of human anaphylaxis, they represent an interesting tool to understand mechanisms implicated in skin allergy and anaphylaxis (*411*).

ASA can be induced by means of active immunization (or sensitization) with an antigen such as bovine serum albumin (BSA) or OVA, but also common allergens such as peanut, usually together with an adjuvant. This immunization induced a specific and polyclonal antibody response. After few days (18-21 days), administration of the same antigen is able to induce the anaphylactic reaction. In ASA models, mice develop an IgE and IgG response before the challenge. Anaphylaxis is generally thought to rely on allergen-specific IgE, mast cells and histamine release. To understand the role of effector cells, mouse models have been developed with surprising results. Notably, in ASA models employing adjuvants (*i.e.* ALUM) in the sensitization phase, studies have reported that the reaction can still occur in the absence of IgE (*89*), FcεRI (*90*) and even mast cells and basophils (*90, 91*). Moreover, another study showed that anaphylaxis was suppressed after treatment with an anti FcγRII/III antibody, suggesting a role of this activating receptors in an OVA-induced ASA (*412*). Nevertheless, in a recent study, they hypothesized that adjuvant use might mask mast cell and IgE contributions. Authors developed an adjuvant free ASA mouse model and found that anaphylaxis manifestations were partially but significantly reduced in FcεRI or mast cell deficient mice, confirming key roles of mast cells and FcεRI in their setting. However, anaphylaxis manifestations were reduced to a higher extent in FcγRIII deficient mice or after depletion of monocytes/macrophage indicating that additional mechanisms of anaphylaxis contributed to a significant extent to the pathology in their model. Finally, they demonstrated that the reaction was dependent on histamine and

PAF, with mast cells and monocytes/macrophages representing the main source respectively (*413*).

Additionally, intestinal anaphylaxis models have been also developed, where mice are systemically sensitized with the allergen (including OVA but also peanut) and orally challenged (oral gavage). In these models, mice undergo a systemic anaphylaxis which is accompanied with gastrointestinal symptoms, such as diarrhea. In a mouse model of peanut-induced anaphylaxis in C57Bl/6 mice, authors confirmed mast cell contribution in mast cell deficient mice with a partial IgE contribution (*104*). Another study confirmed IL-4 and IL-13 contribution in a peanut-induced anaphylaxis model in BALB/c mice and showed a reduced intestinal allergy and inflammation response in mice treated with an anti-IL-13Rα2 (type 2 IL-13 receptor) (*414*). In this study, authors also showed a significant allergen-specific IgG1 and IgG2a increase, which was FcεRI independent.

Interestingly, mice with a mutation on the immunoreceptor tyrosine–based inhibitory motif at position 709 (Y/F) of IL-4Rα have been generated (*415, 416*). These mice present a marketed allergic phenotype, with increased IgE levels and susceptibility to allergen-induced airway inflammation. Even if there is no human equivalent for the F709 mutation, it is a prototypic of a number of human IL-4Rα polymorphisms that promote receptor signaling and are associated with atopy, including IL-4Rα I75V or Q576R mutations (*417*).

1.6.3. Humanized mice

Although there is a significant overlap in the disease phenotype between mice and humans, there are inherent differences between human and mouse immune systems limiting finding translation from one host to the other. Moreover, in many cases, the evaluation of a certain new drug requires development of a mouse counterpart in order to test its efficacy in a relevant model. Humanized mouse models, where a cell population or a protein has been replaced by the human version, increase the translational potential of results obtained in mouse.

A significant advance in the research field of humanized mice was the generation of genetically modified mice: the NOD-SCID IL-2Rγ^{null} (NSG or NOG) strain (*418-420*). Advances in the ability to generate humanized mice is highly related to the study of genetic modifications leading to development of an immunodeficient mice. First, the severe combined

immunodeficiency (SCID) mutation on CB17 mice, highly affecting both T and B lymphocytes development, was discovered (*421*). Immunodeficient non-obese diabetic (NOD) mice-SCID mice development led to the observation of a higher human PBMCs engraftment level, mainly because of NK cells activity reduction (*422*). Finally, the definitive advance came with mice deficient for an important receptor in both innate and acquired immunity, the IL-2 receptor common gamma chain (IL-2Rγ) which is a crucial component in six different cytokine receptors, including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (*423, 424*). Absence of IL-2Rγ chain leads to severe impairments in T and B cell development and functions, and completely prevents NK-cell development. Importantly, and after sublethal gamma-irradiation, NSG or NOG strains exhibited impressive engraftment rate and hematopoiesis improvement following CD34+ HSC transplantation, illustrated by the presence of human mature T and B cells detected in peripheral organs.

Human PBMC-engrafted models have been used for allergic disorders study, by exploring the human IgE mediated response. For example, Weigmann *et al*. used NSG mice transplanted with PBMCs from allergic donors, and showed that B cells coming from the donor were able to produce allergen specific IgE antibodies after allergen administration, and the inflammation was blocked after Omalizumab administration (*425*). In addition, Burton *et al*. used NSG mice transplanted with human hematopoietic stem cells and showed that following peanut gavage, mice mounted a peanut specific IgE response. The anaphylactic response was abrogated with Omalizumab treatment (*426*).

However, there are several limitations of using this type of model including the fact that human FcεRI-expressing mast cells and basophils are rarely induced after PBMC transplantation (*427*). The use of PBMCs engrafted mice for new candidate treatment molecules study can also be challenging because PBMCs transplantation is an inadequate protocol for long-term evaluation due to severe xenogenic graft-versus-host diseases (*428*). Novel protocols to reconstitute human immune cells including mast cells and basophils have been established using a new generation of this humanized mouse that also expresses human IL-3 and GM-CSF genes. NOG IL-3/GM-CSF^{Tg} mice allowed the development of human FcεRI-expressing mast cells and basophils after human HSC transplantation (*429*). Very recently, NOG IL-3-GMCSF^{Tg} mice were shown to develop human mast cells that can mediate a human IgE-driven PCA reaction upon antigen challenge (*429*). In addition, these mice present several asthmatic diseases symptoms after

intratracheal IL-33 administration, such as inflammation involving human lymphocytes, eosinophils, mast cells, and basophils, hyperplasia of goblet cells, AHR and production of human IL-5, IL-13 and periostin (*430*). Moreover, Burton *et al*. developed a peanut-induced anaphylactic reaction in NSG mice expressing membrane–bound stem cell factor (SCF) supporting functional mast cell development and showed that the reaction was reduced in Omalizumab treated mice (*426*). Finally, the SGM3 model developed on the NSG background including additional expression of human IL-3, GM-CSF and SCF have been generated and showed that after human CD34+ HSC injection, large population of mast cells was generated. They demonstrated that mast cells degranulated and an IgE-dependent PSA was developed (*431, 432*).

Another approach to increase usefulness of the mouse as a model system for understanding human immune responses is the mouse alteration at the genetic level. In such animals, the immune system is more similar to the human system. Using this strategy, it is possible to replace genes that encode important immune functions with their human homologues.

In the field of allergic research, an important tool was generated by Dombrowicz and colleagues (*433*). To obtain a humanized animal model responsive to human IgE in allergic reactions, transgenic mice expressing the alpha-chain of the human FcεRI receptor (FcεRIα) instead of the mouse protein have been generated. Interestingly, authors also included the regulatory human region to recapitulate the expression pattern observed in humans. While mouse FcεRI expression is restricted to mast cells and basophils, human FcεRI is expressed in additional cell populations, including dendritic cells and monocytes/macrophages (*266, 434*). Human FcεRI^{Tg} mice were able to develop systemic anaphylaxis in response to intravenous sensitization with human IgE. In addition, passive cutaneous anaphylaxis was also induced when mice were sensitized with human IgE (*435*). Moreover, human FcεRI^{Tg} mice also exhibited cutaneous anaphylaxis after intradermally sensitization with serum from patients with peanut allergy and intravenous challenge with peanut (*436*). Mice expressing the humanized FcεRI provide an important tool for investigating numerous aspects of FcεRI biology, as well as molecular mechanisms underlying the tissue-specific FcεRI distribution and evaluation of therapeutic agents.

Notably, mouse IgE is able to bind both human and mouse FcεRI, while human IgE does not bind the mouse receptor (*437*). Thus, IgE-targeted therapies development is limited by a lack of model to assess human IgE (hIgE) *in vivo*. Some attempts to obtain mice expressing human IgE have been realized. Laffleur *et al*. set up the εKI mutation replacing Sμ with a human epsilon heavy chain (Cε) encoding both secreted IgE and membrane IgE (*438*). Nevertheless, heterozygous wt/εKI mice were derived, but no B cell expressing IgE appeared. More importantly εKI /εKI mice presented a complete B cell lymphopenia and neither murine Ig nor human IgE were detected in blood. In a mouse pre-B cell line transfected Cε, authors showed IgE expression on the cell surface suggesting that B cell lymphopenia is not due to defective association of human epsilon heavy chain with mouse might chain. They suggested an apoptotic phenotype of membrane IgE+ cells. This phenotype would be directly imposed by membrane IgE BCR expression prior to any antigen encounter and may explain the short life span and *in vivo* rarity of membrane IgE+ cells.

2. Summary and objectives

Most of the current therapeutic options for allergies and asthma are symptomatic. Allergen-specific immunotherapy is limited (for most types of allergies) by high rates of adverse reactions, and poor long-term efficiency. In recent years, several recombinant monoclonal antibodies have been developed with promising results, such as Omalizumab (anti-IgE) and Dupilumab (anti-IL4Rα). However, use of recombinant antibodies is limited by high cost, the need to perform repeated injections, and potential risks of appearance of anti-drug antibodies (ADAs) limiting their efficacy, or other adverse reactions. Therefore, while IL-4, IL-13 and IgE are promising therapeutic targets for treatment of allergies, there is a clear need to improve current strategies aimed at blocking these molecules, in order to reach long-term therapeutic effects. Therapeutic vaccine could represent an alternative treatment promoting an endogenous, long-lasting neutralizing antibody response against a given cytokine or protein.

This thesis is inscribed within a direct collaboration between an INSERM laboratory located at Institut Pasteur and a private company, NEOVACS SA. Taking advantage of the expertise of all partners, the thesis is focused on the preclinical development of different vaccine candidates. We make the hypothesis that vaccination against IL-4, IL-13 or IgE using the kinoid strategy would promote long-term benefits in allergic diseases. Active immunization with kinoids triggers a long-lasting anti-cytokine or anti-protein neutralizing polyclonal antibody response, with no T-cell memory response towards the cytokine or protein (*387*). The general aim is to synthesize and characterize IL-4, IL-13 and IgE kinoids, and subsequently evaluate their immunogenic properties in order to identify the best kinoid candidates, *i.e.* kinoids eliciting strong neutralizing antibody response *in vivo*. Selected candidates' efficacy is then evaluated in clinically-relevant mouse models of allergy. To do so, the work has been divided in two main projects:

1. **Development and characterization of anti-IL-4 and IL-13 vaccine (Chapter 3)**

IL-4 and IL-13 present a similar structure, share one receptor subunit (IL-4Rα) but are also thought to play some non-redundant functions in allergy and asthma. In order to develop a vaccine strategy able to neutralize endogenous IL-4 and IL-13 overproduction, we first worked on the development of mouse IL-4/IL-13 vaccines, in order to test vaccine candidates in a relevant *in vivo* model. We produced different vaccine candidates and chose the best candidates, based on manufacturing yields, coupling efficacy and reproducibility. Best candidates were

then tested *in vivo* to study their immunogenicity in mice. Since Dupilumab is now approved for moderate-to-severe asthma, we elected to test the efficiency of IL-4 and IL-13 kinoids in mouse models of house dust mite (HDM)-induced chronic allergic asthma. This chronic mouse model reproduces key features of allergic asthma observed in clinics, including airway hyperresponsiveness (AHR), airway eosinophilia, mucus overproduction and airway remodeling, thus representing a suitable model to test the vaccine candidates. Finally, to assess the translational potential of our approach, we also developed and characterized kinoids against human IL-4 and IL-13 for vaccination studies in mice humanized for IL-4, IL-13 but also for their common receptor chain IL-4Rα.

2. Development and characterization of hIgE/hFcεRI humanized mice and anti IgE vaccine (Chapter 4)

IgE plays a major role in allergies. Ideally, an IgE vaccine should induce "Omalizumab-like" antibodies which can block free IgE but not IgE already bound to the surface of mast cells and basophils. Indeed, such a binding would induce "crosslinking" of FcεRI-bound IgE and potential cell activation. Binding of Omalizumab to IgE locks IgE in its closed conformation, thus inhibiting IgE interaction with FcεRI. We think that an IgE vaccine promoting antibodies which, similarly to Omalizumab, could trap circulating (free) IgE in its closed conformation would be very efficient at blocking binding of IgE to FcεRI, and thereby inducing long-term protection against allergic reactions. Based on these observations, we thought that the IgE fragment linked to the carrier protein for the generation of the IgE kinoid (IgE-K) should comprise the entire Cε3 domain (since Omalizumab epitopes are spread along the entire Cε3 portion) and the Cε4 domain (required to stabilize Cε3 domain conformation). In addition, we inserted a Cys335 mutation which creates an additional disulfide bridge between the two Cε3-4 fragments to "lock" these fragments into a closed conformation recognized by Omalizumab but unable to bind FcεRI. Such design would favor "Omalizumab-like" neutralizing antibodies generation, while also precluding direct binding of the kinoid to FcεRI-bearing skin mast cells. To test this vaccine candidate *in vivo*, we generated $hIgE^{KI}$ $hFc\varepsilon RI^{Tg}$ mice which both produce and respond to human IgE. The availability of this humanized mouse model provided us a tool to directly test the human IgE kinoid *in vivo*, preventing from the need to develop a mouse surrogate. Given the central role of IgE in human anaphylaxis, and the absence of treatment which could prevent such life-threatening reaction, we elected to test whether IgE kinoids could

induce long-term protective effects in a model of IgE-mediated systemic anaphylaxis developed in $hIgE^{KI}$ $hFc\varepsilon RI^{Tg}$ mice.

3. Chapter 3. Development and characterization of anti-IL-4 and IL-13 vaccine

The first part of this thesis has been focused on the development and characterization of anti-IL-4 and anti-IL-13 vaccines using Kinoid strategy. Initial homology studies were carried out and revealed a low interspecies similarity of IL-4 (44%) and IL-13 (55%) between mice and human. Due to these observations, mouse IL-4-K (mIL-4-K) and mouse IL-13-K (mIL-13-K) were first produced. Kinoids were prepared by crosslinking of recombinant mIL-4 or recombinant mIL-13 to a carrier protein: CRM_{197}. It is a non-toxic mutant form of diphtheria toxin (DT) (without activity due to a single base substitution, in DT toxic domain, from glycine to glutamate in position 52). In order to select the best conjugation protocol, different strategies to chemically couple mIL-4 or mIL-13 with CRM_{197} were compared in terms of manufacturing yield, coupling efficacy and reproducibility. To maximize the generation of heteroconjugates (*i.e.* conjugates comprising cytokine and carrier protein), directed conjugation strategies have been evaluated using different heterobifunctional linkers in different reaction conditions. Once the best coupling strategy was identified, we evaluated immunogenic properties of selected mIL-4-K and mIL-13-K candidates, alone or in combination. To do so, BALB/c mice were intramuscularly immunized with the kinoids emulsified with a squalene-based adjuvant. Anti-mIL-4 and anti-IL-13 neutralizing antibodies were detected in sera from immunized mice. An HDM-induced asthma mouse model was established. In order to replicate human allergic airway inflammation features, mice were intranasally sensitized and challenged with HDM, which is one of the most important allergens, affecting more that 50% of allergic patients. mIL-4-K, mIL-13-K and the combination of both were administered in a prophylactic protocol to compare their efficacy in reducing IgE levels, airway hyperresponsiveness (AHR), eosinophilia and mucus production. Since dual IL-4/IL-13 kinoid prophylactic vaccination prevented or strongly reduced all key features of HDM-induced asthma in mice, we assessed the efficacy of therapeutic vaccination on mice with established asthma. In parallel to that, to assess the translational potential of the vaccine strategy, we also developed and characterized human IL-4-K and IL-13-K. Vaccination studies were finally conducted in a novel mouse strain humanized for IL-4, IL-13 but also for their common receptor chain IL-4Rα, confirming the efficacy of the human dual vaccine in a relevant preclinical model.

Paper I:

Dual vaccination against IL-4 and IL-13 protects against chronic allergic asthma in mice

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Running title

Dual IL-4/IL-13 vaccination in asthma

Abstract

Allergic asthma is characterized by elevated levels of IgE antibodies, type 2 cytokines such as interleukin-4 (IL-4) and IL-13, airway hyperresponsiveness (AHR), mucus hypersecretion and eosinophilia. Approved therapeutic monoclonal antibodies targeting IgE or IL-4/IL-13 can reduce asthma symptoms but require costly life-long administrations. We developed conjugate vaccines against IL-4 and IL-13, and demonstrated their prophylactic and therapeutic efficacy in reducing IgE levels, AHR, eosinophilia and mucus production in mouse models of asthma without any detectable adverse effect. The human version of the IL-4/IL-13 vaccine was also efficient at neutralizing human IL-4 and IL-13, and reducing IgE levels in mice humanized for IL-4, IL-13 and their common receptor subunit IL-4Rα. Thus, dual IL-4/IL-13 vaccination represents a promising, cost-effective, long-term therapeutic strategy for the treatment of allergic asthma.

Introduction

Asthma is the most common chronic lung disease, affecting more than 300 million people worldwide, and with at least 250,000 deaths attributed to the disease each year[1]. An estimate of 20 % of asthma patients suffer from uncontrolled, moderate-to-severe asthma[2], presenting with persistent symptoms, with reduced lung functions and recurrent exacerbations, despite the use of high-dose pharmacologic therapy[3]. The heterogeneity of asthma phenotypes represents a challenge for adequate assessment and treatment of the disease[4]. However, type 2 inflammation characterized by high levels of cytokines such as interleukin-4 (IL-4) and IL-13, high levels of IgE antibodies, and airway eosinophilia occurs in approximately 50 % of patients with asthma[1,5].

Even tough IL-4 and IL-13 present similar structures and share one receptor subunit (IL-4Rα)[6], IL-4 and IL-13 are also thought to play some non-redundant functions in allergy and asthma[7]. In particular, IL-4 is considered to act predominantly in the early phase of asthma development, through its role in regulating T cell proliferation and survival, and IgE synthesis[6]. In contrast, IL-13 would predominantly be involved in late phases of allergic reactions, such as airway remodeling and mucus hypersecretion[6].

Phase 3 studies indicated that dupilumab - a monoclonal antibody (mAb) against IL-4Rα that blocks both IL-4 and IL-13 signaling[8] - is efficient at decreasing the rate of severe exacerbations, and at improving lung function in patients with moderate-to-severe asthma[9]. Dupilumab was approved in 2018 as an add-on maintenance treatment in moderate-to-

severe asthma with an eosinophilic phenotype or with oral corticosteroid-dependent asthma. However, use of this (or any other) mAb in chronic asthma is limited by high cost and the need to perform injections overs years to lifelong. Therefore, while IL-4 and IL-13 are now clinically validated therapeutic targets for the treatment of asthma, there is a clear need to improve current strategies, with the goal of reaching long-term cost-effective therapeutic effects.

Conjugate vaccines called kinoids can elicit an endogenous, long-lasting neutralizing antibody response against a given cytokine[10], and could be a favorable alternative to therapeutic mAb administration. Vaccination against mouse IL-4 partially reduced IgE levels and eosinophilia with minor effects on mucus hypersecretion in a mouse asthma model[11]. A recombinant mouse IL-13 peptide-based virus-like particle vaccine had significant effects on mucus production without, however, affecting IgE levels[12]. Based on these partial results, and on the superior clinical efficacy in human asthma of targeting both IL-4 and IL-13 signaling (i.e. dupilumab) rather than targeting either IL-4 or IL-13 alone[13-15], we hypothesized that a dual vaccination against IL-4 and IL-13 would be particularly potent at reducing the severity of chronic asthma.

Results

Anti-mouse IL-4 and IL-13 kinoids induce potent and long-lasting neutralizing responses

We developed mouse IL-4 and IL-13 kinoids (IL-4-K and IL-13-K), by coupling these cytokines with diphtheria 'cross-reactive material 197' (CRM_{197}, a non-toxic mutant of diphtheria toxin used as a carrier protein in a number of approved conjugated vaccines[16]) using a thiol-maleimide conjugation (**Supplementary Fig. 1 and 2**). Mice were immunized intramuscularly with IL-4-K and IL-13-K alone or in combination (or the carrier protein CRM_{197} alone as a control), combined 1:1 (v:v) with SWE, a squalene oil-in-water emulsion adjuvant[17] (**Fig. 1a**). We did not observe visible adverse effects of the vaccines, as mice had normal behavior, and vaccination with kinoids had no effect on body weight (**Supplementary Fig. 3**). Immunization with IL-4-K and/or IL-13-K induced high anti-IL-4 and/or anti-IL-13 antibody titers, respectively, detectable already 6 weeks after primary immunization (**Supplementary Fig. 4, a and b**). As expected, all mice exposed to CRM_{197} or kinoids developed anti-CRM_{197} antibodies (**Supplementary Fig. 4, c**). Importantly, anti-cytokine antibodies generated upon vaccination with the kinoids exhibited strong neutralizing capacities against the respective cytokine in more than 90 % of mice starting 6 weeks after primary immunization (**Fig. 1, b and c**). Such neutralizing capacity could still be detected in more than 60 % of the mice over one year after primary immunization (**Supplementary Fig. 5**). Importantly, conjugation between cytokine and carrier protein was mandatory for potent antibody responses and for neutralizing activity (**Supplementary Fig. 6**). Altogether, these data indicate that efficient long-term neutralization of both IL-4 and IL-13 can be achieved through vaccination with kinoids.

Combined vaccination against IL-4 and IL-13 protects against chronic asthma in mice

We then tested the prophylactic efficacy of these vaccines in a chronic asthma model. Mice received a total of twelve intranasal administrations of *Dermatophagoides farinae* house dust mite (HDM) extract (one of the major allergens in human asthma[18]) over a period of 6 weeks, a protocol known to reproduce key features of human chronic asthma[19] (**Fig. 1a**). We assessed airway responses in this asthma model using invasive plethysmography[20]. HDM-treated control mice exhibited marked increase in lung resistance and elastance upon exposure to aerosolized methacholine (a bronchoconstrictor used as part of the diagnostic of asthma in human[21]), as compared to PBS-treated control mice (**Fig. 1, d and e**). These two features were significantly reduced in mice vaccinated with the IL-4-K, and absent in mice vaccinated with the IL-13-K or the combination of IL-4-K and IL-13-K (**Fig. 1, d and e**). The superior benefit of the dual IL-4/IL-13 vaccine on airway hyperresponsiveness (AHR) was even more apparent when using non-invasive whole-body plethysmography[22] (**Supplementary Fig. 7**). Our data suggests that AHR may be more dependent on IL-13 than IL-4 in this chronic asthma model and can be prevented upon prophylactic dual vaccination against IL-4 and IL-13.

Next, we assessed the effect of the kinoids on airway eosinophilia and inflammation. HDM-treated mice demonstrated elevated eosinophil numbers in bronchoalveolar lavage (BAL) fluid that were reduced 5- and 6-fold in mice vaccinated with IL-4-K or IL-13-K, respectively, and reduced even further (21-fold) upon dual vaccination (**Fig. 1f**). The benefit of the dual vaccination was even more evident when assessing eosinophil numbers

that reduced 8-fold in lung tissue, whereas single vaccination with IL-4-K or IL-13-K alone had no effect on eosinophil numbers (**Fig. 1g**). Neither single nor dual kinoid vaccination, however, altered eosinophil levels in circulation (**Supplementary Fig. 8**), indicating that the reduced airway eosinophilia observed after dual vaccination was a consequence of reduced eosinophil recruitment to the lungs rather than systemic effects on eosinophil numbers or progenitors. This HDM-induced asthma model also leads to pronounced peribronchiolar inflammation and mucus production [19]. Single or dual kinoid vaccination reduced peribronchiolar inflammation to a similar extent (**Fig. 1h**; scoring in **Supplementary Fig. 9a**), whereas IL-13 vaccination but not IL-4 vaccination was necessary to profoundly reduce mucus production (**Fig. 1i**; scoring in **Supplementary Fig. 9b**), confirming the key role of IL-13 in mucus hypersecretion[6].

Dual vaccination against IL-4 and IL-13 reduces IgE and mast cell numbers

As IgE antibodies play an important role in allergic asthma[23,24], we next assessed the effects of IL-4-K and IL-13-K on IgE levels in the HDM-induced asthma model. Compared to PBS-treated (naive) mice, control immunization of CRM_{197} in squalene-base adjuvant lead to low but detectable IgE levels in circulation but not, as expected, to detectable HDM-specific IgE (**Fig. 2, a and b**). HDM-treated mice had higher total IgE and significant HDM-specific IgE levels in circulation. Single IL-4 and dual kinoid vaccination markedly reduced total and HDM-specific IgE levels, with more pronounced effects than anti-IL-13 vaccination (**Fig. 2, a and b**), highlighting the prominent role of IL-4 in IgE production[6]. Noteworthy, HDM-treated mice also had elevated levels of HDM-specific IgG antibodies

that were not affected by single or dual kinoid vaccination (**Fig. 2c and Supplementary Fig. 10**).

Mast cells are the main IgE effector cells in the lung[23]. In patients with allergic asthma, inhalation of an aeroallergen leads to crosslinking of membrane bound allergen-specific IgE, inducing rapid release of mast cell mediators such as histamine and tryptase [23]. As expected, the chronic intranasal exposure to HDM of the asthma model we use herein resulted in a marked increase in the numbers of lung mast cells, as compared to PBS-treated animals (**Fig. 2, d and e**)[19]. This mast cell recruitment was abolished following IL-4-K or dual kinoid vaccination, and reduced ~2.5-fold following IL-13-K vaccination (**Fig. 2, d and e**). Importantly, in addition to restoring basal numbers of mast cells in the lungs of HDM-treated mice, kinoid vaccination also markedly reduced IgE levels on mast cells (**Fig. 2, f and g**), indicating that both vaccines induce successful mast cell "desensitization". This vaccination-induced desensitization was observed at the systemic level, as membrane-bound IgE levels were also markedly reduced on blood basophils (which also express the high-affinity IgE receptor FcεRI) and peritoneal mast cells upon vaccination with kinoids (**Supplementary Fig. 11, a and b**).

Since dual IL-4/IL-13 kinoid prophylactic vaccination prevented or strongly reduced all key features of HDM-induced asthma in mice, whereas single IL-4-K or IL-13-K vaccination affected only a subset of these features (**Fig. 1 and 2**), we assessed the efficacy of therapeutic vaccination only using dual IL-4/IL-13 kinoid vaccination on mice with established asthma (**Fig. 3a and Supplementary Fig. 12**). Mice were pre-exposed to HDM for three weeks before the first injection of kinoids, and remained exposed to HDM once a

week for a total of 15 weeks thereafter (**Fig. 3a**). Dual vaccination induced high levels of neutralizing antibodies against both IL-4 and IL-13 whether mice were pre-exposed or not to HDM (**Fig. 3, b and c** and **Supplementary Fig. 13**), suggestive of potential efficacy in a therapeutic setting. Indeed, dual therapeutic vaccination demonstrated a profound reduction in key features of asthma, including a ~2-fold reduction of total and HDM-specific IgE levels (**Fig. 3, d and e**), of AHR to inhaled methacholine (**Fig. 3, f and g**), of airway eosinophilia (**Fig. 3h**), and a ~6-fold reduction in mucus production (**Fig. 3, i and j**).

A human IL-4/IL-13 vaccine induces neutralizing responses in humanized mice

Low interspecies similarity of IL-4 (~44 %) and IL-13 (~55 %) between mice and men would render mouse IL-4-K and IL-13-K highly immunogenic in humans, and less potent to generate neutralizing responses. We therefore developed and characterized kinoids eliciting an immune response against human IL-4 and IL-13 (hIL-4-K and hIL-13-K) (**Supplementary Fig. 14**) for vaccination studies in mice humanized for IL-4, IL-13 and for their common receptor chain IL-4Rα (hIL-4^{KI}; hIL-13^{KI}; hIL-$4R\alpha^{KI}$ mice). Dual hIL-4-K/hIL-13-K vaccination induced neutralizing responses against both human IL-4 and IL-13 in all immunized mice (**Fig. 4, a-c** and **Supplementary Fig. 15**). Confirming the efficacy of the human dual vaccine, mice showed a >2.5-fold reduction in circulating IgE readily detectable 5 weeks post-primary vaccination (**Fig. 4d**), as well as a decrease in membrane-bound IgE on basophils (**Fig. 4e**). Mast cell number and IgE level were very low in the lungs of these mice which had not been previously exposed to allergens. However, we could efficiently detect IgE in most skin mast cells from control hIL-4^{KI}; hIL-

204 13^{KI}; hIL-4Rα^{KI} mice, and such IgE levels were reduced ~2.5-fold upon dual vaccination

205 with hIL-4-K and hIL-13-K (**Fig. 4, f and g**).

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Discussion

Recent clinical data highlight the fact that IL-4 and IL-13 are important therapeutic targets in asthma[9]. However, targeting these cytokines or their receptors through the use of therapeutic mAbs is associated with high costs, and the need to perform frequent reinfusions in order to maintain clinical effects. Our current study provides a proof-of-concept that long-term neutralization of IL-4 and IL-13 can be achieved through vaccination with kinoids, which thus could represent a cost-effective alternative to therapeutic mAbs. We demonstrate that vaccination against IL-4 and IL-13 is well tolerated and protects against key features of chronic asthma in mice, including AHR, eosinophilia and mucus overproduction, after both prophylactic or therapeutic vaccination protocols.

We observed different effects of prophylactic vaccination against IL-4 or IL-13 on asthma features, highlighting the fact that IL-4 and IL-13 can have important non-overlapping functions in asthma[15]. In particular, vaccination against IL-4 had more pronounced effects on IgE levels and lung mast cell numbers than vaccination against IL-13. By contrast, AHR and mucus overproduction were reduced to a greater extent by the IL-13 vaccine. These results are in full agreement with previous observations in mice lacking IL-4 or IL-13[6,25-27]. However, maximal therapeutic effect for all these asthma features required dual vaccination against IL-4 and IL-13. This provides a potential explanation for the superior clinical efficiency of dupilumab (which blocks both IL-4 and IL-13 signaling) over various therapeutic anti-IL-4 or IL-13 mAbs in asthma[13-15], and prompted us to focus on the dual vaccine for further evaluation in a therapeutic protocol.

Levels of IL-4 and IL-13 neutralization obtained upon vaccination with kinoids will likely never reach levels observed directly after injection of high dose of a therapeutic mAb in human, or upon genetic ablation of IL-4 or IL-13 in mice. This was apparent in the therapeutic vaccination protocol in which IgE levels were reduced, but still detectable, in all vaccinated mice. Besides IgE, mice which fully lack IL-4 or IL-4Rα have markedly reduced IgG1 levels[28]. In addition, treatment of mice humanized for IL-4 and IL-4Rα with dupilumab also leads to important decrease in IgG1[29]. However, we found no difference in HDM-specific IgG1 levels between mice vaccinated with IL-4 and IL-13 kinoids or CRM_{197} alone. This indicates that residual cytokine activity in mice vaccinated with kinoids might sustain IgG production, while reducing the pathogenic functions of IL-4 and IL-13 in asthma.

Besides their detrimental role in allergies, IL-4 and IL-13 also play important protective and immunoregulatory functions. In particular, these cytokines can induce host defense responses against helminths infections, and have been implicated in the promotion of anti-inflammatory and tissue repair phenotypes in macrophages[30-32]. Thus, even though we did not observe apparent side effects of the vaccines in a one-year follow-up study in mice, further work is required to evaluate whether the residual IL-4 and IL-13 activity in mice vaccinated with kinoids is sufficient to sustain protective type 2 immune responses.

Altogether, our results indicate that long-term neutralization of both mouse and human IL-4 and IL-13 can be achieved through vaccination with kinoids. Dual vaccination could protect against key features of chronic asthma after both prophylactic or therapeutic vaccination protocols. These results pave the way for the clinical development of an

255 efficient long-term vaccine against asthma and other IL-4- and IL-13-mediated allergic
256 disorders.

Methods

Mice. Female BALB/cJRj mice at 5-6 weeks of age were purchased from Janvier Labs, and maintained in a specific pathogen-free facility at Institut Pasteur or Institut Jacques Monod. hIL-4^{KI}; hIL-13^{KI}; hIL-$4R\alpha^{KI}$ mice were generated by Dr Beverly Koller (University of North Carolina, USA) and maintained in a specific pathogen-free facility at Institut Pasteur. All animal care and experimentation were conducted in compliance with the guidelines and specific approval of the Animal Ethics committee CETEA (Institut Pasteur, Paris, France) registered under #170043, and by the French Ministry of Research. The protocol also received the authorization number EU0285 - Institut Jacques Monod PHEA - APAFiS - Autor. APAFiS #165.

Vaccination with kinoids. Mice were immunized intramuscularly with IL-4 and/or IL-13 kinoids combined 1:1 (v:v) with SWE, a squalene-in-water emulsion adjuvant (Vaccine Formulation Laboratory, University of Lausanne, Switzerland) in PBS on days indicated in the protocol outlines on Fig. 1, 2 and 4, at two initial doses of 30µg followed by boosts of 10µg. As controls, groups of mice were injected with the same schedule with CRM_{197} alone with two initial doses of 15µg followed by boosts of 5µg (these doses were defined based on the weight ratio of CRM_{197} used to generate kinoids; as shown in Fig. S1, S11 & S13, immunization with CRM_{197} alone induced slightly higher levels of anti-CRM_{197} antibodies than immunization with kinoids combined with SWE, or PBS alone. In the experiments depicted in fig. S5, mouse IL-4 and CRM_{197} or mouse IL-13 and CRM_{197} were also co-injected without prior conjugation following the same immunization schedule.

Mouse models of house dust mite (HDM)-induced allergic asthma. In the prophylactic protocol (Fig. 1 and 2), BALB/c mice were immunized with kinoids at day 0, 7 and 28. Starting at day 39, allergic asthma was induced with intranasal (i.n.) exposure to crude house dust mite extracts (HDM; *Dermatophagoides farinae*; lot number: 307244, Greer laboratories). Lightly isoflurane-anesthetized mice were sensitized three times by i.n. exposure with HDM (100 µg in 30 µl PBS), or PBS alone as control, with a 3-day interval between each administration. Lightly isoflurane (3 % in air)-anesthetized mice were challenged i.n. with 25 µg of HDM in 30 µl PBS, or PBS alone as a control, twice a week starting 3 days after the last sensitization, for a total of 9 administrations. Mice were sacrificed 24 h after the last challenge with HDM or PBS.

In the therapeutic protocol (Fig. 3), BALB/c mice were sensitized three times with HDM (100 µg in 30 µl PBS), and PBS as control, with a 3-day interval between each administration. Lightly isoflurane (3 % in air)-anesthetized mice were then challenged i.n. with 25 µg of HDM in 30 µl PBS, or PBS alone as a control, twice a week for a total of 18 challenges, starting 3 days after the last sensitization. Vaccination with mouse IL-4-K and mouse IL-13-K was initiated four days after the third HDM challenge. Mice were sacrificed 24 h after the last challenge with HDM or PBS.

Statistical analysis. Statistical significance was determined using the unpaired Student's t test (unpaired Mann Whitney U test) or test 2-way ANOVA followed by a Tukey posttest. $P \leq 0.05$ was considered statistically significant. Calculations were performed using the Prism® 7.0 software program (GraphPad Software).

304 See the Supplementary Methods for the description of all other experimental procedures.

305

306 Data availability

307 All data generated or analyzed during this study are included in this published article (and
308 its Supplementary information files).

309

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311

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Competing interests

E.C, R.B, P.B, N.C, VS, G.G-V and L.L.R are inventors on patent related to this work (WO2019228674 (A1)), and/or related to Kinoid technology. E.C, R.B, J.B, N.C, F.C, S.H, V.S, G.G-V are currently or were previously employees of NEOVACS and company stocks owners.

Author contributions

Experimental design, E.C, R.B, G.G-V and L.L.R; Investigation, E.C, R.B, B.B, J.B, J.S, R.H, N.G and L.L.R; Formal analysis, E.C, R.B, P.B, R.H., N.G., G.G-V and L.L.R; Writing (original draft), E.C and L.L.R; Writing (review and editing), all authors.

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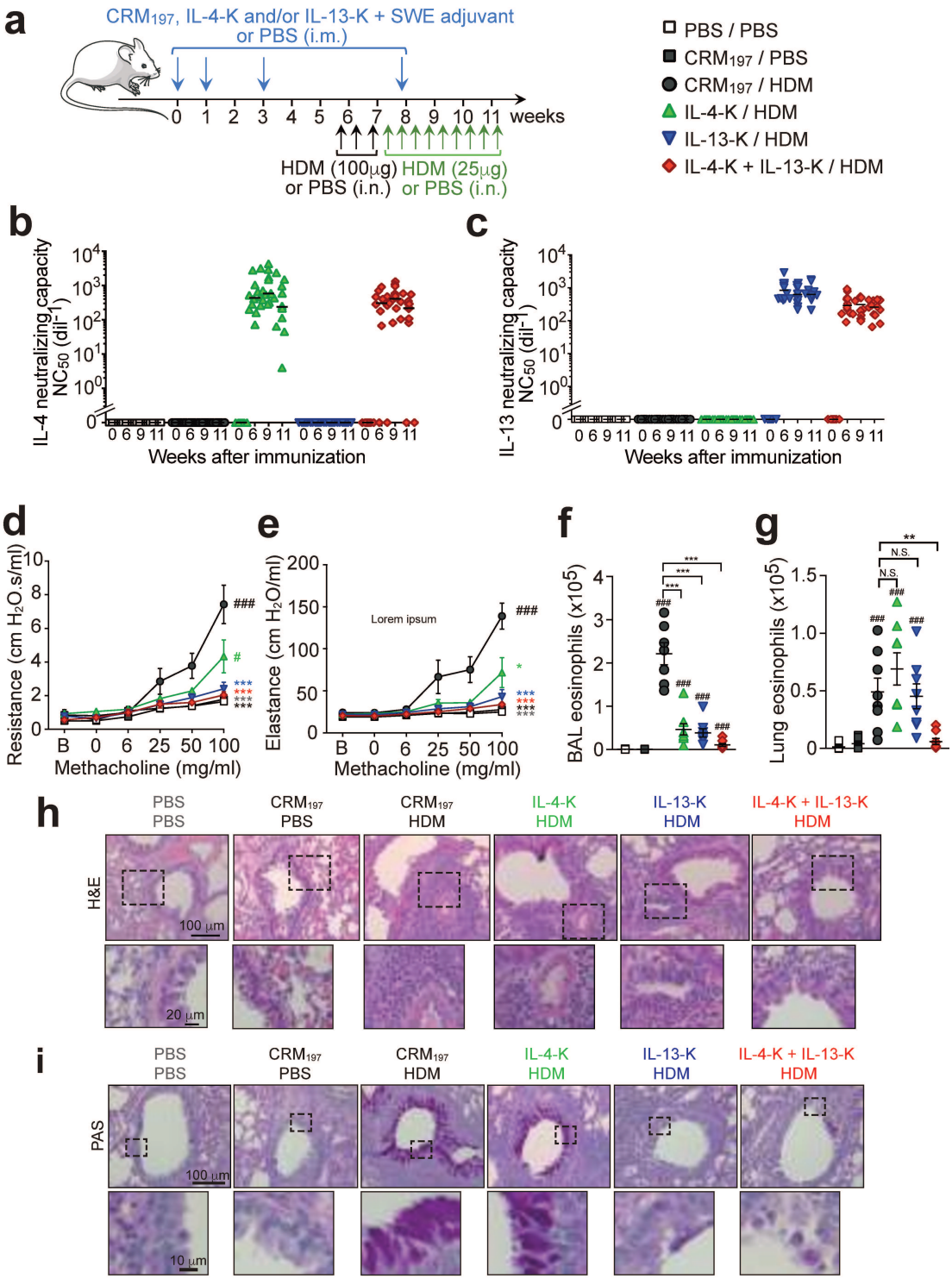

a
CRM197, IL-4-K and/or IL-13-K + SWE adjuvant or PBS (i.m.)
0 1 2 3 4 5 6 7 8 9 10 11 weeks
HDM (100µg) or PBS (i.n.)
HDM (25µg) or PBS (i.n.)
PBS / PBS
CRM197 / PBS
CRM197 / HDM
IL-4-K / HDM
IL-13-K / HDM
IL-4-K + IL-13-K / HDM
b
IL-4 neutralizing capacity NC50 (dil-1)
Weeks after immunization
c
IL-13 neutralizing capacity NC50 (dil-1)
Weeks after immunization
d
Resistance (cm H2O.s/ml)
Methacholine (mg/ml)
e
Elastance (cm H2O/ml)
Lorem ipsum
Methacholine (mg/ml)
f
BAL eosinophils (x10^5)
g
Lung eosinophils (x10^5)
N.S.
h
PBS PBS
CRM197 PBS
CRM197 HDM
IL-4-K HDM
IL-13-K HDM
IL-4-K + IL-13-K HDM
H&E
100 µm
20 µm
i
PAS
100 µm
10 µm

419

Figure 1. Dual vaccination with IL-4-K and IL-13-K reduces features of chronic asthma. a. Protocol outline. Mice were vaccinated with IL-4-K and/or IL-13-K (or PBS or CRM197 as controls), combined with the adjuvant SWE. At day 39, mice were sensitized and challenged with HDM or PBS, as indicated. **b-c**. Anti-IL-4 (**b**) and anti-IL-13 (**c**) neutralizing capacity in sera collected at the indicated time-points. Data show values from individual mice (n=12/group) with bars indicating median, from a single experiment representative of three independent experiments. **d-e**. Lung resistance (**d**) and (**e**) elastance in response to inhaled methacholine 24 h after the last HDM challenge. Data represent mean ± SEM from two independent experiments (n=8-10/group). **f-g**. Numbers of eosinophils in BAL fluid (**f**) and lung tissue (**g**) 24 h after the last HDM challenge. Data show values from individual mice (n=7-8/group) with bars indicating mean ± SEM, from a single experiment representative of two independent experiments. **h-i**. Representative lung sections stained with haematoxylin and eosin (H&E; revealing leukocyte infiltration) (**h**), or periodic acid-Schiff (PAS; revealing mucus-producing goblet cells in dark purple) (**i**). Lower panels in h and i represent magnifications of the dashed areas. *, ** or ***, $P <$ 0.05, 0.01, or 0.001 *vs*. CRM197/HDM group (d-e, 2-way ANOVA followed by a Tukey posttest; f-g, Mann-Whitney U test). # or ###, $P < 0.05$ or 0.001 *vs*. CRM197/PBS (2-way ANOVA followed by a Tukey posttest). N.S. not significant.

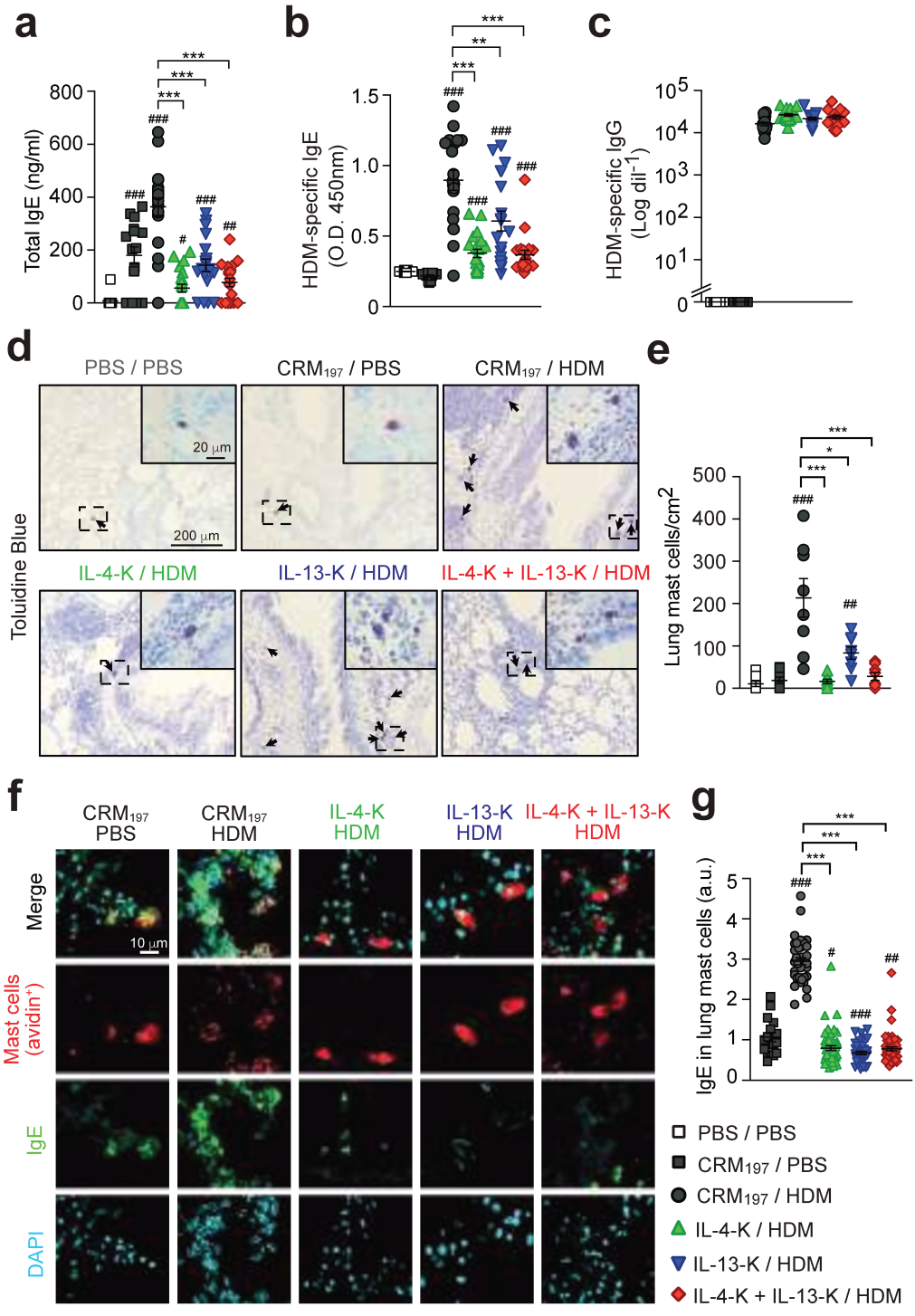


Figure 2. Dual prophylactic vaccination with IL-4-K and IL-13-K prevents elevated IgE levels and lung mast cell numbers after HDM challenges. a-c. Levels of total IgE (**a**), HDM-specific IgE (**b**) and HDM-specific IgG (**c**) 24 h after the last HDM challenge. Results show values from individual mice with bars indicating mean ± SEM from *n*=12-

16 mice (PBS groups) or *n*=19-20 mice (HDM groups) pooled from two independent experiments. **d-e**. Quantification of toluidine blue-positive lung mast cells (**d**) and representative lung sections stained with toluidine blue (**e**), demonstrating mast cells (arrows) 24 h after the last HDM challenge. Insert represent magnifications of the dashed areas. Results in e show values from individual mice with bars indicating mean ± SEM from *n*=8 mice. **f**. Representative lung sections stained with avidin (which stains mast cells, red), anti-IgE (green), and DAPI (blue) 24 h after the last HDM challenge. **g**. Quantification of IgE levels in avidin-positive lung mast cells. Results show values from individual avidin-positive mast cells with bars indicating means ± SEMs. *, ** or ***: $P < 0.05$, 0.01 or 0.001 *vs*. CRM_{197}/HDM group; $^{\#}$, $^{\#\#}$ or $^{\#\#\#}$: $P < 0.05$, 0.01 or 0.001 *vs*. CRM_{197}/PBS group (Mann-Whitney *U* test). a.u.: arbitrary units.

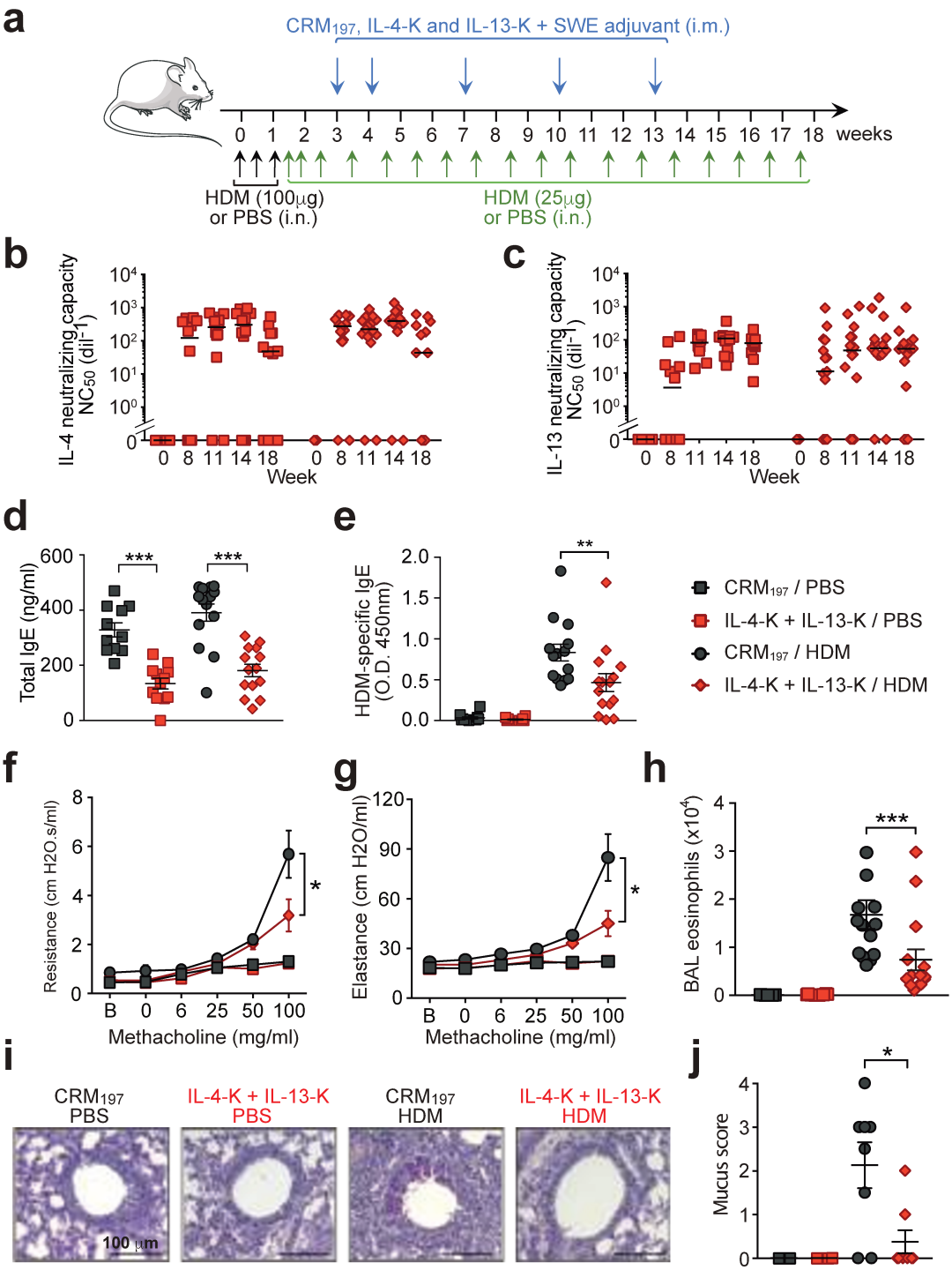


Figure 3. Dual therapeutic vaccination with IL-4-K and IL-13-K ameliorates chronic asthma. a. Protocol outline. Mice were sensitized and challenged with HDM extract (or PBS as a control), as indicated. After the third challenge, mice were vaccinated with IL-4-

K and IL-13-K (or CRM_{197} as control), combined with the adjuvant SWE. **b-c**. Anti-IL-4 (**b**) and anti-IL-13 (**c**) neutralizing capacity in sera collected at the indicated time-points. **d-e**. Levels of total IgE (**d**) and HDM-specific IgE (**e**) 24 h after the last HDM challenge. **f-g**. Lung resistance (**f**) and elastance (**g**) in response to inhaled methacholine 24 h after the last HDM challenge. **h**. Eosinophil numbers in BAL fluid 24 h after the last HDM challenge. **i**. Representative periodic acid-Schiff (PAS) staining of lung sections, demonstrating mucus-producing goblet cells (dark purple). **j**. Quantification of mucus-producing goblet cells. Data show median (b-c) or mean ± SEM (d-h and j) from n=16 mice per group pooled from two independent experiments. Each symbol represents individual mice (b-e, h, j). *, ** or ***: $P < 0.05$, 0.01 or 0.001 *vs*. indicated group (Mann-Whitney *U* test in b-e, h and J, 2-way ANOVA followed by a Tukey posttest in e and f).

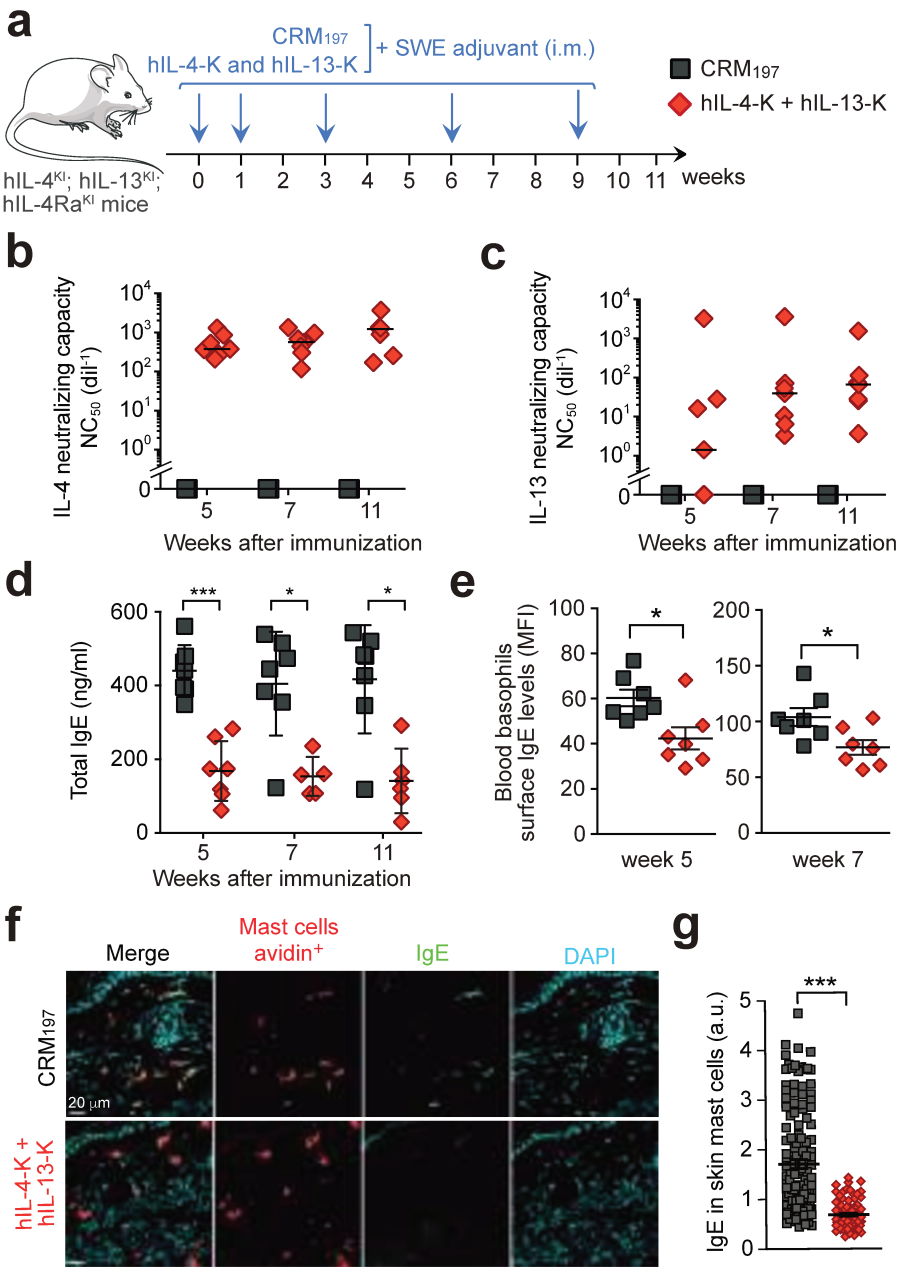


Figure 4. Efficient vaccination with human IL-4 and IL-13 kinoids in IL-4/IL-13/IL-4Rα-humanized mice. a. Vaccination protocol outline. hIL-4h^{KI}; hIL-13^{KI}; hIL-4Rα^{KI} mice were vaccinated with hIL-4-K and hIL-13-K in combination (or CRM_{197} as control), combined with the adjuvant SWE. **b-c**. Anti-human IL-4 (**b**) and anti-human IL-13 (**c**) neutralizing capacity in sera collected at the indicated time-point. Results show values from

individual mice (n=7/group) with bars indicating medians. **d-e**. Levels of total IgE (**d**) in sera or on the surface of blood basophils (**e**) at the indicated time-points. Results show values from individual mice (n=7/group) with bars indicating means ± SEMs. *, ** or ***: $P < 0.05$, 0.01 or 0.001 (Mann-Whitney U test). **f**. Representative ear skin sections stained with avidin (which stains mast cells, red), anti-IgE (green), and DAPI (blue) 24 h after the last HDM challenge. **g**. Quantification of IgE levels in avidin-positive mast cells. Results show values from individual avidin-positive mast cells with bars indicating means ± SEMs. ***: $P < 0.001$ (Mann-Whitney U test). a.u.: arbitrary units.

Supplementary methods

Synthesis and characterization of IL-4 and IL-13 kinoids. Mouse IL-4 (214-14), human IL-4 (200-04), mouse IL-13 (210-13) and human IL-3 (200-13) were purchased from PeproTech. CRM_{197} was purchased from Pfenex. IL-4 and IL-13 were modified with N-γ-maleimidobutyryl-oxysuccinimide ester (sGMBS; Thermo Fisher scientific, 22324), a maleimide-containing agent reacting with primary amines. Cytokines were dissolved in modification buffer (70 mM phosphate buffer, 150 mM NaCl, 5 mM EDTA, pH=7.2) at 1 mg/ml. A solution of 10 mM of sGMBS was prepared and added to the cytokine at a 1:30, 1:20 or 1:10 molar ratio, for human IL-4, mouse IL-4 and human IL-13 or mouse IL-13 modification, respectively, and incubated during 1 hour at room temperature (protected from light). Excess sGMBS was removed and buffer exchanged against modification buffer using Zeba™ desalting spin column (Thermo Fisher scientific). Sulfhydryl moieties were introduced on the carrier protein CRM_{197} with SATA (N-succinimidyl-S-acetylthioacetate; Sigma Aldrich, A9043). CRM_{197} was diluted in modification buffer at 2 mg/ml and a freshly prepared solution of 100 mM SATA (dissolved in DMSO) was added at a 1:80 molar ratio and incubated 30 minutes at room temperature (protected from light). Excess SATA was removed and buffer exchanged against modification buffer using Zeba desalting spin column. SATA modified CRM_{197} was incubated with a solution of hydroxylamine hydrochloride (Thermo Fisher scientific, 26130) at a 50 mM final concentration, at room temperature for 2 hours, protected from light. Excess hydroxylamine was removed and buffer exchanged against modification buffer using Zeba desalting spin column. After CRM_{197} and IL-4 or IL-13 functionalization, protein content of each preparation was determined by Bradford assay according to manufacturer's instructions (Thermo Fisher scientific).

Functionalized CRM_{197} was added to functionalized IL-4 or IL-13 at a molar ratio of 1:2 (for mouse IL-4) or 1:4 (for mouse IL-13, human IL-13 and human IL-4) and a final concentration of 0.4 mg/ml. The mixture was incubated 16 hours at 4 °C, protected from light, and subsequently buffer exchanged against modification buffer using Zeba desalting spin column. Protein content was determined by Bradford assay. Resulting IL-4 and IL-13 kinoids were then 0.22 µm sterile filtered and stored at 4 °C. Kinoids were characterized using different *in vitro* methods. To analyze the profiles of the kinoids obtained, SDS-PAGE and western blots were performed against mouse IL-4 (using AF-404-NA as a detection antibody at 0.1 µg/ml, R&D systems), human IL-4 (AF-204-NA at 0.1 µg/ml, R&D systems), mouse IL-13 (AF-413-NA at 0.1 µg/ml, R&D systems), human IL-13 (AF-213-NA at 0.1 µg/ml, R&D systems) and against CRM_{197} (AbD serotec, 3710-0956 at 860 ng/ml). Size exclusion (SE)-HPLC using a Bio SEC-5 column (2000 Å, 5 µm, 7.8*300 mm, Agilent) and a Bio SEC-3 column (300 Å, 3 µm, 7.8*300 mm, Agilent) in serie. SE-HPLC analysis were performed in the isocratic mode at 1 ml/min with column temperature at 25 °C. After filtration (0.22 µm-cut-off), samples were injected at 100 µl and analyzed at 280 nm. The total run time was 35 min.

To confirm coupling between the cytokines and the carrier protein, and to evaluate epitope preservation, antigenicity was analyzed by sandwich ELISA. Briefly, capture antibody (mouse monoclonal anti-diphtheria toxin, AbD serotec, 3710-0100) was coated overnight at 1 µg/ml. After each step, plates were washed three times with PBS 0.01 % Tween® 20. Then plates were blocked with casein 2 % dissolved in PBS. Kinoid samples were added at 250 ng/ml and 2-fold serially diluted in 100 µl final volumes. After incubation, bound kinoids were detected using biotinylated anti-mouse IL-4 antibody (polyclonal goat IgG, R&D systems, BAF-404), biotinylated anti-human IL-4 antibody (polyclonal goat IgG, R&D systems, BAF-204), biotinylated anti-mouse IL-13

antibody (polyclonal goat IgG, R&D systems, BAF-413) or biotinylated anti-human IL-13 antibody (polyclonal goat IgG, R&D systems, BAF-213) at 250 ng/ml, and then revealed with streptavidin-HRP and an OPD substrate. The reaction was stopped adding 1 M H_2SO_4 after 30 min of OPD incubation at room temperature protected from light, and absorbance was subsequently recorded at 490 nm.

Measurement of airway reactivity to methacholine. Twenty-four hours after the last challenge with HDM or PBS, responses to aerosolized methacholine were measured using whole-body plethysmography (EMKA technologies). Responses to inhaled methacholine were assessed by recording Penh over 5 minutes after each dose of aerosolized methacholine (Baseline, 0, 3, 5, 7 and 14 mg/ml). Invasive measurements were also performed in anesthetized, tracheostomized, mechanically ventilated mice using flexiVent™ (Scireq). Aerosolized methacholine was administered in increasing concentrations (Baseline, 0, 6, 12.5, 25, 50 and 100 mg/ml). Lung resistance (R) and tissue elastance (E) were computed by assuming a constant phase model.

Flow cytometry analysis of leukocytes in blood, bronchoalveolar lavage fluid, peritoneal lavage and lung tissue. Bronchoalveolar lavages (BALs) were performed 24 h after the last challenge with HDM in anesthetized mice (187.5 mg/kg ketamine and 18.75 mg/kg xylazine). After semi-excision of the trachea, a plastic canula was inserted, and airspace was washed with 1 ml of PBS containing 2.6 mM EDTA and 2.5 % (v/v) FBS. This operation was repeated for a total of 3 times. For the analysis of leukocytes in lung tissue, right lung lobes were harvested 24 h after the last challenge with HDM, and transferred into gentleMACS™ C tubes (Miltenyi) containing lung dissociation kit (Miltenyi). Tubes were attached upside down on a gentleMACS dissociator

(Miltenyi). After a washing step, red blood cells were lysed with Ammonium Chloride Potassium (ACK) lysing buffer (Thermo Fisher scientific), and single cell suspensions were 0.22 µm-cut-off filtered. Single cell suspensions of total right lung tissue and BAL fluid were stained with anti-CD45-FITC (clone # REA737, Miltenyi), anti-Ly6G-PE (clone # 1A8, BD Pharmingen), CD11c-VB (clone # N418, Miltenyi), SiglecF-PECy7 (clone # REA798, Miltenyi), B220-APC (clone # RA3-6B2, Miltenyi) and CD3ε-APC (clone # 145-2C11, BD Pharmingen). Eosinophils were gated as $CD45^{+}$, $CD11c^{-}$, $B220^{-}$, $CD3\varepsilon^{-}$, $Ly6G^{-}$, $SiglecF^{+}$, SSC^{high}. Blood was collected on heparin. Red blood cells were lysed with ACK lysis buffer (Thermo Fisher scientific). Cells were stained with anti-SiglecF-PECy7 (clone # REA798, Miltenyi), anti-CD49b-BV421 (clone Dx5, eBioscience), anti-IgE-FITC (clone # R35-72, BD Pharmingen) and anti-CD131-PE (clone #REA193, Miltenyi). Blood eosinophils were gated as $SiglecF^{+}$, SSC^{high}, and blood basophils as $CD49b^{+}$, $CD131^{+}$, IgE^{+}. To harvest peritoneal cells, 5 ml of PBS were injected into the peritoneal cavity and the abdomen was massaged gently for 20 seconds. Fluid containing peritoneal cells was collected and cells were stained with anti-c-KIT APC (clone # 2B8, Bioscience) and anti-IgE-FITC (clone # R35-72, BD Pharmingen). Peritoneal mast cells were gated as $c\text{-}KIT^{+}$, IgE^{+}. Samples were acquired on Miltenyi MACSQUANT 10 and 16. Data was analyzed using FlowJo software.

Quantification of antibodies against mouse and human IL-4 and IL-13, and CRM_{197}. The immunogenicity of the kinoids was assessed by evaluating antibodies against mouse IL-4, human IL-4, mouse IL-13, human IL-13 and CRM_{197} in sera collected at different time-points after vaccination. Mouse IL-4, human IL-4, mouse IL-13, human IL-13 or CRM_{197} were coated and incubated overnight at 4 °C at 1 µg/ml. After each step, plates were washed three times with PBS Tween 20 0.01 % (v/v). After blocking with casein 2 % (w/v) in PBS, serum samples were added,

a two-fold serial dilution was conducted starting at 500 dil^{-1} (diluted in PBS, casein 1 % (w/v), Tween 20 0.01 % (v/v)). After 90 min of incubation at 37 °C, bound antibodies were detected with HRP-conjugated anti-mouse IgG (Invitrogen), and plates were revealed using an OPD substrate. Reaction was stopped with 1 M H_2SO_4 after 30 minutes of OPD incubation at room temperature protected from light, and absorbance was subsequently recorded at 490 nm. Samples were analyzed starting at dilution 500 dil^{-1} up to 256 000 dil^{-1}, except for pre-immune sera analyzed only at 500 dil^{-1}. The titers were defined as the dilution of the serum where 50 % of the ODmax minus OD of corresponding pre-immune sample in the assay was reached. Titers were expressed as serum dilution factors (dil^{-1}). The limit of titer quantification is the lowest dilution tested in the assay: 500 dil^{-1}.

Assessment of the neutralizing capacity against IL-4 and IL-13 in sera from vaccinated mice. Neutralizing capacities of the anti-mouse IL-4 antibodies were evaluated using CTLL-2 cells proliferation assay (ECACC, Ref. 93042610, batch number: 12K006.). Cells were grown in presence of human IL-2 (Sigma-Aldrich; 10 ng/ml). For neutralization bioassays, human IL-2 was replaced by mIL-4 (Peprotech; 2 ng/ml). Dilution series of serum samples from mice vaccinated with IL-4 kinoids were mixed with mouse IL-4 (2 ng/ml). After 1 h incubation, 20 000 CTLL-2 cells were added to pre-incubated samples. After 48 h, cell viability was quantified by MTS/PMS assay (Promega), according to the manufacturer's instructions. Neutralizing capacities of anti-human IL-4, anti-mouse IL-13 and anti-human IL-13 antibodies were evaluated using a HEK-Blue™ IL-4/IL-13 reporter gene cell line bioassay (InvivoGen, hkb-il413, batch number: X14-37-01) by monitoring STAT6 pathway activation, adapted from the manufacturer's instructions. When activated with human IL-4, mouse IL-13 or human IL-13, this cell line produces secreted

embryonic alkaline phosphatase (SEAP) which can be quantified using QUANTI-Blue™ medium (InvivoGen). Briefly, dilution series of serum samples from mice vaccinated with kinoids were mixed with mouse IL-13, human IL-13 (PeproTech; 2 ng/ml) or human IL-4 (PeproTech; 0.25 ng/ml), and then added to 40 000 HEK-Blue™ IL-4/IL-13 cells. After 24 h, supernatants were harvested and mixed with QUANTI-Blue™. The IL-4/IL-13 neutralizing capacity 50 (NC_{50}) result was expressed as the serum dilution factor (dil^{-1}) neutralizing 50 % of IL-4 or IL-13 activity.

Quantification of total IgE levels and HDM-specific IgE and IgG. Total IgE levels were quantified using a commercial ELISA kit (E90-115; Bethyl Laboratories) according to the manufacturer's instructions. HDM-specific IgE in sera were measured by ELISA, using a protocol adapted from (*28*) and (*29*). First, HDM was biotinylated using NHS-PEG4-biotin (molar ratio NHS-PEG4-biotin/HDM: 20/1) in phosphate buffer 70 mM, NaCl 150 mM, pH 7.2. The mixture was allowed to react for 30 min, at room temperature with a modification concentration of 0.6 mg/ml. Excess NHS-PEG4-biotin was removed using Zeba desalting spin column. Protein content was determined by Bradford assay. Effective HDM biotinylation was confirmed by direct ELISA: plate was coated with HDM-biotin, detected with poly-HRP streptavidin and revealed using an OPD substrate. HDM-specific IgE were detected by ELISA. Goat polyclonal anti-mouse IgE antibody (STAR110, Bio-rad) was coated and incubated overnight at 4 °C at 2 µg/ml in PBS. After each step, plates were washed three times with PBS Tween 20 0.01 % (v/v). After blocking with casein 2 % (w/v) in PBS for 90 min at 37 °C, serum samples were added at a 1:50 final dilution (diluted in PBS, casein 1 % (w/v), Tween 20 0.01 % (v/v)) and incubated for 2 h at 37 °C. Then, HDM-biotin (prepared as described hereabove) was added at a 1:400 final dilution and incubated for 2 h at 37 °C. Bound HDM-specific IgE antibodies were detected with poly-HRP streptavidin

(N200; Thermo Fisher scientific; dilution 1:10 000, 60 min at 37 °C incubation) and plates were revealed using an OPD substrate. Reaction was stopped with 1 M H_2SO_4 after 30 minutes of OPD incubation protected from light, at room temperature and absorbance was subsequently recorded at 490 nm.

HDM-specific IgG, IgG1 and IgG2a levels in sera were measured by ELISA. HDM was coated in 96-well plates and incubated overnight at 4°C at 5 µg/mL. After each step, plates were washed three times with PBS Tween 20 0.01%. After blocking with BSA 1 % in PBS for 90 min, serum samples were added, a two-fold serial dilution was conducted starting at 1:2000 (in PBS, BSA 0.5%, Tween 20 0.01%), 1:4000 (in PBS, BSA 1 %) or 1:500 (in PBS, BSA 1 %) for IgG, IgG1 or IgG2a respectively. After 90 min of incubation, bound antibodies were detected with HRP-conjugated anti-mouse IgG (Invitrogen) at 1/5000, goat HRP-conjugated anti-mouse IgG1 (Southern Biotech) at 1/8000 or goat HRP-conjugated anti-mouse IgG2a (Southern Biotech) at 1/8000, and plates were revealed using an OPD substrate. Reaction was stopped with 1 M H_2SO_4 after 30 min of incubation at room temperature and absorbance was subsequently recorded at 490 nm.

Lung histology. Left lungs were excised from mice post mortem, fixed with 4 % paraformaldehyde (PFA) for 24 h at room temperature, and preserved in 70 % ethanol. Longitudinal sections were done and stained with Hematoxylin and Eosin (H&E) (for assessment of leukocyte infiltration), periodic acid Schiff (PAS) staining (for assessment of goblet cells hyperplasia and mucus production) or toluidine blue (for quantification of mast cell numbers) (all from Sigma). The severity of inflammation on H&E-stained lung sections was graded semi-quantitatively in a blind manner for the following features: 0: normal, 1: few cells, 2: a ring of inflammatory cells, 1 cell

layer deep, 3: a ring of inflammatory cells 2-4 cells deep, 4: a ring of inflammatory cells of > 4 cells deep (adapted from (*30*)). The extent of mucus production was also quantified in a blind manner on PAS-stained lung sections by a score according to the percentage of goblet cells in the epithelial cells: 0: no goblet cells, 1: less than 25 %, 2: 25-50 %, 3: 50-80 %, 4: more than 80 % (*31*). Mast cell quantification on toluidine blue-stained lung sections was performed using Zen Software.

IHC section and acquisition. For lung and ear skin IHC, the bigger lobe from the right lung or the ears were excised post mortem fixed with 1 % PFA for 24 h at room temperature. Tissue dehydration was performed in sucrose gradient baths (10%, 20% and 30%), then tissues were embedded in OCT compound.

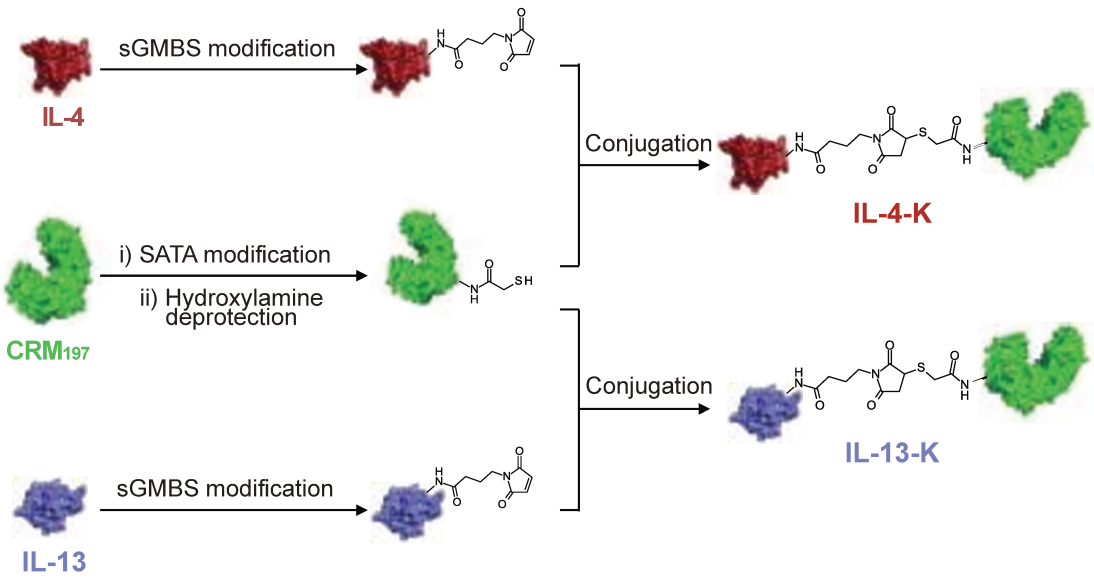
8 µm-thick sections of lung tissue sections were treated using a heat-induced epitope retrieval method as previously described (*32*). Tissue sections were blocked and permeabilized with PBS 0.5 % (w/v) BSA (Sigma-Aldrich), 0.3 % Triton X-100 (Merck) for 1 h at room temperature, then incubated with an anti-IgE antibody coupled to AF488 (Biolegend, UK), at 10 µg/ml overnight at 4 °C in the dark. Tissue sections were then washed three times in PBS 0.5 % (w/v) BSA, and incubated with DAPI (Thermo Fisher scientific) and avidin-sulforhodamine at 5µg/ml (Sigma-Aldrich) (to stain mast cells (*33*)) in PBS 0.5 % (w/v) BSA, for 2 h in the dark. Finally, samples were mounted in Mowiol medium (Sigma-Aldrich) and sealed with nail polish. 512 x 512 pixels Z-Stack images were acquired using a confocal microscope SP8 (Leica Microsystems) equipped with a HC PL APO CS2 with 20X NA 0.75 Dry or a HC PL APO CS2 60X/NA 1.40 oil objective. In our study, a digital zoom of 3 or 6 was applied for 60X or 20X objective, respectively. A Maximum Intensity Projection (MIP) was used for generating 2D images. Individual Mean

Intensity Analysis of anti-IgE staining on avidin-sulforhodamine^{+} mast cells was performed using ImageJ (NIH). Scale bar: 10 µm.

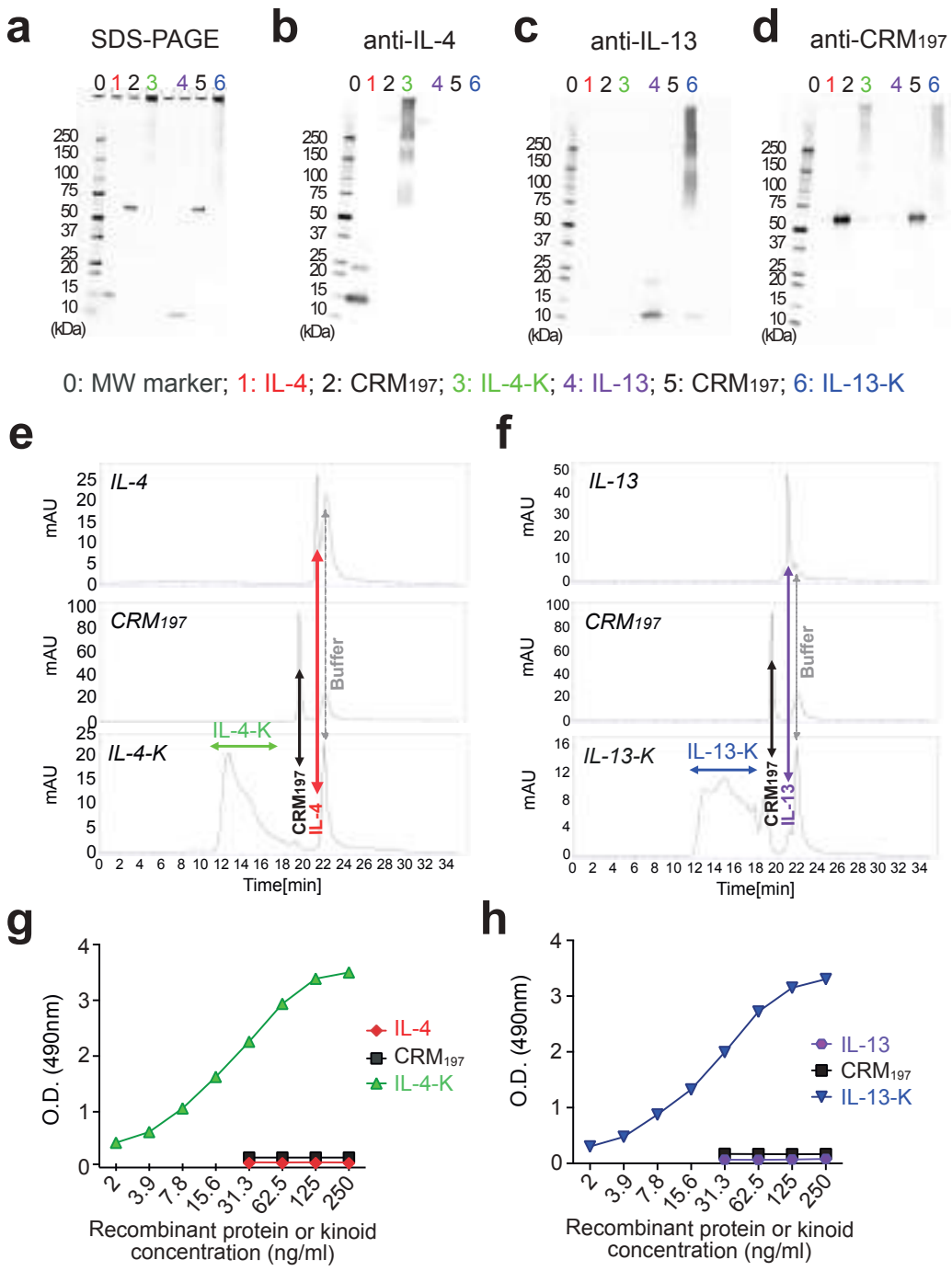
8 µm-thick sections of ear skin sections were blocked and permeabilized with PBS supplemented with 0.5 % (w/v) BSA (Sigma-Aldrich) and 0.1% Saponin (Sigma-Aldrich), for 30 min at room temperature, and incubated with an anti-IgE antibody coupled to AF488 (Biolegend, UK),), at 10 µg/ml overnight at 4 °C in the dark. Tissue sections were then washed three times in PBS 0.5 % (w/v) BSA and 0.1% Saponin, and incubated with DAPI and avidin-sulforhodamine in PBS 0.5 % (w/v) BSA and 0.1% Saponin, for 2 h at room temperature in the dark. Finally, samples were mounted in Mowiol medium (Sigma-Aldrich). 512 x 512 pixels Z-Stack images were acquired using a confocal microscope LSM710 (Zeiss) equipped with a HC PL APO 40X/NA 1.30 oil objective. A Maximum Intensity Projection (MIP) was used for generating 2D images. Individual Mean Intensity Analysis of anti-IgE staining on avidin-sulforhodamine+ mast cells was performed using ImageJ (NIH). Scale bar: 20µm.

ANTIBODY	SOURCE	CLONE	IDENTIFIER	Dilution
anti-mouse IL-4 polyclonal goat IgG	R&D systems	n/a	AF-404-NA	0.1 µg/ml
anti-human IL-4 polyclonal goat IgG	R&D systems	n/a	AF-204-NA	0.1 µg/ml
anti-mouse IL-13 polyclonal goat IgG	R&D systems	n/a	AF-413-NA	0.1 µg/ml
anti-human IL-13 polyclonal goat IgG	R&D systems	n/a	AF-213-NA	0.1 µg/ml
anti-diphtheria toxin polyclonal goat IgG	AbD serotec	n/a	3710-0956	860 ng/ml
monoclonal mouse anti-diphtheria toxin IgG1	AbD serotec	8G1	3710-0100	1 µg/ml
biotinylated anti-mouse IL-4 polyclonal goat IgG	R&D systems	n/a	BAF-404	250 ng/ml
biotinylated anti-human IL-4 polyclonal goat IgG	R&D systems	n/a	BAF-204	250 ng/ml
biotinylated anti-mouse IL-13 polyclonal goat IgG	R&D systems	n/a	BAF-413	250 ng/ml
biotinylated anti-human IL-13 polyclonal goat IgG	R&D systems	n/a	BAF-213	250 ng/ml
anti-Ly6G-PE rat IgG_{2a}	BD Pharmingen	clone 1A8	561104	1:100
anti-CD3-APC armenian hamster IgG_1	BD Pharmingen	clone 145-2C11	561826	1:100
anti-CD45-FITC human IgG_1	Miltenyi Biotec	clone REA737	130-110-658	1:100
anti-SiglecF-PECy7 human IgG_1	Miltenyi Biotec	clone REA798	130-112-334	1:100
anti-CD11b-VG recombinant human IgG_1	Miltenyi Biotec	REA713	130-110-559	1:100
anti-B220-APC rat IgG_{2a}	Miltenyi Biotec	clone RA3-6B2	130-102-259	1:100
anti-CD11c-VB, hamster IgG	Miltenyi Biotec	clone N418	130-102-797	1:100
anti-CD49b- BV421, Rat IgM	BD Horizon	clone Dx5	563063	1:50
anti-IgE-FITC, Rat IgG_1	BD Pharmingen	R35-72	553415	1:50
anti-CD131-PE	Miltenyi Biotec	REA193	130-118-456	1:100
anti-c-KIT APC	eBioscience	2B8	17-1171-82	1:200
rabbit polyclonal HRP-conjugated anti-mouse IgG	Invitrogen	n/a	61-6520	1:5000
Goat polyclonal Anti-Mouse IgG1 Human ads-HRP	Southern Biotech	n/a	1070-05	1:4000
Goat polyclonal Anti-Mouse IgG2a Human ads-HRP	Southern Biotech	n/a	1080-05	1:4000
goat polyclonal anti-mouse IgE antibody	Bio-rad	n/a	STAR110	2 µg/mL
goat polyclonal HRP-conjugated anti-mouse IgG	Bethyl Laboratories	n/a	A90-131P	1:5000
anti-IgE antibody coupled to AF488, Rat IgG1, κ	Biolegend	RME-1	406910	10 µg/ml

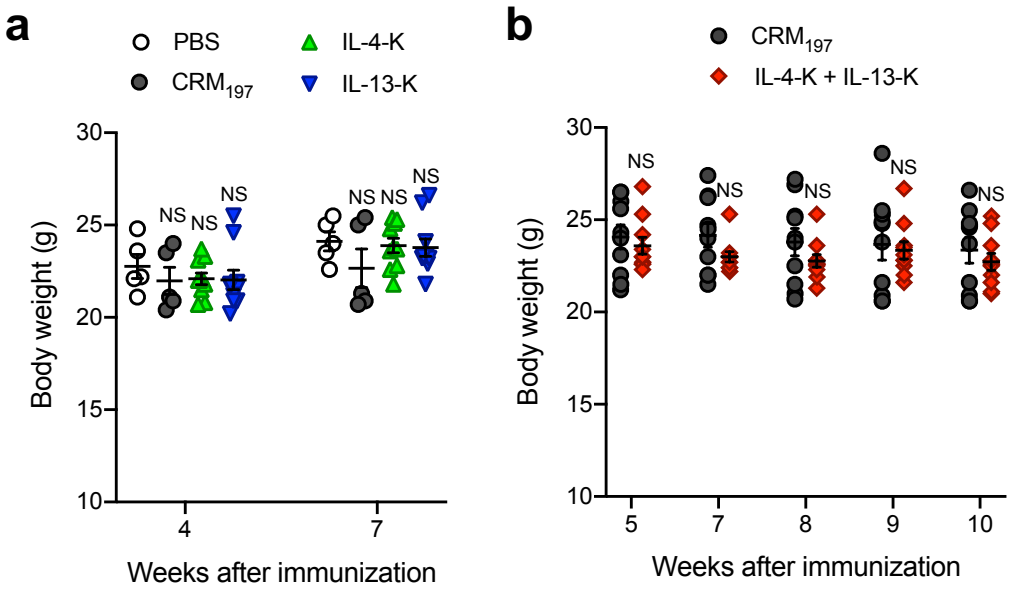
Supplementary Table 1. List of antibodies used in this study.



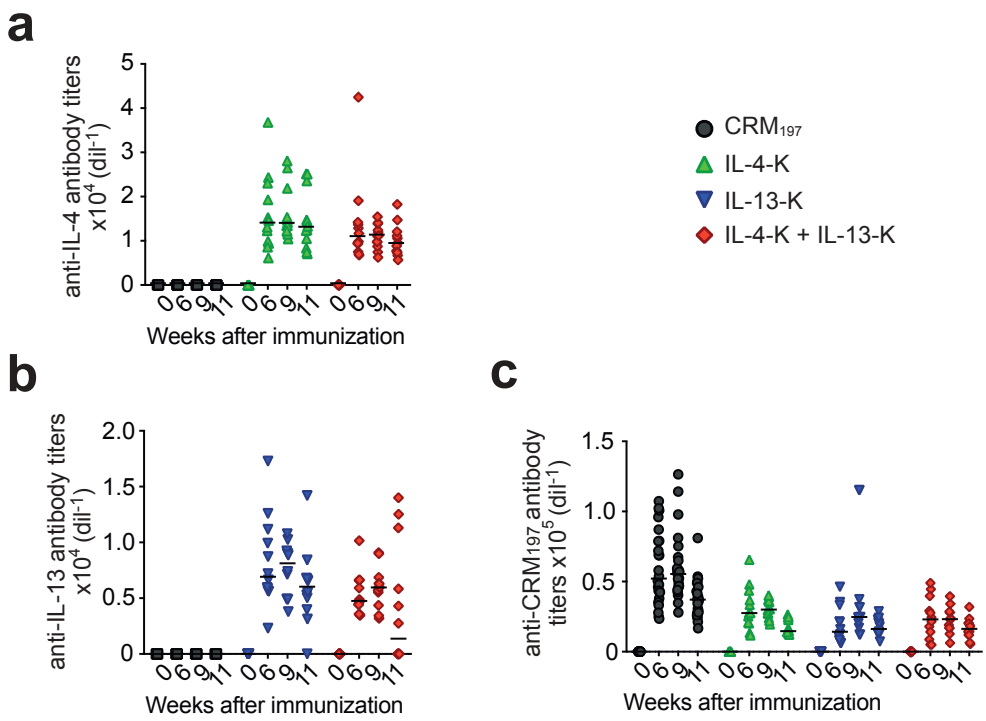
Supplementary Figure 1. Synthesis of IL-4-K and IL-13-K using a thiol-maleimide conjugation. Recombinant mouse IL-4 and IL-13 were modified with N-γ-maleimidobutyryl-oxysuccinimide ester (sGMBS), a maleimide-containing agent reacting with primary amines. Sulfhydryl moieties were introduced on the carrier protein, CRM_{197}, with SATA (N-succinimidyl-S-acetylthioacetate). Functionalized CRM_{197} was added to functionalized IL-4 or IL-13 at a molar ratio of 1:2 (CRM_{197}-IL-4) or 1:4 (CRM_{197}-IL-13), to generate the IL-4 kinoid (IL-4-K) and IL-13-K, respectively.



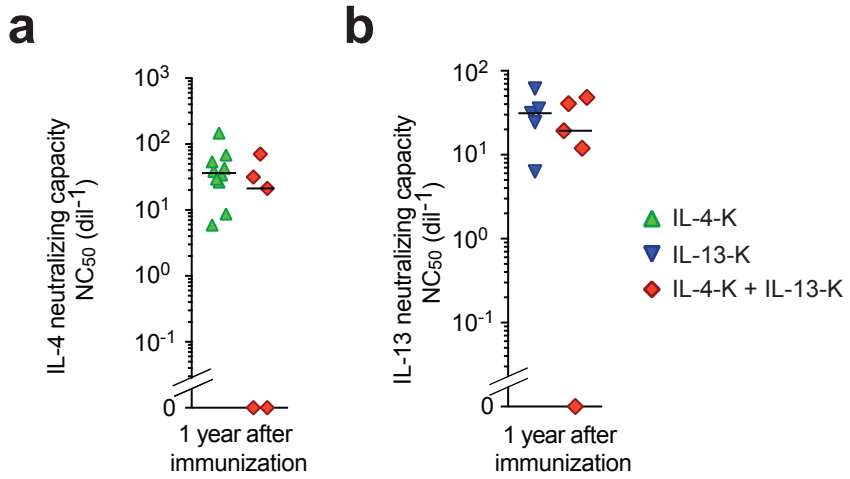
Supplementary Figure 2. Characterization of mouse IL-4-K and IL-13-K. Generation of high molecular weight kinoids upon conjugation of IL-4 or IL-13 to CRM_{197} was confirmed using SDS-PAGE (**a**), western blots against IL-4 (**b**), IL-13 (**c**) or CRM_{197} (**d**), and size exclusion (SE)-HPLC (**e** and **f**). Epitope preservation and antigenicity of IL-4-K and IL-13-K was analyzed by sandwich ELISA using plates coated with anti-CRM_{197} antibodies and revealed with anti-IL-4 (**g**) or anti-IL-13 (**h**) antibodies.



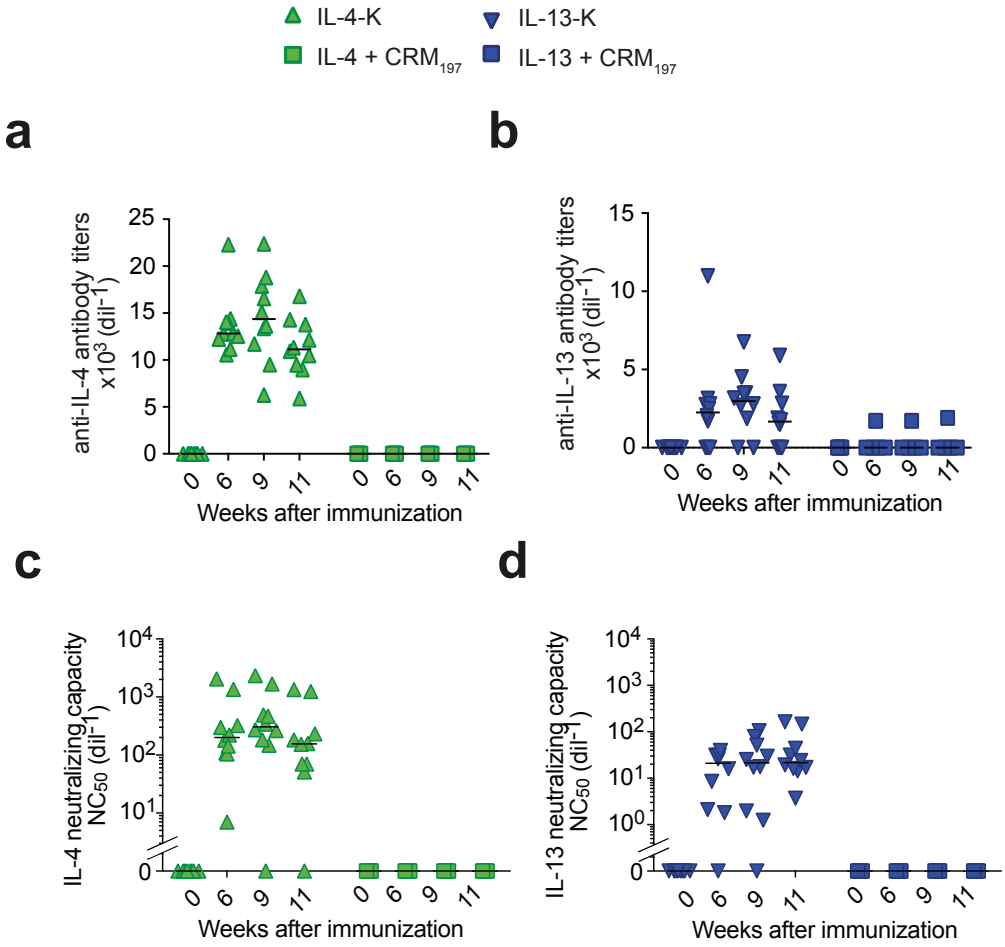
Supplementary Figure 3. Changes in body weight following vaccination with kinoids. Mice were immunized with IL-4-K, IL-13-K, CRM_{197} or PBS as control (**b**), or IL-4-K and IL-13-K in combination or CRM_{197} (**b**), as outlined in Figure 1A. Body weight was measured at the indicated time points after first injection of kinoids. Results show values from individual mice with bars indicating mean values ± SEMs, and are pooled from two independent experiments. NS: not significant (P>0.05).



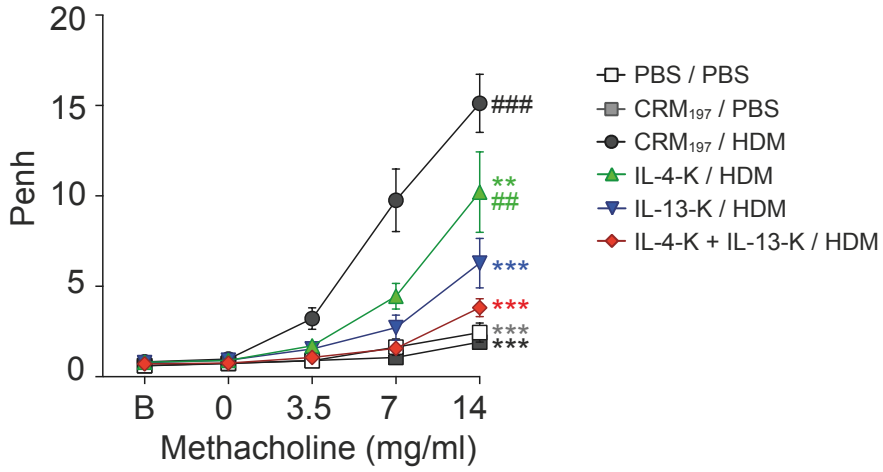
Supplementary Figure 4. Anti-IL-4, anti-IL-13 and anti-CRM$_{197}$ antibody titers in mice vaccinated with kinoids. a-c. Anti-IL-4 (**a**), anti-IL-13 (**b**) and anti-CRM$_{197}$ (**c**) antibody titers in sera at 6, 9 and 11 weeks after first injection of kinoids. Results show values from individual mice with bars indicating medians. Data are from a single experiment with *n*=12 mice per group, representative of two independent experiments.



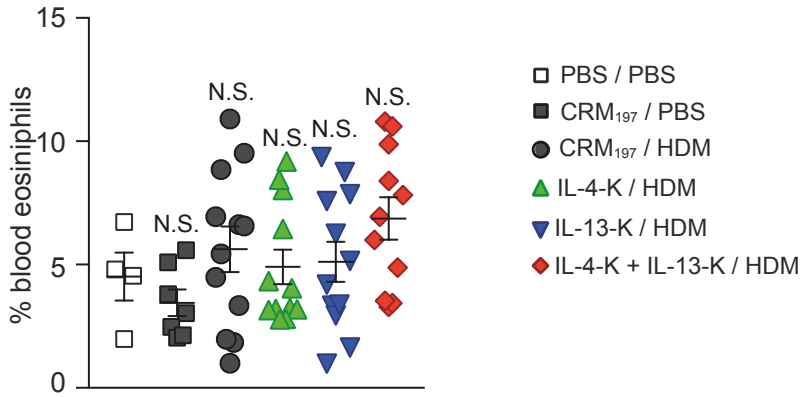
Supplementary Figure 5. Anti-IL-4 and anti-IL-13 neutralizing capacity one year after vaccination. Anti-IL-4 (**a**) and anti-IL-13 (**b**) neutralizing capacity in sera collected one year after the first injection of IL-4-K and/or IL-13-K. Results show values from individual mice with bars indicating medians, and are expressed as the serum dilution factor (dil^{-1}) neutralizing 50 % of IL-4 or IL-13 activity (0 indicates no neutralizing capacity). Data are from a single experiment (*n*=5-10 mice per group).



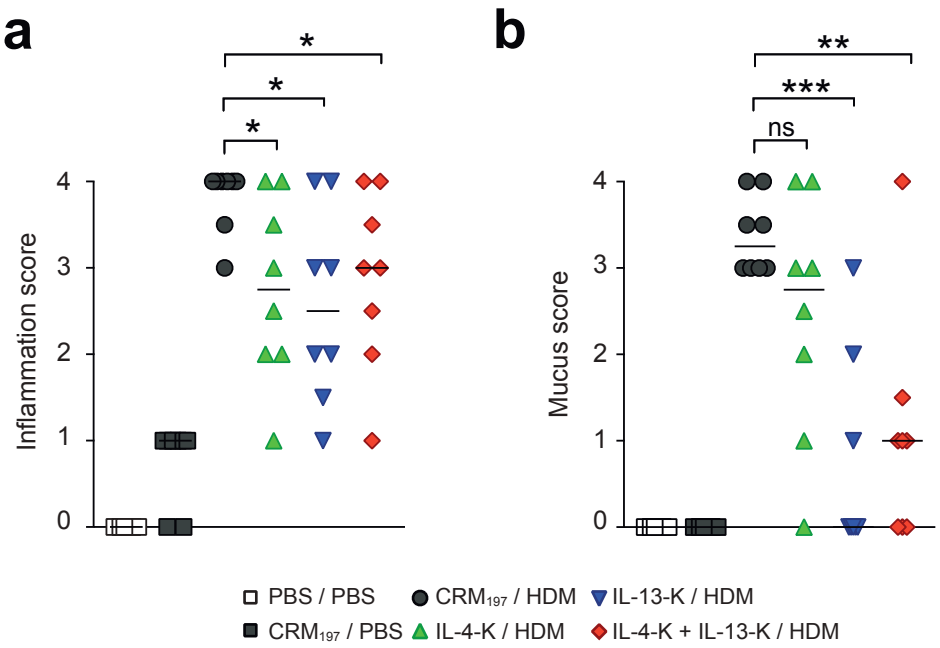
Supplementary Figure 6. Conjugation between IL-4/IL-13 and CRM_{197} is mandatory to mount a potent antibody response. a. Anti-IL-4 antibody titers in sera from mice immunized with IL-4-K or with a mixture of soluble IL-4 and CRM_{197} without prior conjugation. **b**. Anti-IL-13 antibody titers in sera from mice immunized with IL-13-K or with a mixture of soluble IL-13 and CRM_{197} without prior conjugation. Results in **a** and **b** show values from individual mice with bars indicating medians. **c**. Anti-IL-4 neutralizing capacity in sera from mice immunized with IL-4-K or with a mixture of soluble IL-4 and CRM_{197} without prior conjugation. **d**. Anti-IL-13 neutralizing capacity in sera from mice immunized with IL-13-K or with a mixture of soluble IL-13 and CRM_{197} without prior conjugation. Results in **c** and **d** show values from individual mice with bars indicating medians, and are expressed as the serum dilution factor (dil^{-1}) neutralizing 50 % of IL-4 or IL-13 activity (0 indicates no neutralizing capacity).



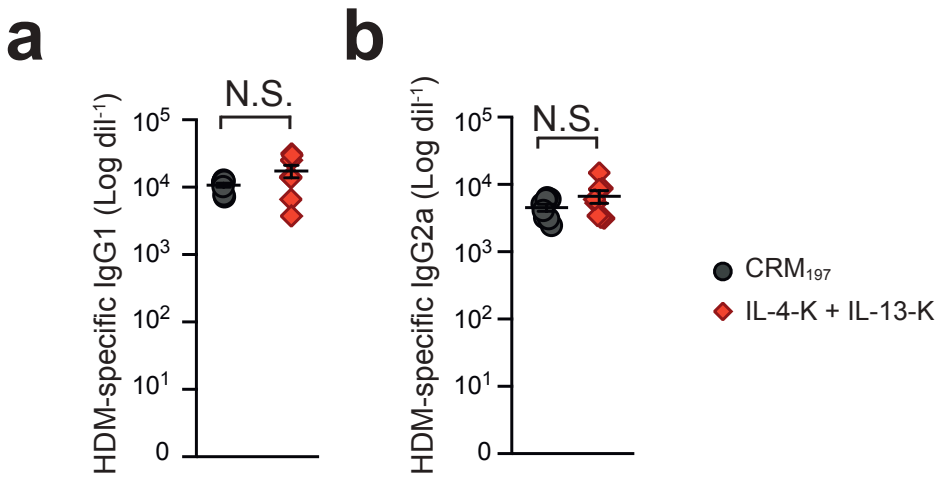
Supplementary Figure 7. Effects of IL-4-K and/or IL-13-K on HDM-induced airway hyperresponsiveness measured using non-invasive plethysmography. Mice were vaccinated with IL-4-K and/or IL-13-K (or PBS or CRM_{197} as controls) followed by sensitization and challenges with HDM (or PBS as a control), as outlined in Figure 1A. Penh responses to increasing doses of methacholine was measured using non-invasive plethysmography 24 hours after the last challenge. Data represent mean values ± SEMs from n=8-12 mice (PBS groups) or n=15-16 mice (HDM groups) pooled from two independent experiments. ** or ***: $P < 0.01$ or 0.001 *vs*. CRM_{197}/HDM group; $^{\#\#}$ or $^{\#\#\#}$: $P < 0.01$ or 0.001 *vs*. CRM_{197}/PBS group using a 2-way ANOVA test followed by a Tukey posttest.



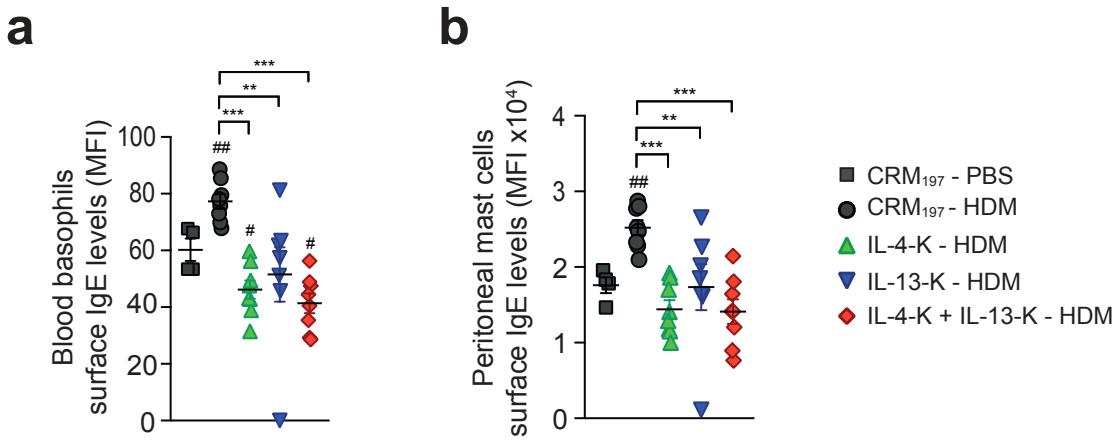
Supplementary Figure 8. Effect of vaccination with IL-4-K and/or IL-13-K on blood eosinophils. Mice were vaccinated with IL-4-K and/or IL-13-K (or PBS or CRM_{197} as controls) followed by sensitization and challenge with HDM (or PBS as a control), as outlined in Figure 1A. Percentage of IgE^+ $CD49b^+$ blood eosinophils 24 h after the last HDM challenge. Results show values from individual mice with bars indicating means ± SEMs from n=4 mice (PBS/PBS control group) or 8 mice (CRM_{197}/PBS control group) or n=11-12 mice (all other groups) pooled from two independent experiments. N.S.: not significant; $P > 0.05$ *vs*. PBS/PBS group using Mann-Whitney *U* test.



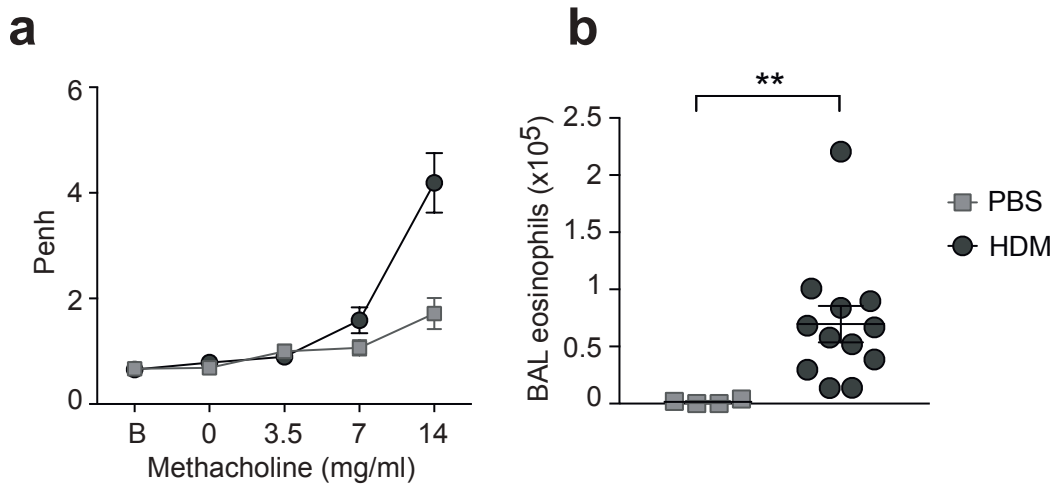
Supplementary Figure 9 Effects of IL-4-K and/or IL-13-K on lung inflammation and mucus production in an asthma model. Mice were vaccinated with IL-4-K and/or IL-13-K (or PBS or CRM_{197} as controls) followed by sensitization and challenge with HDM (or PBS as a control), as outlined in Figure 1a. **a**. The extent of leukocyte infiltration was scored in the H&E-stained lung tissue sections shown in Fig.1H obtained 24 h after the last challenge with HDM. **b**. The extent of mucus production was scored according to the percentage of goblet cells in bronchial epithelium from the PAS-stained lung tissue sections shown in Fig. 1I obtained 24 h after the last challenge with HDM. ns: not significant; *, ** or ***: $P < 0.05$, 0.01 or 0.001 *vs*. indicated group using Mann-Whitney *U* test; ns: not significant ($P > 0.05$).



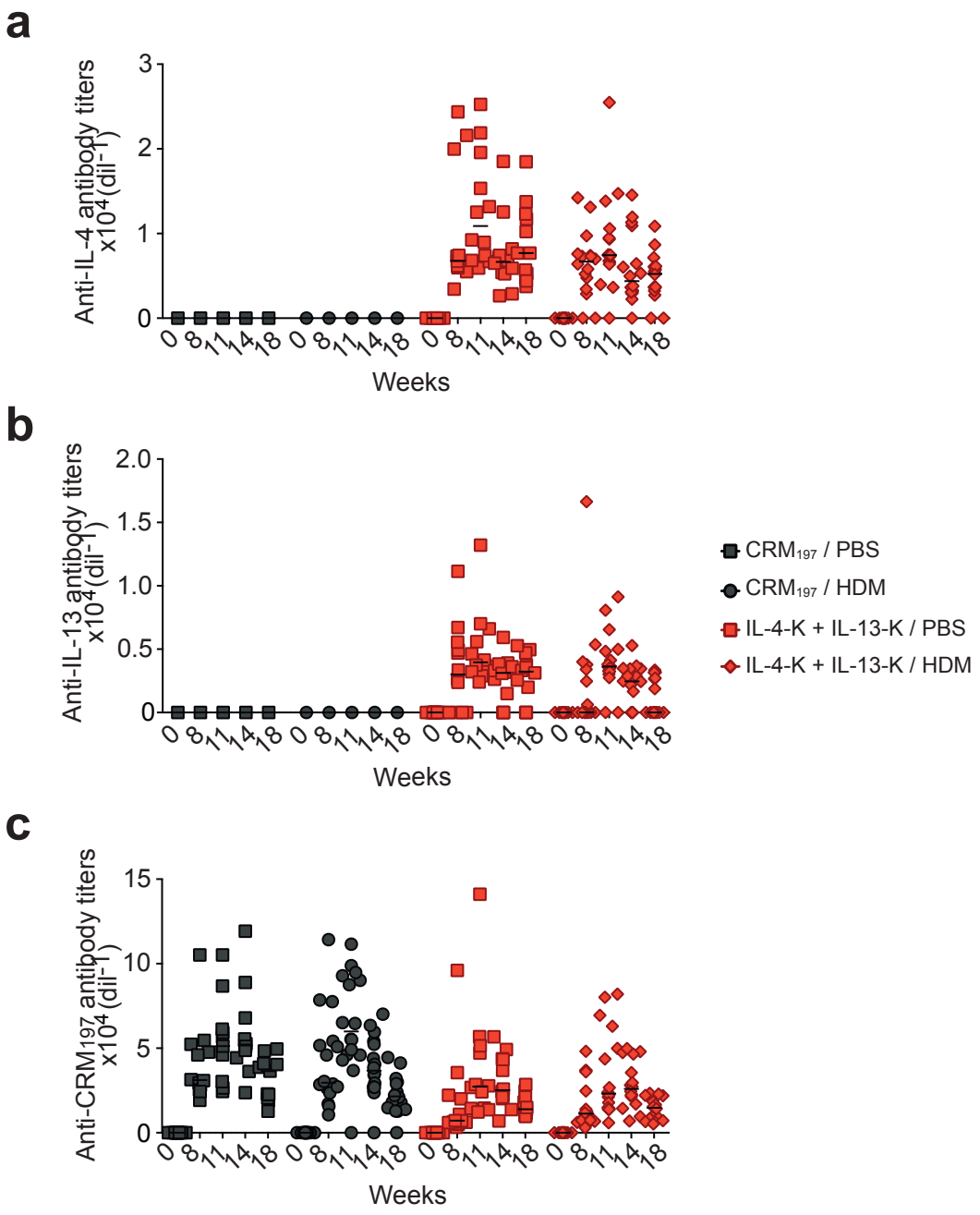
Supplementary Figure 10. Levels of HDM-specific IgG1 and IgG2a in mice vaccinated with IL-4-K and IL-13-K. Levels of HDM-specific IgG1 (**a**) and HDM-specific IgG2a (**b**) 24 h after the last HDM challenge in mice vaccinated with IL-4-K and IL-13-K, or CRM_{197} alone as a control. Results show values from individual mice with bars indicating mean ± SEM. Data are from a single experiment with n=8 mice per group, representative of two independent experiments. N.S.: not significant ($P > 0.05$ using Mann-Whitney U test).



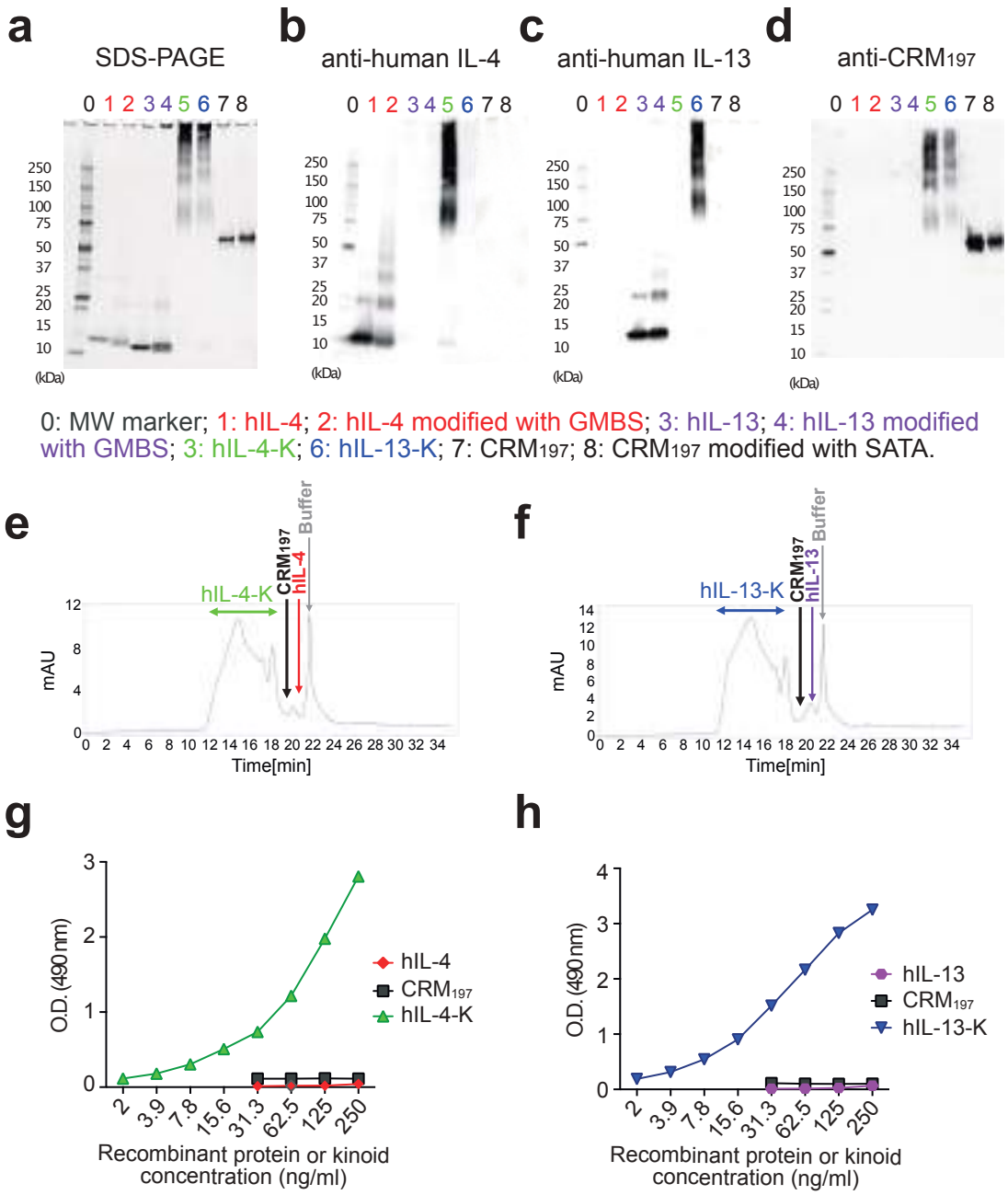
Supplementary Figure 11. Effects of IL-4-K and/or IL-13-K on surface IgE levels in blood basophils and peritoneal mast cells. Mice were vaccinated with IL-4-K and/or IL-13-K (or PBS or CRM_{197} as controls) followed by sensitization and challenge with HDM (or PBS as a control), as outlined in Figure 1A. Levels of IgE on the surface of IgE^+ $CD49b^+$ blood basophils (MFI: mean fluorescence intensity) (**a**), and levels of IgE on the surface of $CD117^+$ peritoneal mast cells (MFI) (**b**) 24 h after the last HDM challenge. Results show values from individual mice with bars indicating means ± SEMs from *n*=4 mice (CRM_{197}/PBS control group) or *n*=7-8 mice (all other groups) pooled from two independent experiments. ** or ***: $P < 0.01$ or 0.001 *vs*. indicated group; # or ##: $P < 0.05$ or 0.01 *vs*. CRM_{197}/PBS group using Mann-Whitney U test.



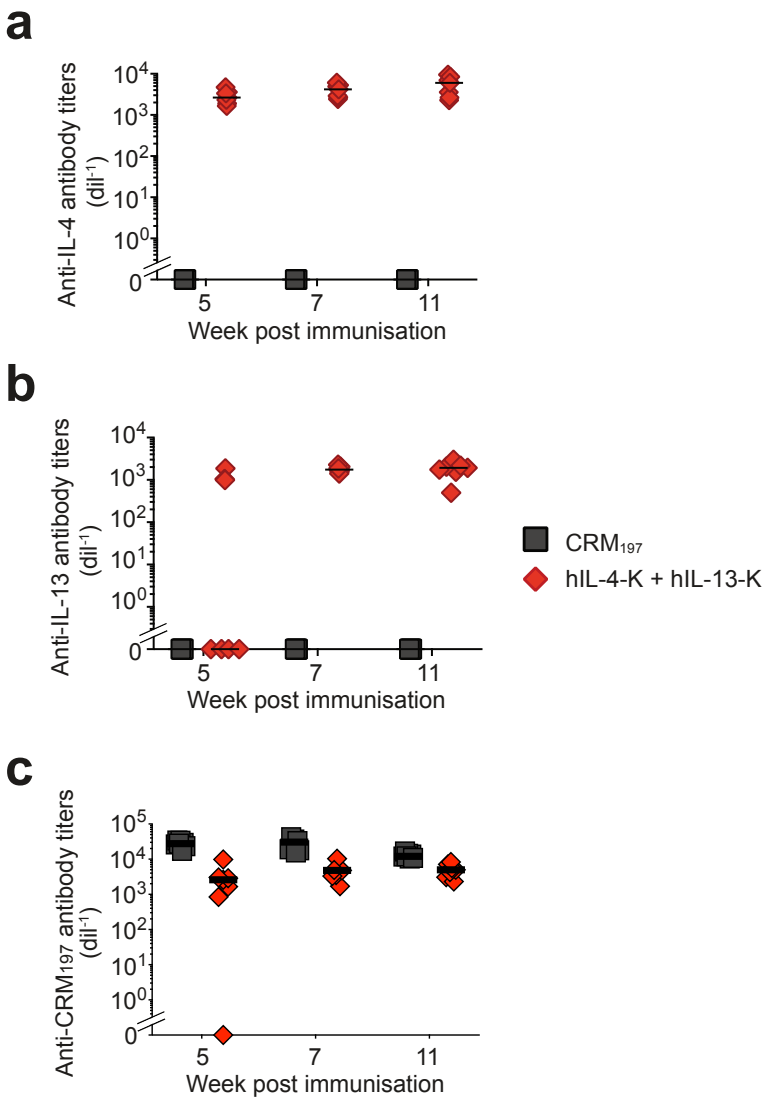
Supplementary Figure 12. Evidence of established airway hyperreactivity and eosinophilia before the start of therapeutic vaccination. Mice were sensitized and challenged with HDM or PBS as a control, as outlined in Figure 3A. Penh responses to increasing doses of methacholine measured using non-invasive plethysmography (**a**), and numbers of eosinophils in BAL fluid (**b**) 24 hours after the third challenge (*i.e.* immediately before vaccination with IL-4-K and IL-13-K). Data represent mean values ± SEMs from n=4 mice (PBS group) or n=12 mice (HDM group) pooled from two independent experiments. **: $P < 0.01$ vs. CRM_{197}/PBS group using Mann-Whitney U test.



Supplementary Figure 13. Anti-IL-4, anti-IL-13 and anti-CRM$_{197}$ antibody titers upon therapeutic dual vaccination with IL-4-K and IL-13-K. Mice were sensitized and challenged with HDM extract (or PBS as a control). After the third challenge, mice were vaccinated with IL-4-K and IL-13-K in combination (or CRM$_{197}$ as control), as outlined in Figure 3A. Anti-IL-4 (**a**), anti-IL-13 (**b**) and anti-CRM$_{197}$ antibody titers (**c**) in sera from mice at indicated time points. Results show values from individual mice pooled from two independent experiments with bars indicating median (n=16 mice per group).



Supplementary Figure 14. Characterization of human IL-4-K and IL-13-K. Generation of high molecular weight kinoids upon conjugation of human IL-4 or human IL-13 to CRM_{197} was confirmed using SDS-PAGE (**a**), western blots against human IL-4 (**b**), human IL-13 (**c**) or CRM_{197} (**d**), and size exclusion (SE)-HPLC (**e** and **f**). Epitope preservation and antigenicity of human IL-4-K (hIL-4-K) and hIL-13-K was analyzed by sandwich ELISA using plates coated with anti-CRM_{197} antibodies and revealed with anti-human IL-4 (**g**) or anti-human IL-13 (**h**) antibodies.



Supplementary Figure 15. Anti-hIL-4, anti-hIL-13 and anti-CRM$_{197}$ antibody titers upon dual vaccination with human IL-4 and IL-13 kinoids in IL-4/IL-13/IL-4Rα-humanized mice. IL-4/IL-13/IL-4Rα-humanized mice were vaccinated with hIL-4-K and hIL-13-K in combination (or CRM$_{197}$ as control), as described in Fig. 4A. Anti-human IL-4 (**a**), anti-human IL-13 (**b**) and anti-CRM$_{197}$ (**c**) antibody titers in sera at the indicated time-points. Results show values from individual mice with bars indicating medians (*n*=7 mice per group).

4. Chapter 4. Development and characterization of hIgE^{KI} hFcERI^{Tg} mice and anti IgE vaccine

The second part of this thesis is focused on the development and characterization of an anti-IgE vaccine also using the kinoid technology. IgE-targeted therapies development is limited by a lack of model to assess human IgE (hIgE) *in vivo*. For this project and to bypass this limitation, we generated an IgE-humanized mouse strain (hIgE^{KI}) in which the Fc porting of the human IgE Cε1-4 is knocked-in into the mouse gene encoding Cε1-4. The IgE antibody repertoire in these mice remains therefore polyclonal, with a variable heavy and light chain encoded by mouse V(D)J genes, light chain constant domains encoded by mouse kappa and lambda chains but IgE heavy chain constant domains chain encoded by the human Fcε knock-in gene. We then crossed hIgE^{KI} mice with mice expressing the human high affinity IgE receptor hFcεRI following its expression pattern in humans, in the absence of its mouse counterpart (mFcεRI^{-} hFcεRI^{Tg} mice). The resulting hIgE^{KI} hFcεRI^{Tg} mice have circulating hIgE as well as hIgE bound on the surface of basophils and mast cells that express hFcεRI. We took advantage of this model to directly test the human IgE-K *in vivo*. IgE-K development followed the same steps defined in the IL-4-K/IL-13-K project. However, an important preliminary work was completed in order to obtain a safe IgE fragment, before development of the conjugation strategy with a carrier protein. As a general approach, we decided to use a closed IgE fragment, which comprises Cε3 and Cε4 domain as well as a small part of the Cε2 domain, with a mutation at Cys335 described in Jardetzy and Wurtzburg's work (*262*), and which creates an additional disulfide bridge between the two fragments to "lock" them into a closed conformation recognized by Omalizumab but unable to bind FcεRI. Such IgE fragment was produced in Expi 293 cells and linked to carrier protein CRM_{197}. To evaluate best candidate's immunogenicity, we vaccinated hIgE^{KI} hFcεRI^{Tg} mice and observed a strong neutralizing antibody response against human IgE, with decreased levels in circulation but also on the surface of hFcεRI-expressing mast cells and basophils, promoting their desensitization. We took a great care at evaluating the safety profile of the IgE kinoid (IgE-K) by following signs of anaphylaxis after each injection, to be sure that IgE-K injection was not triggering mast cell degranulation. Finally, to test the efficiency of the vaccine, we developed an IgE-dependent systemic anaphylaxis model and showed that vaccinated mice were fully protected from hypothermia and death in this anaphylaxis model.

Paper II:

Anti-IgE vaccination prevents human IgE-mediated severe allergic reactions in humanized mice

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Running title

Vaccination against IgE blocks anaphylaxis

ABSTRACT

IgE antibodies play a key role in allergic diseases. Anti-IgE targeting antibodies have been developed and demonstrate potent activity on IgE-dependent pathologies, like allergic asthma and chronic spontaneous urticaria. Their use is however restricted to patients with low-to-moderate IgE levels, due to potent adverse events at high circulating IgE levels, and with moderate-to-severe symptoms, due to high cost. Their main limitation resides in the fact that they require life-long administrations. We developed herein a vaccine strategy against IgE to induce long-term reduction of or protection from IgE-dependent pathologies. A fragment of human IgE was used to generate a conjugate vaccine technology termed kinoid. To assess the efficacy of IgE vaccination, we generated a mouse model humanized for IgE and its high-affinity receptor FcεRI. IgE vaccination induced long-term production of anti-human IgE neutralizing antibodies without any detectable adverse effect. It reduced both circulating and FcεRI-bound human IgE, and protected against severe IgE-mediated allergic reactions in IgE/FcεRI humanized mice, and even in a genetically predisposed allergic variant of this model. Thus, IgE vaccination represents a promising, cost-effective, long-term therapeutic strategy for the treatment of IgE-mediated allergic conditions.

INTRODUCTION

The prevalence of allergic diseases has dramatically increased over the past years, especially in industrialized countries, with more than 30% of children suffering from allergies (1). The most dramatic clinical manifestation of allergy is anaphylaxis, an acute and potentially fatal systemic reaction (2, 3). Immunoglobulin E (IgE) plays a central role in mediating allergic reactions and anaphylaxis (2, 4). Upon exposure to an allergen, such allergen is recognized by allergen-specific IgE bound to their high-affinity receptor FcεRI on the surface of tissue mast cells and blood basophils, which promotes the degranulation of these cells, and the release of both preformed and newly synthesized mediators, including histamine (5, 6). For this reason, clinical diagnoses of allergies are largely based on measurements of allergen-specific IgE (7).

Most treatments for allergies are symptomatic (mostly antihistamines and corticosteroids) (8, 9). In recent years however, several recombinant monoclonal antibodies (mAbs) have been developed for the treatment of allergies. Omalizumab, a humanized anti-IgE mAb, shows clinical benefit for the treatment of allergic asthma (10) and chronic spontaneous urticaria (11). Available clinical data suggest that this mAb could also have benefit for the treatment of other types of allergies (12). However, use of omalizumab (or any other mAb) is limited first and foremost by high cost and the need to perform repeated injections, and also by potential risks of appearance of anti-drug antibodies (ADAs) or other adverse reactions (13, 14). The main medical limitation is patients with levels of IgE higher than 700 IU/ml, that may be of risk of anaphylaxis if treated with omalizumab. A next-generation anti-IgE mAb, ligelizumab, with significantly higher affinity for IgE than omalizumab and potentially reduced adverse effects, has been developed but did not demonstrate improved efficacy over omalizumab in severe asthma patients (NCT02075008). Omalizumab and ligelizumab differ in

the epitopes they bind on IgE and on their ability to interfere with FcεRI-bound IgE or IgE production (15). Therefore, while IgE are promising therapeutic targets for the treatment of allergies and anaphylaxis, there is a clear need to improve current strategies to block IgE, in order to reach long-term therapeutic effects.

Therapeutic conjugate vaccines called kinoids are used in an active immunization strategy to induce neutralizing antibodies against an abnormally highly produced target, to reduce target levels back to baseline or lower (16). We hypothesized that therapeutic vaccination against human IgE using anti-IgE kinoids could promote long-term protection against IgE-mediated allergy and anaphylaxis. Ideally, an IgE vaccine should induce 'Omalizumab-like' antibodies which can block free IgE but do not recognize IgE already bound to the surface of mast cells and basophils, avoiding potential cell activation. IgE has globally the same structure as IgG, with the exception of an additional domain in the heavy chain (Cε2) (17). Two peptide-based IgE vaccine candidates were previously designed using short peptides (16 and 20 aa): one peptide corresponding to a potential sequence recognized by Omalizumab in the Cε3 domain, the other one likely corresponding to part of the FcεRI-binding site. In a first study in cynomolgus monkeys, IgE levels were only partially inhibited by the vaccines, however the degree of inhibition was much less than that seen with Omalizumab (18). Moreover, in a phase 1 study in patients with allergic rhinitis (NCT01723254), the two vaccine candidates were well tolerated and induced antibodies against the Cε3 peptides, but did not significantly lower serum IgE levels, and the development of these vaccine candidates has therefore been discontinued (19). The crystal structure of Omalizumab-bound IgE revealed that the Omalizumab epitope is discontinuous and spread among the entire Cε3 domain (20). It is thus likely that induction of "Omalizumab-like" neutralizing antibodies would require immunization against the entire Cε3 domain. In addition, proper folding of the Cε3 is supported

by the Cε4 domain, which limits the ability of a peptide vaccine to induce potent neutralizing anti-IgE Abs.

Here, we generated an anti-human IgE kinoid consisting of the entire Cε3-4 domains coupled to the non-toxic mutant of diphtheria toxin, CRM_{197} (21). This vaccine induced a long-lasting anti-human IgE neutralizing antibody response without any adverse effects in wt mice or in mice humanized for both IgE and FcεRI (IgE/FcεRI humanized mice). Anti-IgE vaccination reduced both circulating and FcεRI-bound human IgE, and fully protected against IgE-mediated anaphylaxis in IgE/FcεRI humanized mice.

RESULTS AND DISCUSSION

Vaccination with hIgE kinoid induces potent anti-IgE neutralizing antibodies in IgE humanized mice

IgE kinoids (hIgE-K) were generated by coupling human IgE Cε3–4 domains with diphtheria 'cross-reactive material 197' (CRM_{197}, a non-toxic mutant of diphtheria toxin used as a carrier protein in a number of approved conjugated vaccines (21)) using a thiol-maleimide conjugation (**Figure 1A**). We replaced the native glycine residue at position 335 by a cysteine residue into Cε3-4. Consequently, interchain disulfide bonds are formed that locks the IgE fragment into a "closed" conformation retaining high-affinity binding to omalizumab, but not FcεRI (20, 22). We hypothesized that an IgE conjugate vaccine containing this G335C mutation would favor generation of "omalizumab-like" neutralizing antibodies while avoiding potentially harmful binding to FcεRI. SDS-PAGE and HPLC analysis indicated formation of high molecular species upon conjugation of hIgE Cε3-4 G335C to CRM_{197}, confirming efficiency synthesis of hIgE-K (**Figure 1B and 1C**).

To validate the in vivo efficacy of the IgE-K, we developed IgE humanized mice ($hIgE^{KI}$ mice) in which the Fc portion of human IgE Cε1-4 was knocked-in into the mouse gene encoding Cε1-4 (**Figure 2A**). The IgE Ab repertoire in those mice remains therefore polyclonal with variable heavy and light chains encoded by mouse V(D)J genes, the constant domains of the light chain encoded by mouse kappa and lambda chains, but the constant domains of the IgE heavy chain encoded by the human Fcε knock-in gene. Both $hIgE^{KI/+}$ and $hIgE^{KI/KI}$ mice have detectable levels of chimeric IgE (thereafter termed hIgE) in the blood (**Figure 2B**). Moreover, we could detect mouse IgE in the blood of WT and $hIgE^{KI/+}$ mice, but not in $hIgE^{KI/KI}$ mice, confirming efficient disruption of the mouse gene encoding Cε1-4 (**Figure 2C**).

Immunization of $hIgE^{KI}$ mice with hIgE-K in SWE, a squalene oil-in-water emulsion adjuvant (23), induced high anti-hIgE antibody titers, detectable already 5 weeks after primary immunization and still more than 39 weeks after (the latest time-point assessed so far) (**Figure 2D-E**). As expected, all mice exposed to CRM_{197} alone or hIgE-K developed anti-CRM_{197} antibodies (**Figure 2F**). Importantly, anti-hIgE antibodies generated upon vaccination with the kinoid exhibited strong neutralizing capacities in all mice starting 5 weeks after primary immunization (**Figure 2G**). We could detect hIgE in the blood of CRM_{197}-immunized control mice, but not in $hIgE^{KI}$ mice vaccinated with the hIgE-K, confirming the neutralizing capacities of antibodies generated upon vaccination (**Figure 2H**). Altogether, these data indicate that efficient long-term neutralization of hIgE can be achieved through vaccination with hIgE-K in $hIgE^{KI}$ mice.

Efficacy of anti-hIgE vaccine in a model of hIgE-mediated anaphylaxis

Human IgE does not bind mouse FcεRI (24), which does not permit development of functional hIgE-mediated allergy models in $hIgE^{KI}$ mice. We thus crossed $hIgE^{KI}$ mice with $hFc\varepsilon RI^{Tg}$ mice, which express the human IgE receptor FcεRI under the dependency of their human promoter ($hFc\varepsilon RI^{Tg}$ mice) (25), in order to obtain IgE/FcεRI humanized mice which can both produce and respond to hIgE. IgE/FcεRI humanized mice expressed human FcεRI on the surface of blood basophils (**Figure 3A**) and peritoneal mast cells (**Figure 3B**). While surface hIgE levels were very low in naïve mice (data not shown), mice sensitized with the allergen house dust mice (HDM) had detectable levels of hIgE on the surface of both mast cells and basophils (**Figure 3, C and D**). Interestingly, we found that circulating IgE levels were significantly reduced in $hIgE^{KI}$ mice expressing hFcεRI ($hFc\varepsilon RI^{Tg}$) compared with $hIgE^{KI}$ mice deficient for FcεRI (**Figure 3E**). This phenomenon could reflect the fact that most IgE are

trapped at the surface of mast cells and basophils in IgE/FcεRI humanized mice, and/or that hFcεRI contributes to serum hIgE clearance by monocytes/dendritic cells expressing FcεRI as a trimer, as suggested previously (26, 27).

Anti-human IgE vaccination may result in adverse events in human IgE-expressing mice, similarly to omalizumab injections in individuals with high IgE levels (13, 28, 29) or in mice pre-injected with human IgE (30). We therefore carefully monitored mice after each injection of IgE-K vaccine (or CRM_{197} as a control) (**Figure 4A**) and did not observe any detectable adverse effect in IgE/FcεRI humanized mice: neither hypothermia (**Figure C**), the parameter used to follow anaphylactic shock in mice (31), nor diarrhea, distress or lack of vitality over 1 hour following each vaccine injection in IgE/FcεRI humanized mice. This absence of adverse effects suggest that the vaccine does not trigger FcεRI activation through IgE aggregation on the surface of mast cells and basophils. Vaccination with hIgE-K induced high titers of anti-hIgE antibodies in IgE/FcεRI humanized mice, which were already detectable 5 weeks after the first injection of kinoid (**Figure 4B**), similarly to their appearance in $hIgE^{KI}$ mice (**Figure 2H**).

To further assess the safety and efficiency of the hIgE vaccine, we injected a high dose (10μg) of anti-nitrophenyl (NP) hIgE into IgE/FcεRI humanized mice which had been vaccinated with hIgE-K or with CRM_{197} as a control, following the same immunization schedule described above (**Figure 4A**). Again, we observed neither hypothermia, nor diarrhea, distress or lack of vitality over 1 hour following injection of anti-NP-hIgE, confirming that the vaccine does not induce detectable side effects even in the presence of very high levels of circulating hIgE (**Figure 4D**). Importantly, mice vaccinated with the hIgE-K were protected from hIgE-mediated anaphylaxis, whereas CRM_{197}-vaccinated mice injected with anti-NP hIgE and challenged with the NP antigen suffered profound hypothermia and 1 out of 7 mice died (**Figure 4E)**.

Efficacy of anti-hIgE vaccine in a genetically predisposed allergic mouse model

IgE/FcεRI humanized mice demonstrated low levels of circulating hIgE, whereas allergic patients display moderate to high levels of circulating IgE, making the mouse model potentially easier to protect from IgE-induced events following anti-IgE vaccination. To resolve this discrepancy, we crossed IgE/FcεRI humanized mice with mice bearing the gain-of-function Y709F mutation in the gene encoding the interleukin-4 (IL-4) and IL-13 receptor subunit, IL-4Ra, to generate $hIgE^{KI}$; $hFc\varepsilon RI^{Tg}$; F709 IL4Ra mice (**Figure 5A**). The Y709F mutation disrupts the Immunoreceptor tyrosine-based inhibitory motif (ITIM) of IL4Ra, thus enhancing receptor signaling in response to IL-4 and IL-13, amplifying IgE levels and IgE-mediated anaphylaxis (32, 33). We used $hIgE^{KI}$; $hFc\varepsilon RI^{Tg}$; F709 IL4Ra mice to assess the efficiency of the hIgE vaccine in a model in which anaphylaxis is triggered by endogenous hIgE. To do so, we injected $hIgE^{KI}$; $hFc\varepsilon RI^{Tg}$; F709 IL4Ra mice with a high dose of polyclonal anti-hIgE Abs to trigger mast cell activation through crosslink of FcεRI-bound hIgE (33). CRM_{197}-immunized mice, used as controls, developed severe hypothermia (**Figure 5B**) with 100% mortality within 30 min after anti-hIgE injection (**Figure 5C**), confirming that $hIgE^{KI}$; $hFc\varepsilon RI^{Tg}$; F709 IL4Ra mice have sufficient levels of endogenous hIgE bound to hFcεRI to trigger hIgE-mediated anaphylaxis. By great contrast, after anti-hIgE injection IgE-K vaccinated mice displayed only a transient mild hypothermia and suffered no mortality (**Figure 5B-C**).

Altogether, our results indicate that a vaccine against human IgE Cε3–4 domains can be produced using standard industrial methods, and this vaccine can lead to long-term neutralization of hIgE leading to undetectable IgE levels in circulation and reduced FcεRI-bound hIgE. hIgE-K vaccination does not induce any detectable adverse effects in mice humanized for IgE and FcεRI, even after repeated injections. IgE-K vaccination leads to protection from severe IgE-mediated allergic reactions, even in genetically predisposed allergic

mouse models. These results pave the way for the clinical development of an efficient long-term vaccine against hIgE-mediated allergic disorders.

MATERIAL AND METHODS

Mice. hIgE^{KI} mice were obtained inserting human IgE sequence (1080 base pair, located on human chromosome 14: 106,064,224-106,068,065) on mouse chromosome 12 (Chr12:113,147,778). hIgE^{KI} hFcεRI^{Tg} were generated by intercrossing of hIgE^{KI} and mFcεRI$^{-/-}$ hFcεRI^{Tg} mice (25). hIgE^{KI}; hFcεRI^{Tg}; F709 IL4Ra mice were generated by intercrossing of hIgE^{KI}; hFcεRI^{Tg} with F709 IL4Ra mice (32). Mice were maintained in a specific pathogen–free facility at Institut Pasteur. Mice were bred at Institut Pasteur and demonstrated normal development and breeding patterns. All animal care and experimentation were conducted in compliance with the guidelines and specific approval of the Animal Ethics committee CETEA (Institut Pasteur, Paris, France) registered under #170043, and by the French Ministry of Research.

IgE fragments production. The recombinant hIgE Cε3-4 fragment (containing G335C mutation, with C-terminal Strep Twin tag) was synthesized and transiently transfected into exponentially growing Expi-293 cells that were cultured in Expi293™ Expression Medium (Life Technologies) in suspension at 37°C in a humidified 5% CO_2 incubator on a shaker platform rotating at 110 rpm. Twenty-four hours before transfection, cells were harvested resuspended in Expi293™ Expression Medium at a density of 2 x 10^6 cells/ml, and cultured overnight in the same conditions as mentioned above. Twenty-four hours after, 500 µg of expressing plasmids and 1350 µL of Expifectamine were pre-incubated during 5 min in Opti-MEM (Life Technologies) medium and mixed together. After 20 minutes of incubation, the mixture is added to Expi-293 cells at density of 2.9 x 10^6 cells/mL. Twenty hours after the transfection, 25 mL and 2.5 mL of transfection enhancer 1 and 2 (ThermoFisher) respectively were added. Cells were cultured for 5 days after transfection, supernatants were harvested, centrifuged at 4200 rpm for 30 min and filtered (0.2 μm). Proteins were purified by affinity

chromatography using an AKTA pure FPLC instrument (GE Healthcare) and Strep-Tactin® Column (IBA Lifescience).

Synthesis and characterization of hIgE Kinoid. hIgE Cε3-4 was modified with N-γ-maleimidobutyryl-oxysuccinimide ester (sGMBS; Thermo Fisher), a maleimide-containing agent reacting with primary amines. Buffer of hIgE Cε3-4 was exchanged against modification buffer (70 mM Phosphate buffer, 150 mM NaCl, 5mM EDTA, pH=7,2) at 1 mg/mL. A solution of 10 mM of sGMBS was prepared and added to the hIgE Cε3-4 at a 1:30 ratio and incubated during 60 minutes at room temperature (protected from light). Excess sGMBS was removed and buffer exchanged against modification buffer using Zeba desalting spin column (Thermo Fisher). CRM_{197} was purchased from Pfenex (USA). Sulfhydryl moieties were introduced on the carrier protein CRM_{197} with SATA (N-succinimidyl-S-acetylthioacetate.). CRM_{197} was diluted in modification buffer at 2 mg/mL and a freshly prepared solution of 100 mM SATA (dissolved in DMSO) was added at a 1:80 molar ratio and incubated 30 minutes at room temperature (protected from light). Excess SATA was removed and buffer exchanged against modification buffer using Zeba desalting spin column. SATA modified CRM_{197} was incubated with a solution of hydroxylamine at a 50 mM final concentration, at room temperature for 120 minutes, protected from light. Excess hydroxylamine was removed and buffer exchanged against modification buffer using Zeba desalting spin column. After CRM_{197} and Fcε3-4 IgE functionalization, protein content of each preparation was determined by Bradford (Thermo Fisher) assay according to manufacturer's instructions.

Functionalized CRM_{197} was added to functionalized hIgE Cε3-4 at a molar ratio of 1:1 and a final concentration of 0.4 mg/mL. The mixture was incubated 16 hours at 4°C, protected from light, and subsequently buffer exchanged against modification buffer using Zeba desalting spin

column. Protein content was determined by Bradford assay. Resulting hIgE kinoid (hIgE-K) was then 0.22 µm sterile filtered and stored at 4°C.

The hIgE-K was characterized using different *in vitro* methods. To analyze the profiles of the kinoids obtained, SDS-PAGE and western blot were performed against the hIgE Cε3-4 fragment (Strep-TACTIN HRP conjugate (IBA Lifescience)). Size exclusion (SE)-HPLC using a Bio SEC-5 column (2000 Å, 5 μm, 7.8*300 mm, Agilent) and Bio SEC-3 column (300 Å, 3 μm, 7.8*300 mm, Agilent) was also used. SE-HPLC analysis were performed in the isocratic mode at 1 mL/min with column temperature at 25°C. After filtration (0.22 µm-cut-off), samples were injected at 100 µL and analyzed at 280 nm. The total run time was 35 min.

Production of human IgE antibodies. Anti-nitrophenyl hIgE were produced and purified as described previously (30). JW8/5/13 (ECACC 87080706) cells were obtained from Sigma-Aldrich. This cell line produces a chimeric human IgE antibody directed against the hapten 4-hydroxy-3-nitrophenacetyl (NP), and composed of the human Fcε chain and mouse anti-NP variable chain. JW8/5/13 cells were cultured in complete Dulbecco-modified Eagle medium (DMEM, Gibco) containing 2 mM glutamine (Thermo Fisher Scientific) and 10% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific) at $9x10^5$ cells/ml. After 15 days, supernatants were harvested, centrifuged at 4200 rpm for 30 min and filtered (0.2 μm). We purified IgE antibodies by affinity chromatography. Briefly, CNBr-activated Sepharose 4 Fast Flow Beads (GE Healthcare) were coupled with WT anti-IgE using a ratio of 2.5 mg of protein for each gram of beads. Beads were weighted, washed with 15 volumes of cold 1mM HCl and centrifuged for 5 min at 2500 rpm. WT anti-IgE were resuspended in coupling solution (0.1 M NaHCO3 pH 8.3 containing 0.5M NaCl) and mixed with beads overnight at 4°C under agitation. Beads were washed with coupling buffer and non-reacted groups were blocked with 0.1 M Tris-HCl buffer pH 8.0. WT anti-IgE-coupled beads were then washed using alternate low (0.1 M acetate buffer

pH 3) and high (0.1 M Tris-HCl pH 8) pH solutions and stored in Borate buffer (100 mM Borate, 150 mM NaCl pH 8.0) at 4°C until use. For purification of IgE, WT anti-IgE-coupled sepharose beads were packed in XK 16/20 Column (GE Healthcare) and affinity chromatography was performed using an AKTA pure FPLC instrument (GE Healthcare). After purification, IgE antibodies were desalted with HiTrap Desalting Column (GE Healthcare), and stored at 4°C until use.

Vaccination with hIgE Kinoid. Mice were immunized intramuscularly with kIgE-K combined 1:1 (v:v) with SWE, a squalene-in-water emulsion adjuvant (Vaccine Formulation Laboratory, University of Lausanne, Switzerland) in PBS at day 0, 7 and 28 with two initial doses of 30µg followed by a boost of 10µg. As controls, groups of mice were injected with CRM_{197} following the same schedule with two initial doses of doses of 15µg followed by a boost of 5µg (these doses were defined based on the weight ratio of CRM_{197}) combined with SWE.

Quantification of IgG against human IgE and CRM_{197} in sera from vaccinated mice. The immunogenicity of the kinoid was assessed by evaluating antibodies against human IgE and CRM_{197} in sera collected at different time points after vaccination. Human IgE or CRM_{197} were coated at 4°C at 5 or 1 µg/mL respectively in coating buffer (carbonate/bicarbonate buffer pH 9.6) and incubated overnight. After each step, plates were washed three times with PBS Tween 20 0.005%. After blocking with BSA 1% PBS, serum samples were added, a two-fold serial dilution was conducted starting at 2000 dil^{-1} (diluted in PBS, BSA 1%). After 90 minutes of incubation at 37°C, bound antibodies were detected with HRP-conjugated goat anti mouse IgG (Bethyl Laboratories) at 1/10 000 and plates were revealed using an OPD substrate. Reaction was stopped with 1 M H_2SO_4 and absorbance was subsequently recorded at 490 nm. Samples were analyzed starting at dilution 2000 dil^{-1} up to 1 024 000 dil^{-1}. The titers were defined as the

dilution of the serum where 50% of the OD max. Titers were expressed as serum dilution factors (dil^{-1}). The limit of titer quantification is the lowest dilution tested in the assay: 2000 dil^{-1}.

Assessment of the neutralizing capacities of anti-hIgE antibodies produced upon vaccination with hIgE-K. Bone marrow-derived cultured mast cells (BMCMCs) expressing hFcεRI were obtained by culturing bone marrow cells from $mFc\varepsilon RI^{KO}hFc\varepsilon RI^{Tg}$ mice mice in medium containing IL-3 (10ng/ml) for 6 weeks, at which time cells were >95% c-$Kit^{+}hFc\varepsilon RI\alpha^{+}$ (data not shown). To assess the neutralizing capacity of anti-hIgE antibodies produced upon vaccination with hIgE-K, we incubated BMCMCs with dilutions of plasma from mice vaccinated with hIgE-K or CRM_{197} (as a control). We then added FITC-labeled hIgE (produced as described previously (30), and assess binding of FITC-hIgE to hFcεRI on BCMMCs by flow cytometry.

IgE quantification on the surface of basophils and mast cells. Blood was collected with heparine. For peritoneal lavage fluid (PLF), the outer skin of the peritoneum was gently removed. Then 3 mL of cold PBS was injected into the peritoneal cavity using a 27 g needle. After a gently massage of the peritoneum, an incision was performed in the inner skin of the peritoneum and while holding up the skin with forceps, the PLF was recovered.

Red blood cell lysis was carried out to remove red blood cells. Cells coming from blood were staining with anti CD49b-BV421 (clone DX5, eBioscience), anti CD131-PE (clone REA193, Miltenyi) and with anti-human IgE-biotin (clone MHE-18, Biolegend) and anti-Biotin-APC (clone REA746, Miltenyi) or anti-human FcεRI-APC (clone AER-37 (CRA-1), BioLegend). Cells coming from PLF were stained with anti cKIT-APC (clone 2B8, eBioscience) and with anti-human IgE biotin (clone MHE-18, BioLegend) and anti- Biotin-APC (clone REA746, Miltenyi) or anti-human FcεRI-APC (clone AER-37 (CRA-1), BioLegend). Basophils were

gated as CD49b+ CD131+ and mast cells as cKIT+ IgE+ or FcεRI+. Surface expression of human FcεRI and IgE was assessed and expressed by mean fluorescence intensity (MFI).

Total human and mouse IgE quantification. Total human IgE levels were quantify by ELISA. Anti-Cε2 human IgE antibody (clone 8E/5D4, Aviva Systems Biology) was coated and incubated overnight at 4 °C at 5 µg/mL in coupling buffer (carbonate/bicarbonate buffer pH= 9,6). After each step, plates were washed three times with PBS Tween 20 0,005%. After blocking with BSA 1 % in PBS for 1 h30 at room temperature, serum samples were added at 1/10 final dilution (diluted in PBS, BSA 1 % 10% FBS) and incubated for 90 minutes at room temperature. Then, anti-human IgE antibody (A80-108P, Bethyl Laboratories) were added at 1:10,000 during 90 minutes at room temperature. Plates were revealed using OPD substrate. Reaction was stopped with 2 M H_2SO_4 and absorbance was subsequently recorded at 490 nm. Total mouse IgE levels were quantified by ELISA using a commercial ELISA kit (E90-115; Bethyl Laboratories) according to the manufacturer's instructions.

Passive systemic anaphylaxis. In $hIgE^{KI}$; $hFc\varepsilon RI^{Tg}$ mice, purified mouse IgE anti-NP antibodies were administered intravenously (i.v.) at a dose of 10 μg in 100 μL of PBS. Twenty-four hours later, mice were challenged i.v. with 500 μg of NP (21-31)-BSA (Santa Cruz Biotechnology) in PBS. Rectal measurements of body temperature were performed immediately before (time 0) and at different time points for up to one hour after challenge. In $hIgE^{KI}$; $hFc\varepsilon RI^{Tg}$; F709 IL4Ra mice, rabbit anti-hIgE antibodies (Bethyl Laboratories) were administered i.v. at a dose of 250 µg. Rectal measurements of body temperature were performed immediately before (time 0) and at different time points for up to one hour after the injection.

***Statistical analysis*.** Statistical significance was determined using the unpaired Student's *t* test (unpaired Mann Whitney test). $P \leq 0.05$ was considered statistically significant. Calculations were performed using the Prism 7.0 software program (GraphPad Software).

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FIGURES

Figure 1

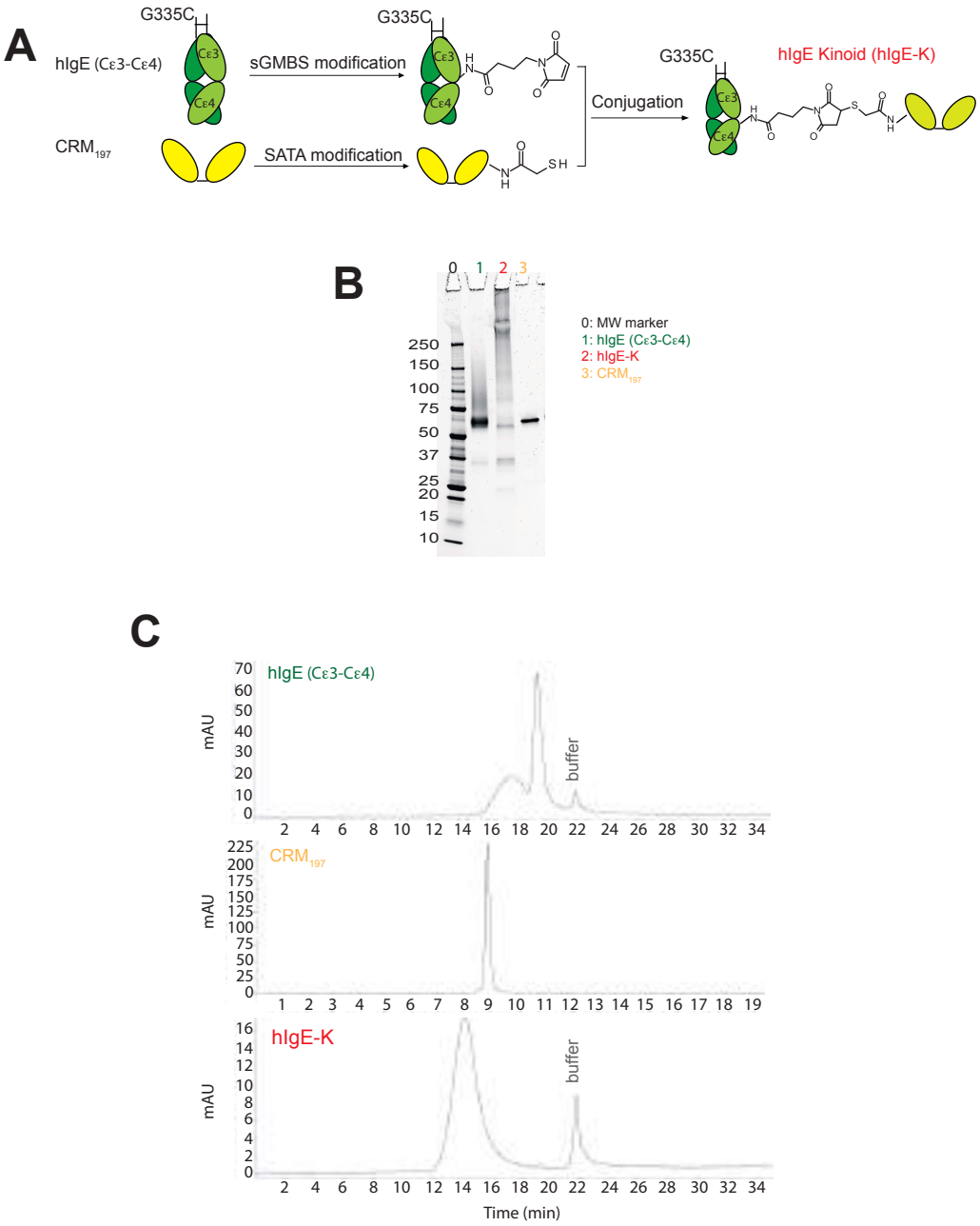

Figure 1. Generation of hIgE Kinoid. (**A**) Synthesis of hIgE-K using a thiol-maleimide conjugation (**B**) Generation of high molecular weight kinoids upon conjugation of IgE to CRM_{197} was confirmed using SDS-PAGE and (**C**) HPLC.

Figure 2

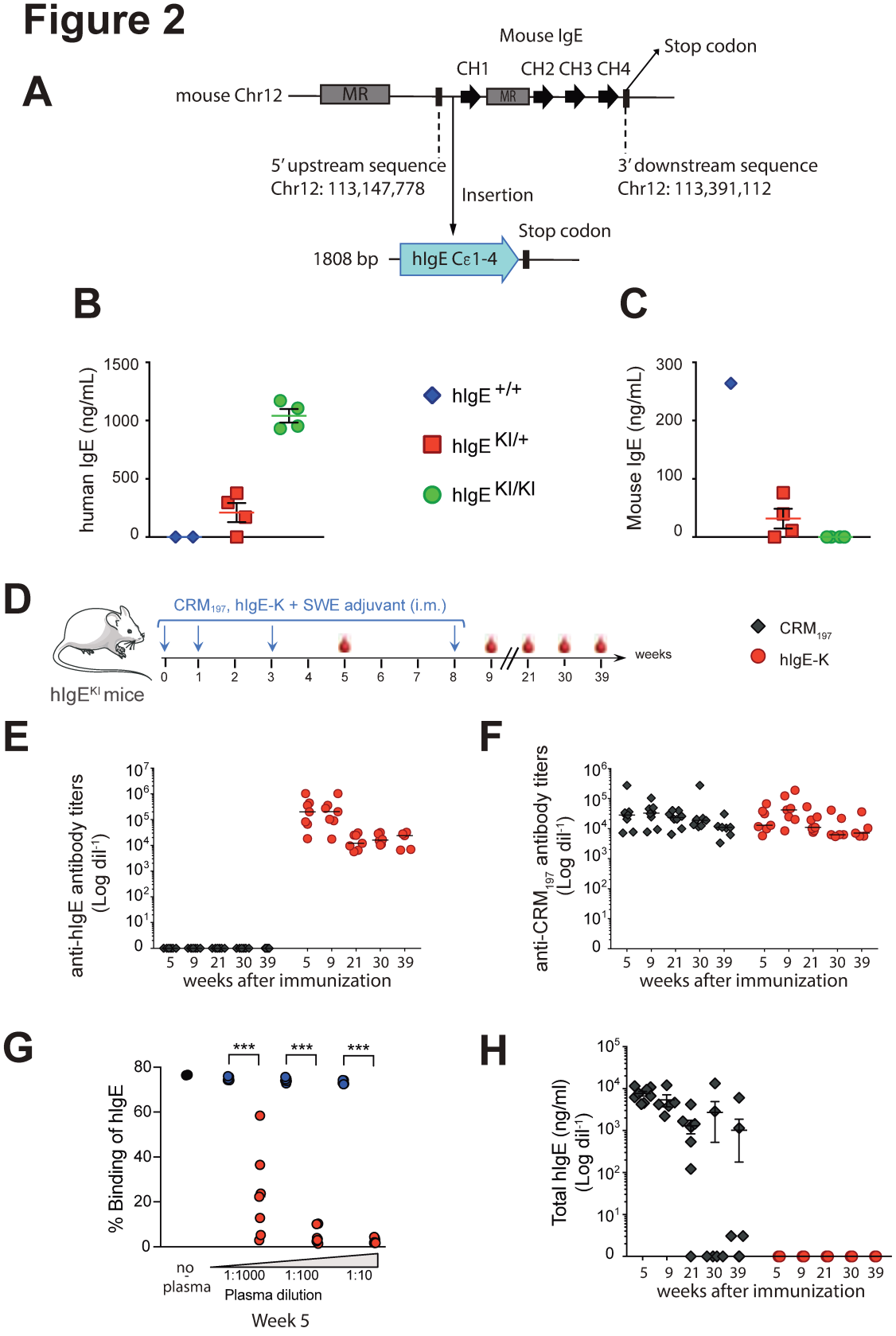
A
Mouse IgE
Stop codon
CH1
CH2 CH3 CH4
mouse Chr12
MR
MR
5' upstream sequence
Chr12: 113,147,778
Insertion
3' downstream sequence
Chr12: 113,391,112
Stop codon
1808 bp
hIgE Cε1-4
B
human IgE (ng/mL)
1500
1000
500
0
hIgE +/+
hIgE KI/+
hIgE KI/KI
C
Mouse IgE (ng/mL)
300
200
100
0
D
CRM197, hIgE-K + SWE adjuvant (i.m.)
hIgEKI mice
0 1 2 3 4 5 6 7 8 9 21 30 39
weeks
CRM197
hIgE-K
E
anti-hIgE antibody titers
(Log dil-1)
5 9 21 30 39 5 9 21 30 39
weeks after immunization
F
anti-CRM197 antibody titers
(Log dil-1)
5 9 21 30 39 5 9 21 30 39
weeks after immunization
G
% Binding of hIgE
***
***
***
no plasma
1:1000
1:100
1:10
Plasma dilution
Week 5
H
Total hIgE (ng/ml)
(Log dil-1)
5 9 21 30 39 5 9 21 30 39
weeks after immunization

Figure 2. hIgE-Kinoid induces neutralizing anti-IgE antibodies. (**A**) Humanization of the IgE gene. Insertion of the constant region of the human hIgE gene (Cε1-4) followed by a stop codon. The total human sequence inserted on mouse chromosome 12 (Chr12:113,147,778) with a total of 1080 base pair (based on human IgE gene on chromosome 14: 106,064,224-106,068,065). MR indicates mouse replication site. Representations are not drawn to scale. (**B**) Circulating human IgE levels and (**C**) circulating mouse IgE levels in sera from mice sensitized with peanut and aluminum salt. Data show values from individual mice with bars indicating mean ± SEM. (**D**) Vaccination protocol outline. $hIgE^{KI}$ mice were vaccinated with hIgE-K (or CRM_{197} as control), emulsified with the adjuvant SWE. (**E**) Anti-IgE (**F**) and anti-CRM_{197} antibody titers in sera 5, 9, 21, 30 and 39 weeks after first injection of kinoid. Results show values from individual mice with bars indicating medians. (**G**) Anti-IgE neutralizing capacity in sera collected at week 5. (**H**) Levels of total IgE in sera collected at week 5, 21, 30 and 39. Results show values from individual mice with bars indicating mean ± SEM. (**E-H**) Data are from a single experiment with n=8 mice per group, representative of two independent experiments. ***, $P < 0.001$ (Mann-Whitney *U* test).

Figure 3

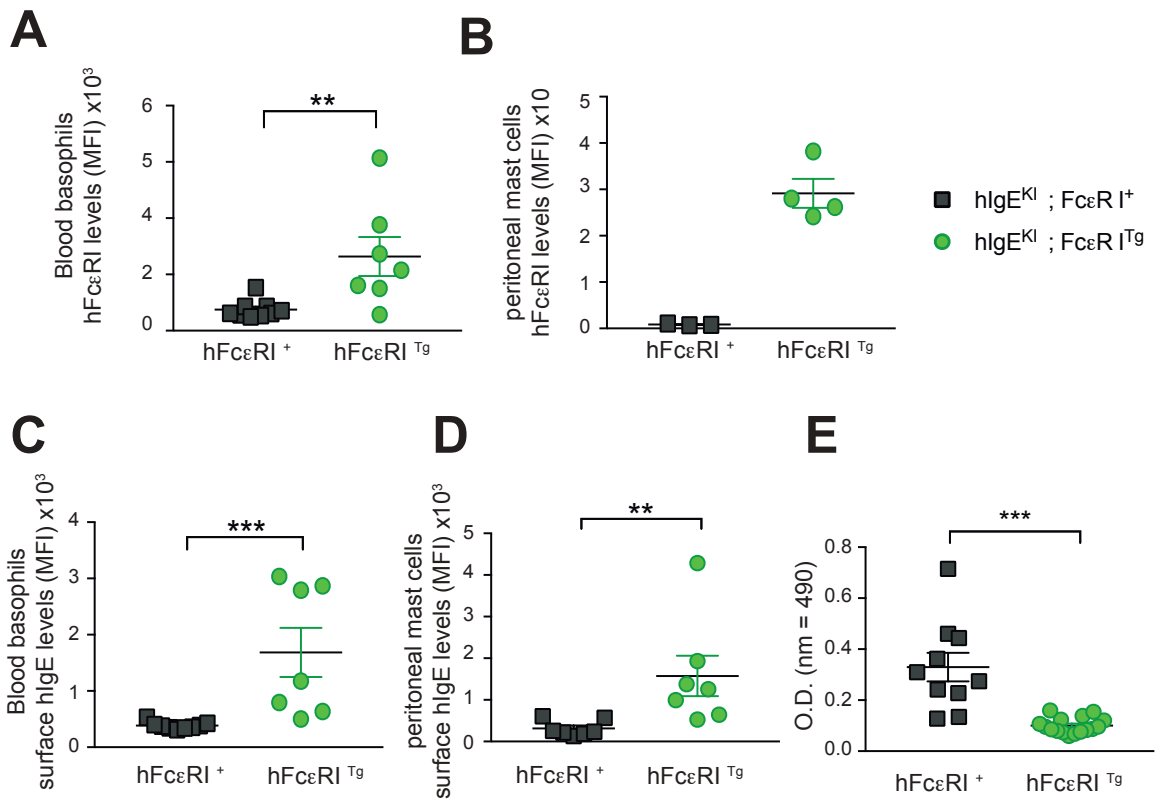

Figure 3. Generation and characterization of IgE/FcεRI humanized mice. (**A**) FcεRI expression on the surface of blood basophils and (**B**) peritoneal mast cells. (**C**) IgE expression on the surface of blood basophils and (**D**) peritoneal mast cells from mice sensitized with the allergen house dust mice (HDM). (**A-D**) Data show values from individual mice (*n*=3-8/group) with bars indicating mean ± SEM, from a single experiment representative of three independent experiments. (**E**) Circulating human IgE levels from mice sensitized with house dust mite (HDM). Data represent mean ± SEM from two independent experiments (*n*=10-16/group). ** or ***, $P < 0.01$, or 0.001 (Mann-Whitney *U* test).

Figure 4

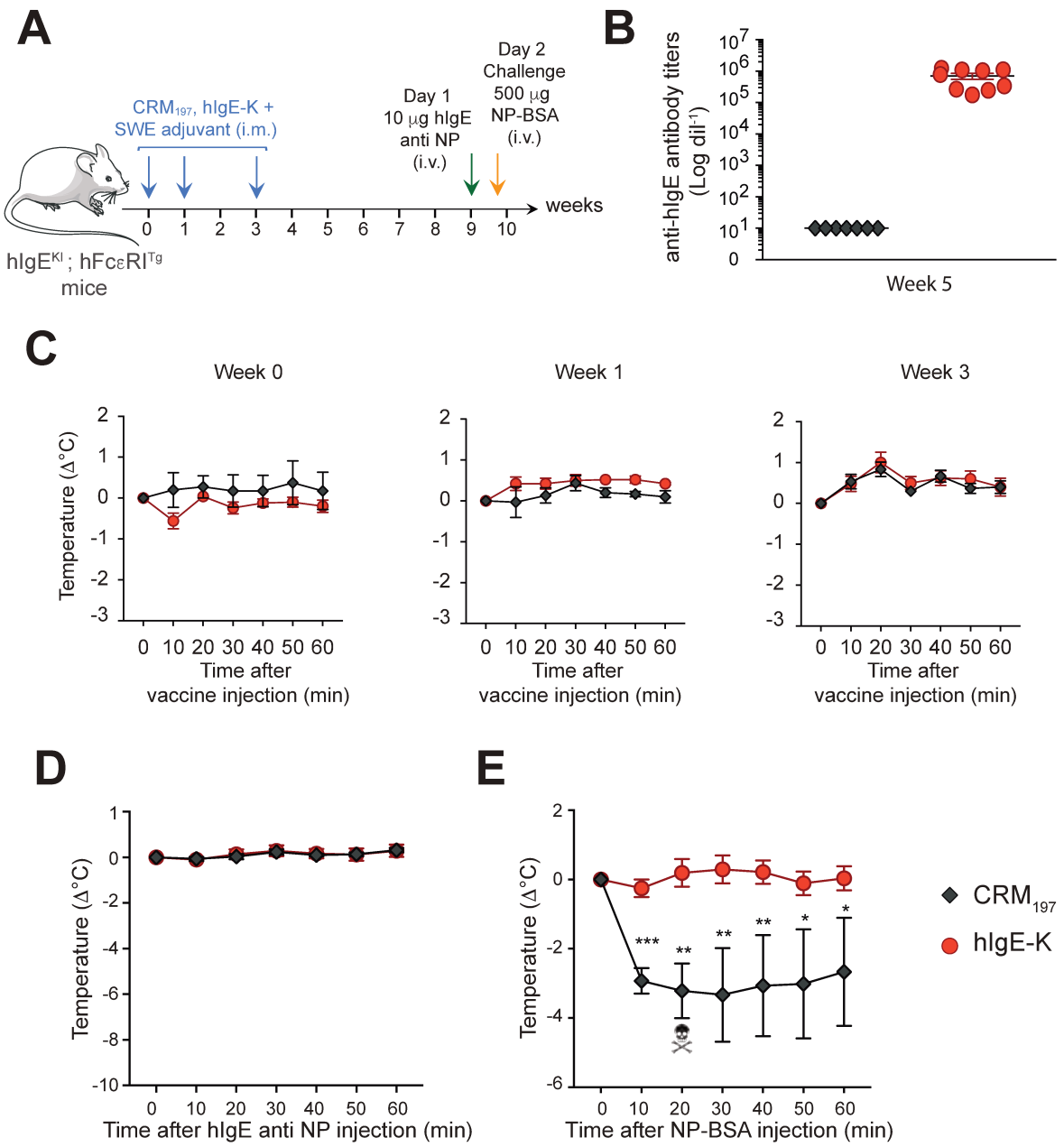
A
CRM197, hIgE-K + SWE adjuvant (i.m.)
Day 1 10 µg hIgE anti NP (i.v.)
Day 2 Challenge 500 µg NP-BSA (i.v.)
weeks
0 1 2 3 4 5 6 7 8 9 10
hIgEKI ; hFcεRITg mice
B
anti-hIgE antibody titers (Log dil-1)
Week 5
C
Week 0
Week 1
Week 3
Temperature (Δ°C)
Time after vaccine injection (min)
D
Temperature (Δ°C)
Time after hIgE anti NP injection (min)
E
Temperature (Δ°C)
Time after NP-BSA injection (min)
CRM197
hIgE-K

Figure 4. Vaccination with hIgE-K prevents IgE-mediated systemic anaphylaxis. (**A**) Protocol outline. $hIgE^{KI}$; $hFc\varepsilon RI^{Tg}$ mice were vaccinated with hIgE-K (or CRM_{197} as control), emulsified with the adjuvant SWE. At week 9, mice were sensitized with hIgE anti-nitrophenyl (NP) and challenged and NP coupled to BSA, as indicated. (**B**) Antibody titers in sera 5 weeks after first injection of kinoid. Results show values from individual mice with bars indicating medians. (**C**) Changes in body temperature (Δ°T, mean ± SEM) after intravenous injection of 10 μg anti-NP hIgE. Data are pooled from two independent experiments with a total of n=7-9 mice per group. (**D**) Changes in body temperature (Δ°T, mean ± SEM) after intravenous injection of 500 μg of NP-BSA. Data are pooled from two independent experiments with a total of n=7-9 mice per group. *, ** or ***, $P < 0.05$, 0.01, or 0.001 (Mann-Whitney *U* test).

Figure 5

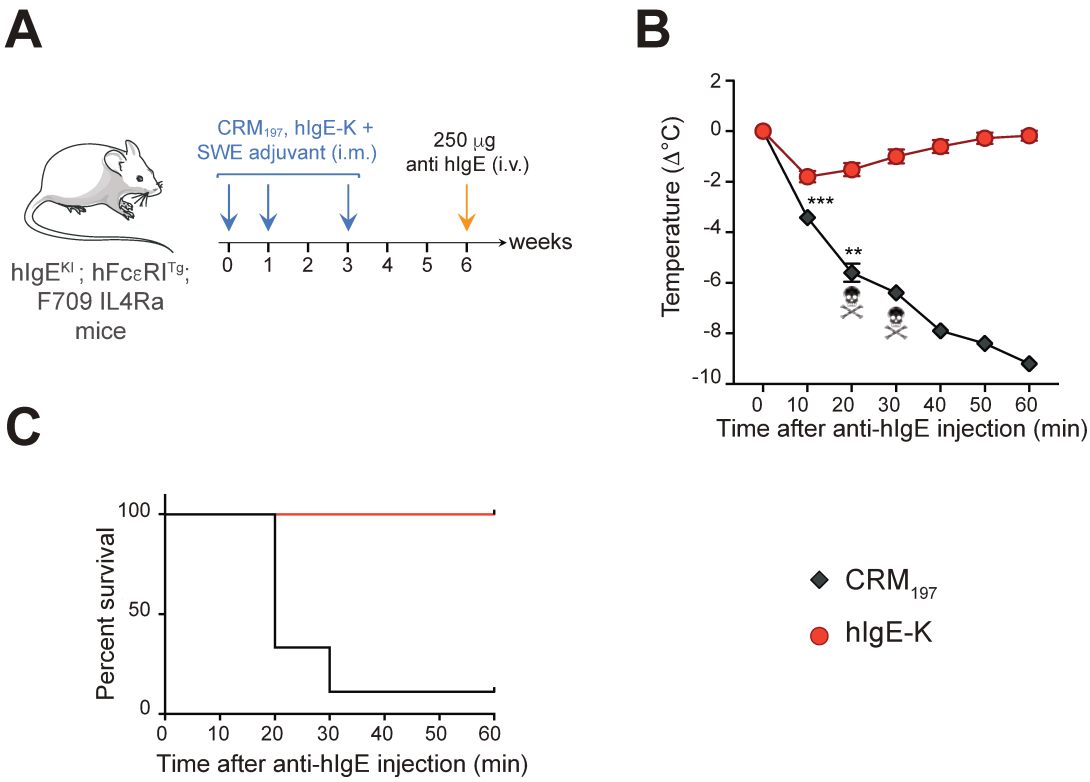

Figure 5. Vaccination with hIgE-K prevents IgE-mediated systemic anaphylaxis in a genetically predisposed allergic mouse model. (**A**) Protocol outline. hIgE^{KI}; hFcεRI^{Tg}; F709 IL4Ra mice were vaccinated with hIgE-K (or CRM_{197} as control), emulsified with the adjuvant SWE. At week 6, mice were injected i.v. with 250 µg of anti-hIgE. (**B**) Changes in body temperature (Δ°T, mean ± SEM) after intravenous injection of 250 µg of anti-hIgE Abs. (**C**) Survival curve after intravenous injection of 250 µg anti-hIgE. Data are pooled from two independent experiments with a total of n=9 mice per group. ** or ***, $P < 0.01$, or 0.001 (Mann-Whitney *U* test).

5. Discussion

This thesis has been focused on the development of a new long-term strategy for allergic disorders treatment. The thesis is divided in two main projects, developing two novel vaccine strategies, either blocking the central type 2 cytokines IL-4 and IL-13 or directly blocking IgE. The discussion chapter will be therefore divided in different sections comparing these different candidates and the results obtained *in vivo*. To do so, I have divided my discussion chapter in different parts: 1) choice of the vaccine strategy, 2) evaluation of the results obtained *in vivo* in terms of efficacy using different mouse models, 3) safety profile evaluation of this therapeutic strategy and I will finish the discussion by opening to 4) future direction for preclinical and clinical development of these vaccine candidates.

5.1.Vaccine strategy: Kinoid technology

Why did we choose IL-4, IL-13 and IgE as targets?

The majority of patients with allergic diseases and asthma, especially with a mild phenotype can achieve disease control by standard controller therapy including corticosteroids (*i.e.* inhaled corticosteroids [ICs] for asthma or topical corticosteroids for rhinitis or atopic dermatitis [AD]), non-sedating H1-antihistamines and SABAs (β2-adrenoceptor agonists) (*282, 439*). Then why go further? Patients with severe uncontrolled asthma have disproportionally high morbidity and healthcare need utilization, and options for these patients have been previously very limited, with unacceptable side effects. Indeed, long term administration of symptomatic treatments such as corticosteroids might cause impaired growth in children or decreased bone mineral density (causing osteoporosis) after years of treatment (*440*). Besides, these treatments are conceived to block symptoms once they appeared. In the case of asthmatic patients for example, it would be more interesting to prevent severe symptoms such as exacerbations than control the exacerbations once they are triggered. Biologic therapies emergence for asthma treatment have thus been considered with growing interest in the scientific community, due to promising results.

The first therapeutic strategy developed was allergen-specific immunotherapy more than one hundred years ago, and clinical results revealed long-term immune modifications in parallel to symptomatic relief (*441*). However, allergen-specific immunotherapy is limited by high adverse reactions rates, and poor long-term efficiency, especially for food allergy, since many patients regain allergy a few years after immunotherapy cessation (*442, 443*).

In the era of monoclonal antibodies, this wider technology has also emerged in the field of allergic disorders as a new strategy to control disease and to impact its natural history, particularly in patients with an endotype driven by Th2 inflammation. The first monoclonal antibody approved for the asthma treatment in the Unites States and European Union was Omalizumab. It has been clinically used for the allergic asthma treatment for more than 15 years and has shown favorable outcomes in several randomized control trials, reducing asthma exacerbations by approximately 25% (*444*). Efforts are still ongoing to better understand specific patient characteristics and identify biomarkers, which would select patients benefitting from the most from Omalizumab treatment. Retrospective analysis suggested a greater reduction in asthma exacerbations for Omalizumab-treated patients with high eosinophil counts and high FeNO levels at baseline (*445*). Because of its key role in type I hypersensitivity reactions, IgE has always been a key downstream biomarker of Th2 cell activation and a prime target for intervention in allergic diseases. Omalizumab success in clinical development might be explained by the target nature. Free IgE represents a biomarker for target engagement, and Omalizumab mode of action is well documented in blocking mast cell and basophil degranulation, resulting in very defined endpoints in clinical studies.

Since then, academic and pharmaceutical laboratories have worked on the development of different monoclonal antibodies targeting diverse molecules. Allergic disorders are complex diseases where an interplay of mediators drive multiple features of potential interest to block. In the case of asthma, researchers and clinicians have worked on the multiple disease phenotypes characterization. This work is extremely important to target specific interesting molecules in each pathological situation. Even then, in the so called “allergic asthma” different targets had emerged, such as IgE but also IL-4, IL-13 or IL-5, with interesting clinical results. While results targeting IL-4 or IL-13 alone have been disappointing, a recent approved monoclonal antibody targeting the common IL-4 and IL-13 receptor, Dupilumab, has been shown to be efficient at controlling allergic asthma. Dupilumab reduces asthma exacerbations, rapidly improves lung function and decreases corticosteroid use for patients with moderate to

severe asthma (*340*). Furthermore, IL-5 play a central role in eosinophil biology, controlling eosinophil maturation, survival and recruitment into the airways. The compelling evidences linking IL-5 to eosinophilic asthma pathology led to IL-5-targeting monoclonal antibodies development. Mepolizumab is now commercialized and shows interesting results reducing asthma exacerbations and corticosteroids dosage. Moreover, a lack of clinical response to Omalizumab does not predict a lack of response to Mepolizumab (*446*).

The majority of biologics clinical trials in patients with uncontrolled severe asthma have demonstrated a significant response to placebo with reductions in exacerbations, improvement in lung function, as well as in patient-reported outcomes. These findings also suggested that "severe asthma" is not intrinsically severe but often poorly controlled. Because no direct comparison between these biologics have been made in the clinics, claims of one biologic superiority over the other are made by indirect comparison and might not be accurate. However, one can speculate that because IL-5 functions are mainly limited to eosinophil biology, it is logical to expect that anti-IL-5 effects would be predominantly seen in patients whose symptoms and disease severity are driven by eosinophils. However, IL-4 and IL-13 roles are more pleiotropic. Therefore, beneficial effects of anti-IL-4 and IL-13 treatment would be expected in a larger patient population, not restricted to those with significant airway eosinophilia. Based on these observations, we thought that it would be more relevant to try to target IL-4 and IL-13 rather than IL-5, as well as IgE.

However, patients suffering from asthma or skin allergies patients show a heterogenous degree of response and there are a substantial number of non-responders in all available biologics. As already introduced, some patients present a Th2 low endotype, characterized by Th17 response and neutrophilia, and do not respond to Th2 pathway blockers. Therefore, we could imagine that this type of patient would not beneficiate from IL-4, IL-13 or IgE blockade. Consequently, not all the patients with asthma or AD would beneficiate from our therapeutic strategy. With Dupilumab and Omalizumab clinical experience, remarkable advances have been already made, but have to be pursued, to characterize patient endotypes trying to link the distinct clinical entities with the molecular events. As shown by other therapies targeting IL-4 and IL-13 that failed in the clinics (*i.e.* AMG317 from Amgen), it is crucial to define patient study population identifying patients with high Th2 endotype. Therefore, only patients with a high Th2 and IgE endotype would probably beneficiate from a treatment using anti-IL-4, -IL-13 or -IgE vaccination.

Product definition

After the definition of our relevant targets, the coupling strategy represents an important challenge in the vaccine development as well as the carrier protein choice. The carrier protein is needed to break B cell tolerance against the self-protein. Traditionally, at NEOVACS SA, the use of keyhole limpet hemocyanin (KLH) has given very satisfactory results in terms of immunogenicity proprieties. However, KLH is a very complex protein of high molecular weight, and final product characterization becomes very challenging. In this project, we decided to study in detail the choice of carrier protein by comparing KLH to CRM_{197}. We wanted to choose a carrier protein already used in vaccinology, in order to take advantage of its safety database. CRM_{197} is a commonly used carrier protein in vaccinology used for the production of pediatric vaccines such as the pneumococcal polysaccharide conjugate vaccine Prevnar 13® or the Haemophilus b conjugate vaccine Hibtiter®. In these cases, the carrier protein is chemically coupled to 13 distinct polysaccharides or to one single oligosaccharide. Studies in BALB/c mice with IL-4 and IL-13 kinoids demonstrated higher antibody production when cytokines were coupled with CRM_{197} compared to KLH (**Figure 7**).

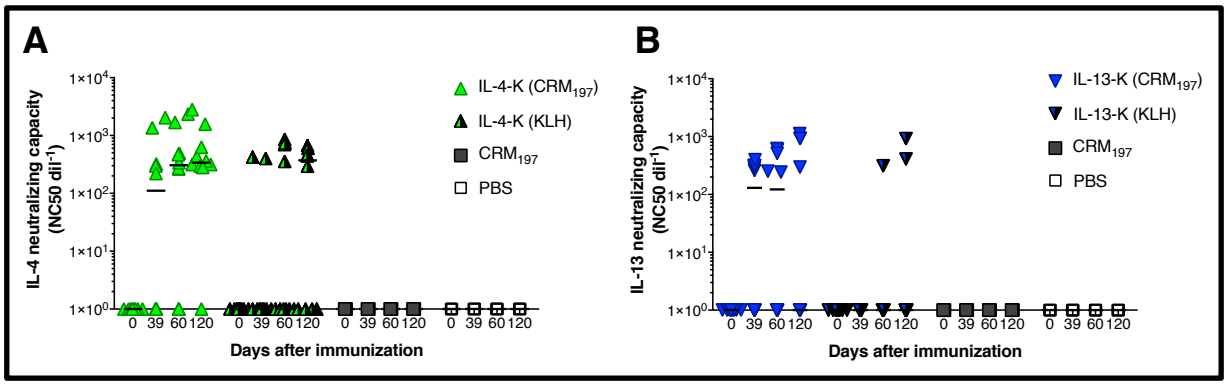

Figure 7. Immunogenicity study using different carrier proteins*. (A) Anti IL-4 neutralizing capacity and (B) anti IL-13 neutralizing capacity in sera from mice immunized with IL-4-K or IL-13-K respectively prepared with CRM_{197} or KLH as carrier protein. Intramuscular injections were done at days 0, 7, 28 and 48 in emulsion with SWE adjuvant. Two control groups were included where mice were immunized with CRM_{197} in emulsion (1:1) with SWE or PBS, following the same immunization schedule. 10 mice/group or 5 mice/control groups.*

In parallel, we tested different conjugation strategies to crosslink both proteins of interest. The most important characteristic of a crosslinker is its reactive chemical group, which guides the chemical modification method and mechanism. Crosslinkers can be classified as homofunctional or heterofunctional, containing at least two reactive groups which target common functional groups found in biomolecules, such as amines or thiols groups. Both primary amines and sulfhydryl groups are known to be significant reactive groups that do not

need a drastic denaturing process. Thus, we focused our efforts on the development of protocols using these chemical functions during kinoids manufacturing. Classical glutaraldehyde protocol has been used at NEOVACS SA for other kinoid programs. Such non-directed chemistry presents several disadvantages, as the resulting kinoid is usually composed by a multitude of entities, including homo and hetero-polymers making product characterization very challenging. In opposition, GMBS / SATA chemistry emerged as a more suitable and direct strategy. This two-step chemistry is based on a stable thioether bond formation between a maleimide and a sulfhydryl group (introduced by protein modification with N-succinimidyl-S-acetylthioacetate (SATA) and N-[γ-maleimidobutyryloxy]-succinimide ester (sGMBS) respectively). In order to guarantee a similar immune response, the manufacturing process reproducibility has to be ensured and the conjugation protocol was in-depth evaluated (pH, ratios, buffers).

Several *in vitro* methods were developed in order to characterize and compare our different candidates. First, we analyzed the conjugation level, guaranteeing heterocomplexes formation between self-protein and carrier protein, necessary to induce an efficient antibody response. In addition, presence of non-coupled free self-protein (cytokine or IgE) was carefully followed. It was important to confirm that no free protein with biological activity was still present in our vaccine which represent a risk for patient. At NEOVACS SA, different methods were developed to follow these parameters, including high exclusion chromatography, western blots, ELISA and bioactivity tests. In addition, we evaluated preserved epitopes presence after modification and conjugation steps. This parameter was assessed by antigenicity assays. While conjugation between cytokine and carrier protein is desired, we also want to maintain native structure of the protein complex avoiding epitope loss. A balance between the modification degree of proteins to allow efficient coupling, and epitope preservation is desired. By this approach, safety should be maintained while antibodies capable of recognizing endogenous proteins overexpressed during the disease should be induced.

Adjuvants are also largely studied in vaccinology increasing the immunogenicity of the product. The ideal adjuvant should maximize vaccine immunogenicity without compromising tolerability or safety. After almost a century, aluminum salts are still one of the most common adjuvants in human vaccines (*447, 448*). This reflects the fact that aluminum adjuvants are extremely effective at enhancing antibody response and are well tolerated. Surprisingly, our

experiments with kinoids injected with aluminum salts showed mitigated effects from this adjuvant. Despite being done at NEOVACS SA with kinoids targeting a different protein (*i.e.* IFN-α) than IL-4, IL-13 or IgE, we could speculate that contrary to traditional vaccines targeting bacterial or viral compounds, kinoid vaccinology requires a strong or at least, a different immune response to break the tolerance against self-proteins.

Emulsions have long been employed as vaccine adjuvants, but were only approved for their human use in the nineties. This can be explained because mineral oils used in the first generation adjuvanted vaccines were not metabolizable and, despite being strong potentiators of the antibody response, their efficacy/safety ratio was not favorable. Development of emulsions such as Montanide ISA™ 51 or squalene-based adjuvants (SWE) using fully metabolizable oils resolved this issue. Based on prior vaccine development at NEOVACS SA, we have thus elected to use SWE as adjuvant for immunogenicity studies in mice with IL-4-K, IL-13-K and hIgE-K. Importantly, MF59®, another squalene based adjuvant commercialized by Novartis and added to the FLUAD flu vaccine, is nowadays used in a panoply of different vaccines, proposed to diverse age populations, such as adults, children even infants at birth and pregnant women and the benefit and safety profiles are now very well established (*449, 450*).

Nevertheless, our results showed an IgE increase after CRM_{197} injection in emulsion with our SWE adjuvant. We studied the specificity of these antibodies and found that they were not specific to CRM_{197}. Results suggested that the increase could be triggered by the adjuvant injection (**Figure 8**), even if additional experiments including groups injected with CRM_{197} alone or SWE alone are required to conclude on that. Squalene-based adjuvants are known to be adjuvants with a "Th2 profile" (*451*).

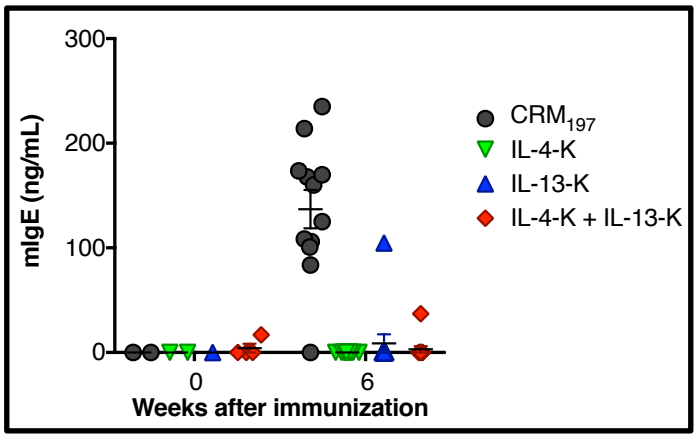

Figure 8. Circulating IgE levels. *Mouse IgE levels after vaccination with CRM197, IL-4-K, IL-13-K or IL-4+IL-13-K, emulsified with SWE (1:1). Mice were intramuscularly injected at days 0, 7, 28. Blood was collected at week 6 after the first injection. Data show values from individual mice with bars indicating mean ± SEM, from a singles experiment representative of three independent experiments.*

Squalene-based adjuvants have been widely used in flu vaccine, since the antibody response is stronger compared with the unadjuvanted vaccine. Conversely, this unspecific increase of IgE has been also been observed by others (*452*). In this study, they compared the safety profile of squalene-based adjuvant with a novel vegetable oil-in-water emulsion adjuvant and showed that vaccination with squalene-based adjuvant induced a significant higher total serum IgE levels. Moreover, an increased rate of allergic reactions was reported in Canada associated with adjuvant system 03 (AS03), a squalene-based adjuvant commercialized by GSK and used in the H1N1 influenza 1 2009 pandemic vaccine (H1N1pdm09) (*453*). The reported rate of all adverse events following immunization with the monovalent adjuvanted H1N1pdm09 vaccine was 50 per 100,000, which was 2.6 times higher compared with the previous six years (19/100,000) when non-adjuvanted trivalent inactivated influenza vaccine (TIV) was used. Symptoms compatible with allergy, such as urticaria and skin rashes, followed the general trend with a reporting rate 3.3-fold greater after H1N1pdm09 vaccination than with previous seasonal TIV. Noteworthy, anaphylaxis was reported 20 times more frequently than in the previous six years, during which 4 cases were passively reported among more than 10.4 million doses of TIV distributed (0.4/million doses). Although the adjuvant was initially considered as the trigger of these adverse events, it was not possible to prove that this reaction was directly due to the adjuvant and adverse events observed in association with AS03-adjuvanted pandemic H1N1 vaccine remain mostly unexplained despite extensive risk factor review (*454*).

Starting from 2016-2017, the influenza vaccine is now emulsified with a new squalene-based adjuvant marketed by Novartis (MF59®). Occurrence of allergic-type responses, such as urticarial rash, allergic bronchospasm, or systemic anaphylaxis, is extremely rare (*449*). These observations lead to think that it is still possible to use a squalene-based adjuvant if it provides an increased benefit in terms of efficacy.

Further studies with IL-4, IL-13 and IgE kinoids are required to confirm results obtained with IFN-kinoid and to confirm that despite the observed IgE increase, SWE emulsions are still the best adjuvant choice in terms of efficacy and safety. Of note, such increase in IgE levels were not observed in mice vaccinated with IL-4-K and/or IL-13-K or hIgE-K. However, the earliest time-point assessed is 39 days after the first injection, a time-point at which anti-IL-4/13 or anti-IgE neutralizing antibodies already have reduced IgE levels. It would be interesting to assess IgE responses at early time-points in vaccinated mice to 1) identify whether early

increase in IgE occurs, and 2) better characterize the speed at which these vaccines can induce neutralizing antibodies which then decrease IgE levels.

Kinoid strategy vs. monoclonal antibodies

As introduced, we are here developing a new therapeutic strategy that tries to circumvent problems encountered by therapies using monoclonal antibodies (mAbs). mAbs against IL-4Rα chain (common chain shared by IL-4 and IL-13 receptor) and IgE have validated these proteins as therapeutic targets. However, very high costs and potential side effects (due to the need of bolus injections of up to 300 mg of the monoclonal antibody) are still limiting the use of these drugs to a minority of patients.

Even if the development of vaccine candidates presented in this thesis are still in the preclinical development, we can conjecture about potential advantages of this technology compared with mAbs. Interestingly, one of the biggest pros of kinoid technology is the long-term response induction. In mice, we observed neutralizing antibodies even one year after first immunization. The treatment would thus be more convenient for patients since less injections would be required to manage the pathology. Based on clinical data obtained with IFN-K, we are expecting that patients would require between 4 and 5 injections in the beginning of the treatment, and eventually a boost every year (*388-390*). This immunization schedule would represent a clear benefit compared with patients treated with monoclonal antibodies, who have to attend to the hospital once or twice a week for their injections.

Additionally, as opposed to passive immunization with mAbs where development of anti-drug antibodies can induce treatment failure (as reported for adalimumab (*455*) or infliximab (*456*)), patients treated with kinoids will produce their own therapeutic antibodies. The kinoid approach has the advantage to generate self-antibodies that will be well-tolerated because of the absence of xenogenic epitopes.

Because it directly impacts treatment efficacy, drug bioavailability is evaluated after mAbs injections. This challenge has raised the question of the administration route. Studies have been conducted in order to find the best administration strategy for monoclonal antibodies in the treatment of asthma, in terms of efficacy and convenience (*457*). They are often administrated by subcutaneous (s.c.) (*e.g.* Dupilumab and Omalizumab) or intravenous (i.v.) routes.

Interestingly, clinical trials analyzing anti–IL-13 monoclonal antibodies have yielded mixed results. As an explanation, administration route has been considered. In order to explore new administration routes, Lightwood *et al*. reported preliminary efficacy of an inhaled anti-IL-13 monoclonal antibody in macaques by using vibrating membrane nebulizer (*458*). Inhaled anti–IL-13 therapy approach could potentially offer several advantages over systemic administration; delivering a high dose directly to the target organ, improving efficacy while also limiting systemic exposure and any associated side effects. Interestingly, studies have revealed that larger amount of IL-13 are released by Th2 cells in lung effector sites, whereas IL-4 is mainly produced by T follicular helper cells in the lymph nodes (*196*). Then, the therapeutic strategy needs to neutralize the cytokine in the distinct sites where we observe its overproduction, endorsing the inhaled route as an interesting route to consider. In order to overcome this constraint, vaccine strategy could be an interesting approach. The therapeutic action of vaccination-induced antibodies might rely on their capability to localize the immune response to inflammation sites.

Finally, therapies using monoclonal antibodies in allergies and autoimmune diseases are more commonly used as therapeutic solutions rather than in a preventive setting. Interestingly, apart from the development as a therapeutic vaccine, we could also think about the possibility of the development as a prophylactic vaccine. As introduced, during childhood, allergic diseases can evolve from eczema, rhinitis and progress to asthma, in what is called 'atopic march'. We could propose a vaccination in those cases, avoiding the disease to evolve to more serious asthma forms. Finally, and considering that some cases of asthma have a high genetic component, it could also be administered in healthy children who are at high risk of developing allergic disease.

Recombinant protein-based vaccine vs. peptide-based vaccines

Traditionally, NEOVACS SA has defined its kinoids as vaccines where the entire cytokine is coupled to a carrier protein. An alternative to the recombinant protein-based vaccine is the use of immunogens composed of small cytokines peptides coupled to the carrier protein. This new strategy present potential advantages in terms of safety. In the specific case of an IgE vaccine, it is not desirable to use whole IgE as vaccine antigen, since induced antibodies could bind to receptor-bound IgE resulting in cross-linking and subsequent anaphylaxis. The use of shorter peptides reduces this risk. However, such peptides have to be carefully selected to be able to

induce a response capable of neutralizing the entire endogenous protein that is overexpressed in the pathology.

A number of peptides have to be rationally designed, using structural information of the whole protein. Indeed, a peptide-based IgE vaccine was developed by Pfizer. Two vaccine candidates were designed using short peptides (16 and 20 amino acids): one peptide corresponding to a potential sequence recognized by Omalizumab in Cε3, the other one likely corresponding to part of the FcεRI-binding site. A first study in cynomolgus monkeys gave very modest results with both vaccine candidates: IgE levels were only partially inhibited, and inhibition degree was much less than what is expected with Omalizumab (*373*). Nevertheless, a phase I study was conducted in patients with allergic rhinitis (NCT01723254). While the two vaccine candidates were well tolerated, little or no lowering of serum IgE was observed in most patients, and vaccine development has therefore been discontinued. Our analysis of the recently described Omalizumab-bound IgE crystal structure revealed that Omalizumab epitope is discontinuous. It is formed by residues belonging to 3 stretches spanning the entire IgE Cε3 domain: 376-380 (loop), 414-419 (beta-strand) and 426-427 (loop) (**Figure 9**). The FcεRI binding domain is mainly located in the Cε3 domain. We could hypothesize that unsatisfactory results obtained with the vaccine from Pfizer were due to peptide selection. We think that by using the entire Cε3 domain, rather that only small peptides from the domain would greatly favor "Omalizumab-like" neutralizing antibodies generation.

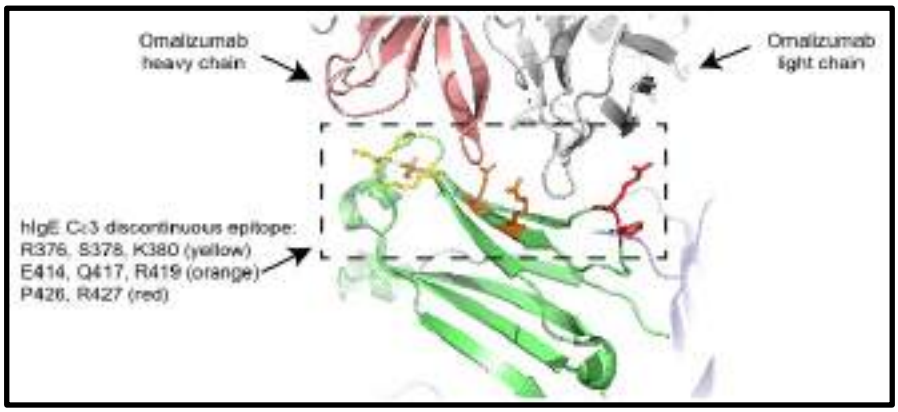

Figure 9*. *The IgE Cε3 epitope for Omalizumab *(analysis of the crystal structure PDB 5HYS)*

Compared with Pfizer strategy, the counterpart of our strategy relies on the safety profile. In comparing the benefits, we should always consider both efficacy as well as safety of the

designed peptide. Design of a closed peptide using the mutation described by Wurtzburg *et al*. seems to be a reliable strategy to overcome this challenge (*262*). Our preliminary *in vivo* results showed no signs of anaphylaxis after vaccine injection, suggesting that the fragment is not able to trigger mast cells and basophils activation. While our data do not suggest major safety concern, the future vaccine development should emphasize safety characterization.

Concerning the development of IL-13 targeting vaccines, a recombinant mouse IL-13 peptide-based virus-like particle vaccine has been developed with significant effects on mucus production without, however, affecting IgE levels. In addition, only partial reduction of AHR has been shown in an acute OVA-induced mouse model of asthma (*368, 459*). Even if blocking IL-13 has not been shown to be the best strategy for blocking IgE production, our IL-13-K candidate has shown partial efficacy for this feature. Interestingly, our IL-13-K effect in controlling AHR increase seems to be superior than the peptide approach, as IL-13-K completely abrogates AHR in HDM-challenged mice. Collectively, even if an accurate comparison of both candidates remains difficult, we can consider that using the whole IL-13 protein as antigen presents a benefit over their peptide-based vaccine in terms of efficacy.

The strategy choice adopted for the development of the vaccine remains critical and is subjected to each singular target, which has its particularities, with consequences in terms of efficacy and safety.

5.2. Evaluation of kinoids efficacy in mouse models

IL-4 and IL-13 kinoids efficacy

In order to investigate and compare IL-4 and IL-13 kinoids efficacy, we produced mouse kinoids in order to test these vaccine candidates in a relevant model. First, we aimed to develop a mouse model of allergic asthma. We adapted a model described by Sibilano *et al*. (*404*). Compared with other acute models, this HDM-induced asthma model enables us to test the different candidates in a clinically relevant model. Indeed, this model reproduces key features of chronic asthma including AHR, airflow obstruction, airway wall remodeling, mucus production and inflammatory responses in the lung, characterized by a marked eosinophil count increase. Moreover, while the widely used allergen OVA is not relevant in clinics, HDM are a

major source of allergens, affecting more than 50% of allergic patients (*460, 461*). Mice are not naturally allergic, but BALB/c mice develop a marked allergic Th2 response, dependent on IL-4 and IL-13, after repeated intranasal HDM challenges, without using any adjuvant (*462*). We followed main readouts: levels of HDM-specific antibodies in serum, levels of total IgE in serum and on the surface of basophils and mast cells, AHR to inhaled methacholine (a bronchoconstrictor used as part of asthma diagnostic in human (*463*)), airway and lung inflammation and mucus production. First, in a prophylactic approach, we compared effects observed after IL-4 and IL-13 blockade alone or in combination. Our results showed that IL-4-K is more effective at blocking IgE production, which was detected at the circulating level but also on basophils and mast cells surface. In opposition, IL-13-K was more effective in blocking AHR after methacholine challenge and mucus production. Finally, IL-4-K and IL-13-K combination was necessary to block inflammation in the airways and lungs, as observed by an important decrease of eosinophil count. Collectively, our results showed that in our mouse model, dual IL-4/IL-13 kinoid prophylactic vaccination prevented or strongly reduced all key features of HDM-induced asthma, whereas single IL-4-K or IL-13-K vaccination affected only a subset of features. These results suggested that IL-4 and IL-13 contribute to disease pathology by driving distinct and overlapping effects. Our results confirm what has also been observed by other research groups in mice (*464*), and could explain the clinical superiority of Dupilumab versus mAbs targeting IL-4 or IL-13 alone in asthma (*50, 328, 340, 465*).

In light with the results obtained using the prophylactic model, we assessed therapeutic vaccination efficacy only using dual IL-4/IL-13 kinoid vaccination on mice with established asthma (confirmed by an increased AHR to inhaled methacholine and eosinophilia 24h after the third allergen challenge). Mice remained exposed to HDM once a week and were injected in parallel with kinoid. Indeed, dual therapeutic vaccination demonstrated a profound reduction in key asthma features, reducing total and HDM-specific IgE levels, AHR to inhaled methacholine, airway eosinophilia, and mucus production. We could observe an interesting correlation between anti- IL-13 neutralizing antibody levels and resistance value at the highest dose of inhaled methacholine (100 mg/mL) recorded in plethysmography experiment (**Figure 10**), indicating that neutralizing antibodies are somewhat associated with mice protection. By great contract, this correlation was not observed with anti-IL-4 neutralizing antibody levels. This supports our observation that IL-13-K was more efficient at reducing AHR than IL-4-K.

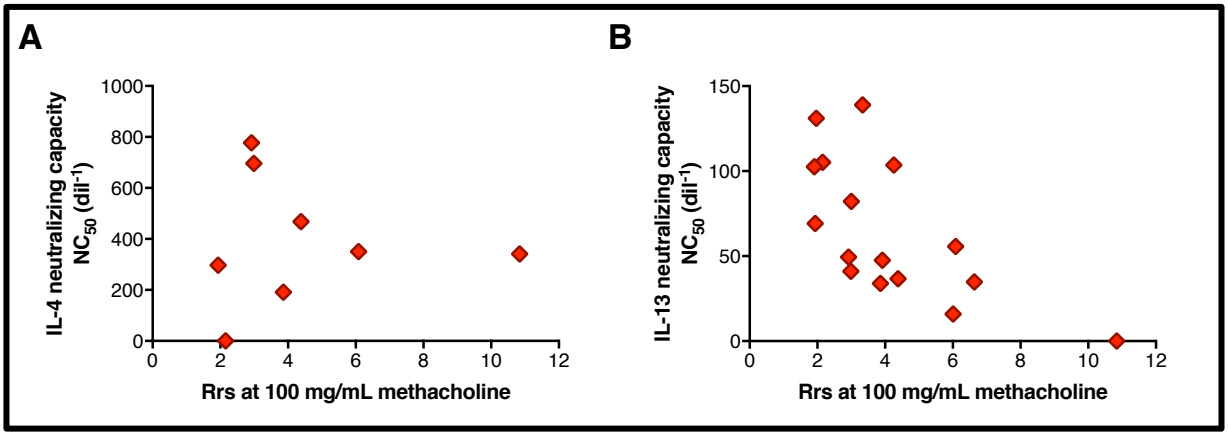

Figure 10. Correlation between neutralizing antibodies and AHR*. Correlation between anti-IL-4 (A) and anti-IL-13 (B) neutralizing antibodies and resistance value recorded after challenge with inhaled methacholine at the highest dose (100 mg/mL).*

The allergic response in CRM_{197} vaccinated and HDM-challenged mice showed differences in terms of response intensity between prophylactic and therapeutic models. Indeed, Th2 response was decreased in the therapeutic model, including a decrease of HDM-specific IgE, AHR, eosinophil numbers and mucus production score. This observation can suggest possible mechanisms of immune regulation and tolerance (*392*). As mentioned in the introduction, OVA delivered via inhalation without a systemic-adjuvanted sensitization can trigger tolerance (*401*). Although HDM has been more successful in asthma mouse models, alterations in the protocol (challenges are less frequent but more numerous) can explain the differences observed between prophylactic and therapeutic models. Such observations may rely on differences in Th2 signature and consequently in the IL-4 and IL-13 model dependency.

Interestingly, while vaccination decrease eosinophilia in the airways, a slight (but not significant) neutrophil increase in BAL from kinoid-vaccinated mice was observed in the therapeutic model (**Figure 11**). Our data suggest a possible switch in the airway inflammation nature. This data is consistent with some studies highlighting the crosstalk between Th2 and Th17 pathway (*466*). The fact that targeting Th2 cytokines might promote corticosteroid-resistant IL-17 dependent neutrophilic airway inflammation was proposed as a mechanism for this effect. In their HDM-induced asthma mouse model, authors showed a strong Th2 stimulus which was also accompanied by an IL-17 induction. However, although IL-4/IL-13 blockage mitigated a wide array of pathological consequences to HDM exposure, these same interventions enhanced IL-17 expression, IL-17 dependent chemokine/cytokine expression

(including a TNF-α increase) and lung neutrophilia. These observations raised the possibility that Th2-targeted treatment may contribute to adverse Th17 permissive environment emergence, limiting therapeutic efficacy over time. In light with these observations, a clinical case report described alopecia in patients shortly after beginning Dupilumab therapy for AD (*467*). Th1 and Th17 pathways play a role in alopecia areata pathogenesis (*468*). Thus, authors proposed that IL-4/IL-13 signaling blockade after Dupilumab treatment can trigger Th1/T17 upregulation. These highlights the importance of understanding Th2 and Th17 responses regulation and interplay for developing and optimizing therapeutic intervention strategies.

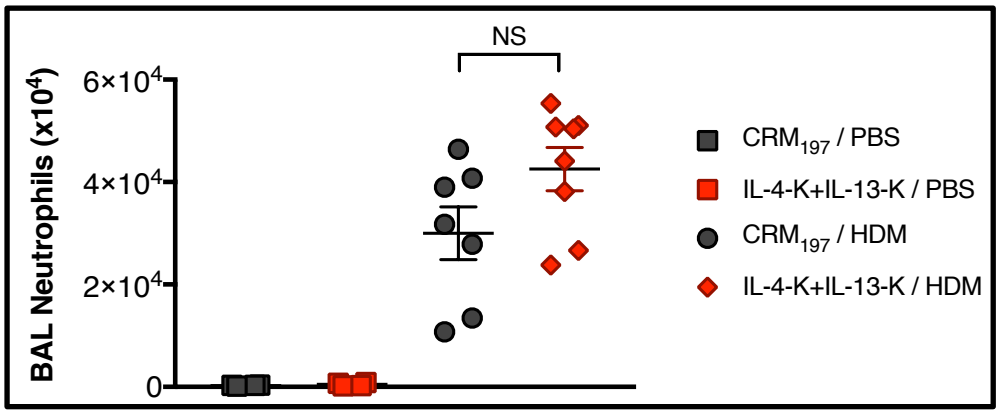

Figure 11. Neutrophil numbers in BAL fluid 24 h after the last HDM challenge*. Data show values from individual mice (n=8/group) with bars indicating mean ± SEM, from a single experiment representative of two independent experiments. NS: no significant (Mann-Whitney U test).*

Finally, as an attempt to better model the human situation with our mouse models, we decided to use mice humanized for IL-4, IL-13 and their common chain receptor IL-4Rα. Low interspecies similarity for IL-4 (~44 %) and IL-13 (~55 %) between mice and men were shown. In order to perform preclinical studies in a relevant model enabling to demonstrate the proof of concept, we decided to start with mouse kinoids development. However, it is crucial to test human vaccines upstream clinical steps. Certainly, in-depth toxicology studies are required before clinical studies (see section 5.4. Future steps in the vaccine development), but human vaccine study in humanized mouse models is a very valuable and interesting strategy at preclinical stages. We had access to these mice thanks to a collaboration with Dr. Beverly Koller (University of North Carolina, USA). Using human kinoids, we were also able to break B cells tolerance against human cytokines in this humanized model. We confirmed human cytokines neutralization efficacy by a decrease in circulating and surface bound IgE levels. Since hIL-4^{KI}, hIL-13^{KI}, hIL-4Rα^{KI} mice are not in a BALB/c background (129S6/ taconic strain background), we are now confirming that it is still possible to induce asthma or other allergy

models in this genetic background. These mice then give the opportunity to test human kinoids efficacy.

IgE-K efficacy

Considering that kinoids have to be tested in relevant models and to avoid mouse kinoid development, we here decided to directly use a humanized system. For this purpose, my laboratory has developed IgE humanized mice in which the Fc portion of human IgE Cε1-4 is knocked-in into the mouse gene encoding Cε1-4. We crossed $hIgE^{KI}$ mice with $hFceRI^{Tg}$ mice in order to obtain $hIgE^{KI}$ $hFceRI^{Tg}$ mice which reproduce production and responsiveness to human IgE as described in humans.

We monitored circulating human and mouse IgE levels, as well as on the surface of mast cells and basophils. While mouse IgE was no longer detected, human chimeric IgE (hIgE) levels detected by ELISA and by flow cytometry on the surface of basophils and mast cells were very low. Interestingly, we compared circulating hIgE in mice expressing or not the human FcεRI and found an increased hIgE signal in mice that did not express the receptor. These results suggest that in our mouse model, FcεRI probably contributes to serum IgE clearance. In order to fully characterize them, mice were HDM-sensitized and challenged. As expected, upon sensitization, mice showed an increase of circulating hIgE levels and on the surface of basophils. However, hIgE levels remain globally low even after sensitization with HDM. Because human IgE is not recognized by mouse CD23 (*437*), we can consider that our $hIgE^{KI}$ $hFc\varepsilon RI^{Tg}$ mouse model is CD23 deficient. Studies using CD23 deficient mice reported an increased antigen specific IgE production (*248*). It is thus somehow surprising to observe low hIgE levels in our humanized mice. However, additional hypotheses have been published which might explain the reason of such low IgE levels: in human and in our humanized mice, hFcεRI is expressed as αγγ trimer on DCs and macrophages (*433*). Studies by the group of J.P. Kinet revealed that this trimer was able to promote IgE endocytosis and subsequent degradation, thereby playing a key role in IgE levels regulation (*243*). Whatever the reason, this observation must be taken in account, especially when analyzing the vaccine safety profile (see below in the safety discussion). In order to increase IgE levels, further work was conducted crossing IgE/FcεRI humanized mice with mice expressing a mutant IL-4Rα form (F709) (*415, 469, 470*). These mice have a gain-of-function mutation in the ITIM motif in IL-4/IL-13 receptor cytoplasmic domain and are incapable of negative signaling. These Th2 biased mice are

particularly susceptible to IgE-mediated allergic reactions (*415*) and could be an interesting tool to increase IgE levels in IgE/FcεRI humanized mice.

Although the potential limitations observed, the availability of this unique humanized IgE/FcεRI model allows us to directly test human IgE kinoids *in vivo*. In paper II, we showed that vaccinated mice displayed high titers of anti-human IgE neutralizing antibodies, and decreased hIgE levels in sera and on cell surface. Of note, we do not know at this point if such observed decrease in IgE levels reflects an actual reduction in IgE protein, or because detection of hIgE by ELISA or flow cytometry is masked by the fact that endogenous hIgE are covered by polyclonal anti-hIgE antibodies induced by the vaccine. But in all cases, this reflects inactivation of endogenous hIgE in IgE/FcεRI humanized mice vaccinated with hIgE-K. Remarkably, IgE-K vaccinated mice were completely protected from an IgE-dependent passive anaphylaxis, highlighting beneficial proprieties of this candidate vaccine in IgE-mediated diseases. The choice of an appropriate mouse model is a key point for a proof of concept success.

We started the project trying to develop an HDM-induced asthma mouse model in $hIgE^{KI}$ $hFc\varepsilon RI^{Tg}$ mice. Preliminary results obtained suggested that mice developed an asthma response, but at a much lower extent compared to BALB/c mice, which thus does not permit proper evaluation of the hIgE-K in asthma models. Indeed, $hIgE^{KI}$ $hFc\varepsilon RI^{Tg}$ are in a mixed C57BL/6N, C57BL/6J, 129 background and the strain is known to present decreased Th2 responses compared to BALB/c mice.

In parallel, we also tried to reproduce an already described peanut mouse model in the humanized mice (*469*) since therapies targeting IgE seem to be a valuable strategy to control food allergies (*471*). Unfortunately, in our hands, mice did not develop visible signs of food allergy (diarrhea, anaphylaxis) upon peanut challenge. We also tested the same protocol in Il4raF709 mice and again experiments were not conclusive. Different studies showed that Il4raF709 mice have an altered microbiota, which is responsible for the increased allergen-specific responses. Differences between animal care facilities might cause alterations in the microbiota with possible consequences in the allergic outcome (*472*). However, the laboratory is now pursuing experiments in order to find a more suited food allergy model, and characterize the effects of the vaccine on allergic intestinal inflammation. Altogether, we think that further

work is needed to properly develop a clinically relevant model (*i.e.* active model) in mice expressing $hIgE^{KI}$ $hFc\varepsilon RI^{Tg}$.

IL-4, IL-13 or IgE?

In this thesis, we have evaluated different candidate vaccines to treat allergic conditions. In paper I, we evaluated and compared the efficacy of blocking IL-4 and IL-13 alone or in combination. The fact that we produced IL-4-K+IL-13-K by mixing the two single kinoids allows us to say that we are developing in parallel three different products. In terms of product development, it would probably arise questions about the financial benefits of such a strategy. However, in terms of research, it is an interesting strategy. This approach would give the opportunity to treat patients, depending of their allergic condition and phenotype. In paper II, we have as well developed an IgE-K vaccine. Although, we haven't yet established a mouse model to formally compare the four different candidate vaccines, we could speculate which vaccine will be the most promising depending on the allergic condition.

Even if we have introduced allergic conditions as a set of diseases, characterized by a common Th2 pathway, each pathology has its own characteristics. Inflammatory responses are influenced by a variety of host and environmental interactions, and are diverse in time and tissue (lung, skin, intestine), leading to their complexity and heterogenicity. The discussion of which therapeutic strategy could be the most efficient in each allergic disease, depends then from the specific disease condition and should be independently considered.

Considering therapeutic solutions for allergic asthma, as already discussed, we considered IL-4/IL-13 as well as IgE as interesting targets. As there are no clinical studies directly comparing biologics targeting IgE and IL-4/IL-13, clinicians suggest that patient phenotype and endotype has to be assessed as best as possible to achieve treatment efficacy and safety (*473*). First, type 2 asthma occurrence has to be checked and subsequently the underlying sub-endotype has to be characterized: allergic-predominant, eosinophilic-predominant or smooth muscle contraction and hyperresponsiveness and mucus predominant.

Nowadays, in patients with IgE levels superior to 100 IU/mL, co-existence of allergic rhinitis, moderate high FeNO (*i.e.* up to 50 ppd) and low number of blood eosinophils (less than 300 cells/μL), Omalizumab could be considered as the first choice due to its proven safety and

efficacy. We could speculate that in this type of patients, IgE-K would be chosen as the vaccinal strategy. Moreover, even if Omalizumab is approved for patients with poorly controlled asthma with serum IgE levels between 30 and 700 IU/mL and positive test results for perennial allergen, its efficacy in patients with IgE levels greater that 700 IU/mL is still unclear. A recent study suggested that Omalizumab was still effective in patients with higher levels, with no major safety concerns (*474*). Our IgE-K vaccine could also be proposed for this type of patients if no major safety issues are observed because of IgE high levels.

Given the well characterized pleiotropic roles of IL-4 and IL-13 in orchestrating Th2 inflammation in allergic diseases, the early failures in treatments blocking these cytokines in asthma were unexpected. Two IL-4 inhibitors and several IL-13 inhibitors generated promising preclinical and preliminary clinical data, but failed to show efficacy in larger clinical trials. With the failed attempts of individual IL-4 and IL-13 blocking, dual cytokine blockage by targeting the shared IL-4Rα represented a novel approach. Currently, in patients with broader clinical signs and symptoms which could be attributed to IL-4 and IL-13 (goblet-cell hyperplasia, mucus secretion, smooth muscle contract and hyperresponsiveness together with eosinophil recruitment), Dupilumab therapy is recommended (*473*). Concretely, Dupilumab efficacy has been demonstrated in patients with moderate to severe asthma and elevated type 2 biomarkers (blood eosinophils ≥ 150 cells/μL and FeNO ≥ 25) (*340*). In addition, as shown in paper I and shown by others (*464*), dual blockage of Il-4 and IL-13 would be required to broadly inhibit type 2 inflammation. In this type of patients, our dual IL-4-K and IL-13-K vaccination would be the elected therapeutic option.

Interestingly, while data from murine models are essential tools to characterize general allergy mechanisms and to attempt to predict a therapeutic strategy effect, some differences are observed between mice and humans. Importantly, the cytokine milieu required for IgE class switch induction differs between both species. While IL-4 alone directs class switching to IgE and IgG1 in mice (*475*), both IL-4 and IL-13 contribute to IgE synthesis in humans (*476*). Then, positive results obtained in our model with IL-4-K for blocking IgE production may be mitigated in humans, reinforcing the need of dual IL-4-K/IL-13-K kinoid vaccination in asthma.

However, Dupilumab's encouraging results came after negative or mitigated results obtained with AM317 from Amgen (mAb against IL-4Rα) and Pitrakinra from Aerovance (mutated IL-

4). Based on available data, several observations may explain these failures. In preclinical studies, it was observed that a monoclonal antibody targeting an IL-13 site inhibiting IL-13 binding to IL-4Rα was more efficient than another antibody blocking IL-13 binding to IL-13Rα1 and IL-13Rα2 (*477*). This data highlights the importance of epitope binding and of antibody design. In the case of nebulized treatments (Pitrakinra), systemic biomarkers of serum IgE or blood eosinophils were not affected. However, subcutaneous formulation of the same molecule was more successful in controlling biomarkers. These examples suggested that the blockers molecular properties as well as the route of administration and drug bioavailability could affect their efficacy. A vaccine strategy inducing a polyclonal antibody response against IL-4 and IL-13 could be an interesting solution to overcome these encountered difficulties. Finally, results obtained in the clinics suggested that to benefit from biological detailed characterization of the patients endotype and biomarkers is needed.

By contrast, in AD, Omalizumab, the first therapy that received regulatory approval for asthma, does not seem to be effective. It seems that a reduction in free serum IgE levels may not be sufficient to achieve good clinical response in this pathology. Patients with moderate AD treated during 16 weeks with Omalizumab did not experience improvements in disease end points (eczema area and severity index) (*478*). Thus, in AD, IgE, does not seem to be a driver pathogenic mediator of the disease. It suggests that this pathology is driven by a broad Th2 inflammation and that IgE may be just a product of this Th2 cell activation. Targeting key proximal mediators of Th2 response, rather than IgE, may be required to control this pathology. In response to Dupilumab treatment, an improvement in the disease was observed in the clinics with decreased type 2 biomarkers. During the treatment, effects on serum IgE levels were observed with a delayed timeframe (*336*). In addition, a prospective study showed that patients stratified by baseline IgE levels (reflecting if the disease was an allergic form or not) similarly respond to treatment suggesting that IgE was not a pathogenic driver in the disease.

In a recent publication, scientist evaluated whether AD development in mice was dependent on IL-4 or IL-13 (*479*). They showed that in an oxazolone- (OX) and 2,4-dinitrofluorobenzene- (DNFB) induced models, two distinct haptens-triggered dermatitis mouse models, disease was dependent on IL-13 signaling, via the type 2 IL-4 receptor (IL-4Rα+ IL-13Rα1), which is expressed on nonhematopoietic cells. Yet, the conclusions of this study have to be taken carefully, since IL-13Rα2 expression is at least partially dependent on IL-13Rα1, as observed

in mice deficient for IL-13Rα1 (*462*). This study is consistent with other published data highlighting unique activities for IL-4 and IL-13 and their receptors in AD (*480-482*). Moreover, authors of this study proposed that IgE production was dependent on IL-4 signaling via type 1 IL-4R. As they pointed out that AD would be more dependent on IL-13 and IL-13Rα1, it could explain why Omalizumab is not efficient in treating AD patients. Finally, this observation could also justify why despite the previous failures in targeting IL-13 in asthma, the anti-IL-13 monoclonal antibody, Lebrikizumab has proven efficacy in AD treatment. Altogether, current studies highlighted that AD is distinct from asthma, even if the two pathological conditions share some common features. IL-13 is upregulated in AD, as in asthma, but the resulting biological consequences are probably different. In skin, IL-13 acts on keratinocytes, which are activated, secreting various chemokines and cytokines. IL-4 also has a role in AD, but its significance and importance compared to IL-13 is less clear (*483*).

Taken together, we think that IgE-K would not be the elected kinoid to treat AD. Further work would be needed to determine if IL-4-K and IL-13-K combination is necessary to control AD. We could speculate that while IL-4/IL-13 kinoid combination is required for asthma treatment, IL-13-K could be adequate for controlling AD.

Food allergy and/ or anaphylaxis treatment in current clinical practice is focused on strict avoidance of the offending food(s) or drug(s), and to date there is no regulatory approved therapy for neither of the two manifestations. Clinical studies have addressed this challenge by immunotherapy (mostly in the case of food allergies), including oral, sublingual and epicutaneously delivery routes. While oral immunotherapy has shown the most promising results in terms of efficacy, it has also demonstrated less tolerability and a less favorable safety profile compared with sublingual and epicutaneous immunotherapy. Recent studies combining oral immunotherapy with Omalizumab showed an increase in tolerability. Adverse reactions were markedly reduced during immunotherapy escalation in Omalizumab-treated patients (*304*).

Food allergen exposure induces intestinal mast cells activation. These cells produce cytokines including IL-4 and IL-13, as well as chemokines that are responsible for mast cell recruitment and expansion, participating in the upper-regulation of this response (*416*). Inhibiting IL-4 and IL-13 could reduce mast cell recruitment at the intestinal level, decreasing the clinical

expression of food allergy. In line with this observation, a clinical communication reported a patient with AD and food allergy who, after initiating treatment with Dupilumab, became tolerant to two foods that previously induced allergic reactions, with this tolerance being confirmed by oral challenge (*484*). Dupilumab is now under evaluation in a phase II clinical trial for the treatment of peanut allergy (NCT03793608).

IgE-K could be a very attractive approach to combine with OIT, using similar schedules than with Omalizumab. Additionally, it would be worthy of consideration dual vaccination with IL-4-K and IL-13-K in patients with food allergy, and presenting other allergic diseases, including AD. In this type of patients, a broadly Th2 inflammation reduction could be an interesting therapeutic target to reach.

5.3.Safety considerations

New drugs regulatory approval requires efficacy, but also safety data. In this thesis, we developed a new approach to target central mediators in type 2 immune response. Because of the approach novelty and the targeted mediators' importance, safety questions had emerged since the beginning of the development. Although we are not able to answer all these questions at this preliminary development stage, in this section, I will discuss available data and what can be hypothesized using results obtained with another kinoid developed by NEOVACS SA and tested in a phase II clinical study (IFN-K), as well as safety data obtained with Omalizumab and Dupilumab.

As highlighted in the introduction, IL-4 and IL-13 are pleiotropic cytokines that are 20–25% identical sharing similar effector functions within the type 2 immune response. They are released by a variety of different cell types, including epithelial cells, eosinophils, basophils and mast cells, Th2 cells, and they have a broad range of overlapping and non-overlapping biological roles. IgE is the key immunoglobulin in Th2 immunity, participating in immunity to parasites and allergic diseases. The selective inhibition of type 2 cytokines or IgE via monoclonal antibodies has revolutionized allergic disease treatment. Therapeutic vaccines development, as an alternative to monoclonal antibodies, requires knowledge of potential risk associated with long-term inactivation of such self-proteins.

First, long-term elimination safety can be discussed by the data generated *in vitro* and with our animal models. *In vitro*, we first checked residual biological activity of IL-4, IL-13 and IgE after conjugation with CRM_{197}. Indeed, it is an important point to consider to avoid specific receptors activation after kinoid injection, that would be detrimental especially in allergic patients. We monitored residual activity of IL-4 and IL-13 using CTLL-2 cells and HEK-Blue™ IL-4/IL-13 cells (transfected with the human STAT6 gene) respectively. We showed that both kinoids had a reduced activity between 1 log (for mouse IL-13-K, the less inactivated kinoid) and 3 logs, in the case of the human kinoids. Differences observed in these results relied on the specific protocol used for the kinoid manufacturing. Mouse IL-13 is modified with a lower sGMBS rate compared with the other proteins, in order to obtain kinoids with immunogenicity properties. In preliminary mouse IL-13 kinoid batches obtained with higher doses of sGMBS, their residual activity was decreased, but without inducing neutralizing antibodies in mice. Then, a compromise between cytokine activity reduction and preserved epitope is required. Regarding the IgE-K, we introduced a mutation (G335C) which precludes binding of the IgE fragment to FcεRI (*262*). This was done to avoid any activation of skin mast cells upon injection of the kinoid. Indeed, we observed no sign of inflammation or anaphylaxis upon hIgE-K injection in IgE/FceRI humanized mice. We are now doing additional *in vitro* work to further demonstrate that the vaccine does not bind and engage FcεRI (ongoing).

In mice, as a first safety assessment, we first investigated whether mouse behavior in general and body weight were affected by kinoid treatment. We showed that mice vaccinated either with IL-4-K and/or IL-13-K or with IgE-K had a normal body weight and behavior, as compared with mice vaccinated with CRM_{197}. While our data does not anticipate a damaging effect after kinoid injection, the response in allergic patients may be altered, because their increased levels of IL-4, IL-13 and IgE. In order to reproduce this observation, a significant quantity of recombinant IL-4 (twice 10 μg) was injected into immunized mice. In mice, systemic mast cell degranulation which arises during anaphylactic shock induces hypothermia within minutes (*82*). We did not observe changes in body temperature over 1 hour following the injection, which strongly suggest that immune complexes formed with IL-4 and the anti-IL-4 antibodies induced upon vaccination did not trigger detectable mast cell activation.

In addition, even though IL-4-K and IL-13-K safety profile evaluation is a priority, we consider even more critical the IgE-K safety profile. During all the project, we carefully monitored mice after each injection and we did not notice any adversary reaction, suggesting that the vaccine does not trigger FcεRI. However, as already introduced, the low IgE levels represent a bias in the model, leading then to a potential loss of information. Being aware of this possible effect, we also monitored the temperature after i.v. injection of 10 µg recombinant IgE. Our results revealed no differences between both groups, suggesting that in mice with increased IgE levels (which is typically found in allergic patients), IgE-K is still safe. While our data cannot completely preclude possible side effects in humans, we are now very confident arguing that the IgE fragment closed conformation gives a real advantage in terms of safety. We are currently working on the production of a kinoid that will comprise the WT IgE fragment. We will compare both fragments to finally estimate the real impact of the mutation that blocks IgE in its closed conformation.

Another important question concerning IL-4, IL-13 and IgE vaccines safety profile is the potential risk to increase susceptibility to parasites infections. Indeed, despite their crucial role in allergic diseases, IL-4, IL-13 and IgE are also involved in the process of killing or expulsing parasites (such as helminths). IL-4 and IL-13 also contribute to the subsequent tissue and wound repair, as well as in insulin-like growth factor 1 (IGF-1) and IL-10 productions, and in "M2 macrophages" development (*485-488*). Accordingly, modulation of these pathways could potentially increase susceptibility to certain infections.

We haven't addressed this question yet, but we could take advantage of the literature available on mouse models. Studies with genetically altered inbred mice using a pulmonary granuloma model, induced with *Schistosomamansoni eggs*, indicated that although eosinophil infiltration, IgE, and IL-5 production are reduced in IL-4–deficient mice and IL-13–deficient mice, they are abolished in the combined absence of both cytokines (*489*). Unexpectedly, *N. brasiliensis*–infected IL-4/13–deficient mice developed elevated IL-5 and eosinophilia (highly elevated by day 10 post infection), indicating that although significantly delayed, IL-5 producing cells can receive sufficient costimulation to expand in the absence of IL-4 and IL-13 signals and so compensatory mechanisms exist for IL-5 expression, although serum IgE remained undetectable. Authors showed that these compensatory mechanisms were able to decrease worm numbers by day 20 post infection in IL-4 an IL-13 deficient mice.

Moreover, in IgE-deficient mice, multiple studies have shown abnormalities in host response after different worm infections including *Brugia malayi (490)*, *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis (491)*, *Trichinella spiralis (492)*, and *Schistosoma mansoni* (*493*). Then, suggested by mouse models and observed in helminth-infected populations, high IgE titers are observed, indicating that IgE may defend against parasites. Nevertheless, other studies using anti-IgE treatment showed the opposite effect, for example in *Schistosoma mansoni*-infected mice, supporting the possibility of detrimental, rather than beneficial, roles for IgE in Schistosomiasis (*494*). It has been shown that the host genetic background can influence IgE contributions in parasite immunity (reviewed in (*488*)). This observation could explain differences observed in all these studies. Finally, a recent study in *Stronglyoides venezuelensis*-infected mice assessed different immune effector mechanisms contribution. Authors showed that during the primary infection, CD4+ T cells, ILCs cells, IgG and Fcγ receptors are crucial to allow parasite clearance. In addition, they determined that mast cells, IgE, FcεRI and basophils have a smaller contribution during primary infection (*495*). This is an important observation as we found that the IL-4, IL-13 and IgE vaccines have no effects on IgG levels. Thus, IgG production should still allow parasite clearance in vaccinated mice. Such experiments will be performed in a near future.

Clinical data concerning increasing susceptibility of parasite infections after treatment with Dupilumab or Omalizumab is still very preliminary. Pooled analyses in Dupilumab clinical trials haven't revealed parasite infections susceptibility in treated group. However, studies have been mostly conducted in North America and Europe and did not include known endemic areas for parasites (*496*). Some studies have addressed this question for patients treated with Omalizumab. A randomized controlled trial has investigated the geohelminth infection risk in allergic patients receiving Omalizumab in Brazil. This published study has provided some evidence that the monoclonal antibody may not be associated with increased morbidity attributable to these parasites (*497*). In the study, all patients received adequate anthelmintic treatment before the study start, to achieve "parasite-free" status. Patients were followed during the 52 weeks of treatment. Overall, study findings indicated a potential risk of re-infection with common geohelminth parasites following Omalizumab therapy in a population considered to be at a high risk of infection, but the potential increased risk did not appear to be associated with increased morbidity. However, the possible excess infection risk was not considered to be clinically significant and overall clinical outcomes were unaffected. The study also assessed

the parasitological response to anthelmintic treatment. No differences were observed in cure rates between both treatments; suggesting that Omalizumab is unlikely to affect treatment efficacy against parasites. Even if the exact influence on parasite infection after Dupilumab and Omalizumab treatment is still unclear, it is recommended to be cautious in patients at high risk for helminth infections, in particular when travelling to areas where helminthic infections are endemic. It is also important that specific treatments against parasites are still efficient even after IL-4/IL-13 or IgE blockage. Patients vaccinated with kinoids should be carefully supervised if they travelled to endemic areas. Even if it is likely to be extremely rare among tourists who stay for short periods in relatively clean environments, we could think about the possibility to treat them with anti-parasite drugs as a preventive treatment. Finally, it can be hypothesized that individuals with high parasite burden would be relatively unlikely to have a Th2 inflammatory disease condition for which Kinoid therapy would be indicated, due to the suggested existence of an apparent inverse association between allergy and chronic helminth infection (the so-called 'hygiene hypothesis').

Concerning other infections, recent phase III studies with Dupilumab on asthma and AD showed no significant or severe treatment emergent infections due to the treatment. Even if no long-term studies have been conducted, the data is reassuring in terms of safety. However, ocular adverse events including dry eye disease, conjunctivitis or keratitis are frequent in Dupilumab treated patients for the AD indication. In a recent meta-analysis, they identified conjunctivitis as the only adverse effect emerging from present data (occurring in up to 19% patients) (*498*). Intriguingly, the risk of ocular adverse events appears to be circumscribed to AD patients. In addition, patients with AD were more likely to develop conjunctivitis if they had higher baseline AD severity. By contrast, their incidence was very similar to placebo in Dupilumab clinical trials for asthma, rhinosinusitis with nasal polyps and eosinophilic esophagitis (*499*). The exact pathogenesis of conjunctivitis events during Dupilumab treatment remains unclear. However, ocular comorbidities have been related to patients with AD (*500*) suggesting that Dupilumab-treated patient might present pre-existing ocular disorders. Increased ocular events in Dupilumab-treated AD patients could be explained by epithelial barrier dysfunction including abnormalities in keratinocyte terminal differentiation, keratinocyte lipid production and tight junctions, which all contribute to increased transepidermal water loss (*501*).

Moreover, viral infections appear to be slightly increased in AD patients on Dupilumab, with notably increased herpesvirus infection (*496*). It is worth remembering that patients with a history of helminth infections were excluded from clinical trials. In the case of Omalizumab, clinical trials also showed a good safety profile, with no increased risk of infection. Moreover, multiple long-term studies followed Omalizumab-treated patients and showed no increased side effects (*502, 503*). They reported however a case of relapsing *herpes labialis* possibly treatment-related, since discontinuation of Omalizumab resulted in herpes remission. This observation may suggest a possible effect of Omalizumab on the specific viral infection immune control. Overall, the good safety profile observed for Dupilumab and Omalizumab suggested that long-term inactivation of IL-4, IL-13 or IgE is not associated with reduced systemic immunity or opportunistic infections.

Moreover, interesting data have been obtained concerning a benefit blocking IL-4, IL-13 and IgE in some respiratory virus infections. As explained in the Introduction, some studies in mouse have shown that Th2 cytokines may impair immunity to rhinovirus or respiratory syncytial virus and showed that the infection can enhance asthma consequences. Recent studies in mice have suggested that blocking IL-4 or IL-13 may favor treatment of virus-associated lung disease (*504-507*). Clinical studies have addressed this question in Omalizumab-treated children with allergic asthma. In their report, Omalizumab decreased rhinovirus infection duration. These findings provide a direct association between IgE blockage and decreased susceptibility to rhinovirus infections (*508*). It should be interesting to monitor this phenomenon in future clinical trials. Treating children with kinoid vaccination at early ages could give a real advantage in blocking severe forms of asthma, related to respiratory infections.

Finally, an unexpected transient elevation of blood eosinophils was observed in Dupilumab-treated patients (specially in subjects with higher baseline serum eosinophil levels, with at least 300 eosinophils/μL). With the exception of one patient who developed a hypereosinophilic syndrome, most of the observed elevations in eosinophil counts were laboratory findings without clinical consequences or associated adverse events. Several hypotheses can be drawn for these findings to explain the observed phenomenon. First, eosinophilia could be a response to a transient rebound increase in eosinophil-active mediators, such as IL-5, resulting from IL-4 and IL-13 blockade (*509*). However, no transient IL-5 increase has been reported neither in Dupilumab studies or in our mouse model. Second, as IL-4 and IL-13 recruit and facilitate

eosinophil migration into tissues, these cytokines inhibition may cause eosinophil accumulation in the peripheral blood (*510*). This hypothesis reflects the different roles of IL-5 and IL-4/IL-13 in the eosinophil biology. While IL-4 and IL-13 play an important role in the recruitment into the tissues, favoring adhesion molecules expression, IL-5 controls their production. We should carefully monitor this feature in our future clinical trials. However, as shown in paper I, vaccination with IL-4-K and IL-13-K in mouse, had no effect on eosinophils level in the blood. Nevertheless, it is important to note that a prior study in IL-4/IL-4Ra humanized mice showed no effect of Dupilumab treatment on blood eosinophil levels, indicating that the mouse is probably not the proper organism model to study this feature (*464*).

Finally, apart from their role in allergic and parasite diseases, IL-4 and IL-13 have been shown to be even more pleiotropic, participating in other different physiological and pathophysiological events. Their modulation could impact other features that should be considered. Several reports have established a role for IL-4 and IL-13, acting via type II IL-4 receptor (expressed on non-hematopoietic cells), on several physiological functions such as fat biogenesis. Thermoregulation has been proposed to involve an innate type 2 immune response that incorporates ILCs, eosinophils and type 2 cytokines as vital controllers of adipocyte precursor number and fate, as well as overall adipose tissue homeostasis (*511*). Acclimatization to environmental cold is orchestrated by this type 2 immune cellular circuit coordinating with cytokines including IL-4, IL-13 and IL-33, which regulate adipocyte precursors expansion and differentiation into beige adipocytes. Genetic loss of eosinophils or IL-4/13 significantly impaired the development of thermogenic beige fat in mice (*512*). Interestingly, a very recent study showed that IL-13 deficient mice showed a reduced running capacity on a treadmill as well as defective fatty acid utilization and failed to increase mitochondrial biogenesis after endurance training (*513*). Thus, IL-13 signaling appears to be activated after exercise and stabilized by endurance training.

Moreover, type 2 immune response has also been considered as a counterbalance for tissue damaging inflammation initiated by type 1 immune responses (*514*). Wound repair response relies on extracellular matrix generation and deposition, such as collagens and fibronectin around the damaged area. IL-4 and IL-13 are vital for alternately activated macrophages generation, which directly participate to the repair process. In early stages of wound healing, infiltrating macrophages expressed an M2 phenotype, expressing IL-4R and their depletion inhibited the formation of a highly vascularized, cellular granulation, and tissues scar. The vast

majority of allergic reactions occur in the interface of the body with the external environment. Thus, type 2 immune reactions have been related to a protection method against harmful substances. Type 2 immunity are then key regulators of tissue homeostasis and play important roles in eliminating, restricting, and neutralizing noxious environmental substances or triggers as well as repairing the damage caused and minimizing inflammation (*515*). Even if no major issues concerning tissue homeostasis have been reported with Dupilumab or observed in our mouse model, it must be important to monitor these homeostatic functions in long-term studies.

Lastly, Kinoid technology has already been tested in the clinics with no major safety concern. IFN-K, a kinoid targeting IFN-α, was first assessed in a phase I clinical (NCT01058343) in patients with SLE and proved to be well tolerated. Two SLE flares were reported as serious adverse events, one in the placebo group and the other in a patient who concomitantly stopped corticosteroids two days after the first IFN-K dose, due to mild fever not related to infection. SLE patients are prone to viral infections (e.g., influenza, cytomegalovirus, Epstein-Barr virus, and herpes), however, no severe viral infection was documented during the trial (*388*). In a double blind, randomized, placebo-controlled, multi-center, phase IIb clinical trial (NCT01058343), the safety profile of IFN-K was confirmed, as the treatment was well tolerated and serious adverse events were reported more frequently in the placebo group (12.9%) compared to the Kinoid-receiving group (6.6%) (*390*). T cell activation studies were conducted after IFN-K injections in mice, confirming that T cell tolerance against IFN-α was not broken (*387*). Further studies should be conducted with our IL-4-, IL-13 or IgE-targeting kinoids: T cell activation is not desired since it could promote non-desired side effects, because of an uncontrolled response.

Collectively, our preclinical data together with the available preclinical and clinical data for Dupilumab and Omalizumab suggest that blocking IL-4, IL-13 and IgE using kinoid technology may fulfill safety requirements. However, as kinoid vaccines development are still in a very preliminary stage, it is impossible to predict possible side effects caused by long-term blockage. While preclinical development in terms of efficacy is more advanced, further preclinical studies are required to in-depth monitor the safety profile. These steps are required to support the clinical development of the vaccine.

5.4.Future steps in the vaccine development

One of the biggest concerns of this therapeutic approach is probably the consequences of a long-term blockade of key Th2 mediators. As discussed above, our results combined with available data are still incomplete and we cannot preclude possible side effects. It is still unclear how long the antibody response will persist after vaccination with kinoids. Although this likely will depend on the vaccine target (IL-4, IL-13 or IgE), clinical studies performed by NEOVACS with another vaccine candidate against INFα (IFN-K) are very informative. The first clinical study suggested that anti-IFN antibodies can be found in some subjects even after 4 years after the first injection (*388*). Results from a phase IIb clinical trial suggested that maybe this duration was overestimated and a boost every year may be required in order to maintain a neutralizing antibody response. However, regardless of the duration of the antibody response, we think it is critical to design a pharmacologic approach which could be used to rapidly stop the antibody response, shall any major adverse event occur. In this regard, we are now designing pilot experiments in mice aimed at 1) characterizing the phenotype of IL-4/IL-13/IgE specific B cells generated by the vaccine, 2) depleting such B cells in order to reduce the antibody response.

Anti-CD20 monoclonal antibodies are used to achieve B cell depletion, and were initially developed to treat B cell proliferative disorders, including non-Hodgkin's lymphoma and chronic lymphocytic leukemia. This antibody have subsequently been tested and used in the treatment of different autoimmune disorders, including rheumatoid arthritis, based on the rationale that the removal of the autoantibody producing or T cell-activating B cells would lead to clinical improvement (*516*). However, CD20 expression on plasma cells has been revealed to be downregulated (*517, 518*), explaining why anti-CD20 therapy failed removing long lived plasma cells. This population has been identified to be resistant to the treatment and responsible for the remaining autoantibody production responsible for a pathology (*519*).

Subsequently, the selective inhibitor of the 26S proteasome bortezomib has been recently approved for the treatment of relapsed multiple myeloma, a plasma cell neoplasia. Mechanisms of bortezomib action may include inhibition of nuclear factor-kB (NF-kB) and cell death induction as a result of activation of the terminal unfolded protein response (UPR) (*520*). Authors showed a correlation between the amount of immunoglobulin subunits retained within

myeloma cells and the sensitivity to proteasome inhibitors suggesting that proteasome inhibitors induce misfolded endoplasmatic reticulum-processed proteins accumulation. In a study in mice with lupus-like disease, authors showed that bortezomib efficiently treatment efficiently depleted both short-lived and long-lived plasma cells, predominantly as a result of activation of the terminal UPR (*521*). In patients, there is now increasing evidence that bortezomib can also efficiently deplete autoantibodies, resulting in clinical symptoms improvement, as has been described for refractory primary Sjögren's syndrome, refractory systemic lupus erythematosus, thrombotic thrombocytopenic purpura (reviewed in (*522*)). Nevertheless, other studies suggested that Bortezomib used alone showed unexpectedly low efficacy in patients. Concomitant treatment with anti-CD20 mAb increased therapy efficacy *in vitro*, increasing expression of several pro-apoptotic genes (*523*). Another study in mice with lupus-like disease showed that short lived plasma cells were efficiently depleted by anti-CD20 mAb treatment and/or Bortezomib. Conversely, long-lived plasma cells from bone marrow and spleen were only efficiently reduced in a combined treatment of anti-CD20 mAb and Bortezomib (*524*). Interestingly, this double therapeutic strategy has already been tested in the clinics in patients with relapsed follicular lymphoma (*525, 526*) and is currently under study in different autoimmune conditions (NCT04083014, NCT02102594).

Subsequently, we could conceive that a combined anti-CD20 and Bortezomib therapy could be an interesting approach to deplete B cells that are secreting antibodies targeting IL-4, IL-13 or IgE (including long-lived plasma cells that could be generated upon vaccination). Mouse models are an ideal tool to follow the presence of specific memory B cells for IL-4, IL-13 or IgE (*527*). First, it would be interesting to evaluate the presence of antigen-specific memory B cells and/or plasma cells in our vaccinated mice, evaluating when and where they are detectable and to confirm that boost injections are required. As an attempt to evaluate the feasibility of our approach, vaccinated mice would be then treated with a combination of anti-mouse CD20 mAb and Bortezomib as already done by others in the context of autoimmune diseases (*524*). The presence of specific B cells and plasma cells would be then followed in the blood, spleen and bone marrow, as well as specific antibody levels in the serum.

Critically, further preclinical studies should be conducted to in-depth evaluate the safety profile to support planned clinical studies. As required by regulatory agencies, these studies should be carried out in relevant species and should include a comprehensive study of the chosen dose that will be after injected into patients. These analyses have to be complemented with the

identification of potential target organs for toxicity (whether it is reversible or not) and safety parameters for posterior clinical monitoring. A Dose-Range Finding (DRF) study will be conducted to define these parameters in a relevant species, *i.e.* a species in which endogenous IL-4, IL-13 or IgE will be neutralized by the antibodies arising from kinoid immunization. To complement our studies in humanized mice, we identified the non-human primate cynomolgus monkey (*macaca fascicularis*) as the most relevant species for our study. To determine the relevant species, sequence identity analysis was pursued, revealing more than 90% sequence identity between both species for the three proteins. Certainly, the relevant species should develop an immune response following immunization (in our case, a humoral response), similar to the expected response in humans, to identify toxicities related to the pharmacodynamic action of the vaccine. Moreover, selected species should demonstrate a similar immunological effect to the adjuvant used. In our case, it is important to monitor the Th2 response triggered by our adjuvant to ensure no further complications in allergic patients. Supporting our choice, previous toxicology studies of humanized monoclonal antibodies, including Pascolizumab (*326*), anti-IL-13 mAb13.2 antibody (*528*), Dupilumab (*529*) or Omalizumab (*530*) were all performed in cynomolgus monkeys. Concerning the DRF study, we have defined three major goals including:

(1) Evaluation of the immunogenic dose of the kinoid in monkey in order to determine the human equivalent dose (HED) (*531*). Dose extrapolation between species is an important concept for pharmaceutical research. Body surface area, which take in account body weight has to be considered for the estimation.
(2) Identification of the number of injections required. Repeated administrations may result in an increasingly pronounced immune response in humans. Accordingly, in order to provide confidence in the safety of the dosing schedule, the number of administrations in the toxicity study should exceed the number planned for human administration. This is generally referred as the (n+1) rule, which means that at least one more administration should be given than in the proposed clinical scheme (*532*).
(3) To complement our study, we are planning to collect blood to analyze IgE levels, to ensure vaccine efficacy.

In the DRF study, we will include three groups of cynomolgus monkey composed of one male and one female injected with three different doses with a follow-up of 6 months. The vaccine tested must be adequately representative of the vaccine formulation that is intended for clinical

use, and in particular all constituents that modulate the immunological response (adjuvant) must be present. It will therefore also necessary to know the intended clinical use and patient population (especially if we are planning to immunize children, as a preventive strategy), route of administration and formulation, dose level and immunization schedule. Vaccine will be injected intramuscularly in emulsion with SWE adjuvant (same adjuvant used for mice studies) following the same immunization schedule defined in mice, with an extra boost (as explained above). In order to answer our main goals, different parameters can be followed including the mortality and morbidity, clinical signs in the monkeys, the local tolerance at the injection site, blood formula, the body weight as well as the rectal temperature. At sacrifice, macroscopic observation of the brain, liver, lungs, kidney and heart should be performed to ensure no major problems. To provide sufficient safety information is critical to support the planned clinical development of the vaccine.

Finally, even if current rationale for targeting IL-4 and IL-13 is more robust in allergic diseases, blocking IL-4 and IL-13 could be interesting to evaluate in other non-allergic diseases, including idiopathic pulmonary fibrosis, chronic obstructive disease (COPD) or cancer. Pulmonary fibrosis represents a broad spectrum of diseases characterized by different degrees of lung inflammation, excessive proliferation of lung fibroblasts, and increased lung collagen content (*533*). Although fibrosis typically begins as a part of wound healing responses (as described above), excessive accumulation of collagen and other components from the extracellular matrix can lead to the destruction of the normal tissue architecture leading to a loss of function. Signals that down-regulate the repair cycle and prevent this tissue-damaging fibrosis and scar formation are not well understood. Although IL-4 and IL-13 can induce fibrosis, studies in which IL-4 and IL-13 were independently inhibited showed IL-13 as the dominant effector cytokine in several models (*534, 535*). Treatment with an antibody targeting IL-13 has a markedly effect reducing collagen deposition in the lungs in mice (*536*). IL-13-K could be then proposed as an alternative of monoclonal antibody treatment.

In addition, COPD is an obstructive lung disease caused by decades of exposure to smoke from smoke or environmental exposures and it is characterized by significant lung and systemic inflammation (*537*). Although airflow limitation in COPD is not reversible, exacerbations represent, as for asthma, a significant issue. In COPD large airways are inflamed leading to chronic bronchitis in half of patients. Small airways are also inflamed causing a decreased lumen and increased resistance to airflow. Alveoli are destroyed resulting in enlarged airspaces,

emphysema and reduced surface area for gas exchange. COPD is considered a Th1 biased diseases where cigarette smoking causes inflammation predominantly mediated by neutrophils. Evidence for IL-4 and IL-13 expression in COP is contradictory. However, some studies have reported important role of IL-4 and IL-13. Submucosal gland cells expressing IL-4 mRNA are found in patients with COPD (*538*) and IL-13 is detected in induced sputum in COPD patients (*539*), plasma (*540*) and lung tissue (*541*). Taken together, there are some evidences that involve IL-4 and IL-13 in at least a subset of patients. Even if Dupilumab has not been tested yet, we can hypothesize that dual vaccination IL-4/IL-13-K could be proposed in a very defined type of patients suffering from COPD.

Lastly, there are evidences supporting a role for IL-4 and IL-13 in the development of some cancers and both cytokines have been closely associated with malignancy. *In vitro* and animal studies identified a role for IL-4 and IL-13 in different cancer models, but this role varied depending on the tumor type. For example, in a model of colorectal cancer in IL-4Rα deficient mice, a reduction in malignant cells proliferation was observed, as well as an increased apoptosis (*542*). Furthermore, IL-13 and IL-4 signaling protected Hodgkin lymphoma cells from apoptosis (*543*). Conversely, in one model of breast cancer, while IL-4 inhibition had no effect, inhibition of IL-13 signaling slowed tumor development (*544*). In another breast cancer model, inhibition of both cytokines signaling led to a reduction in tumor development and metastasis (*545*). Interestingly, elevated levels of IL-4 (produced by tumor-infiltrating lymphocytes) have been documented in patients with progressive prostate cancer (*546*). Studies using cells from prostate cancer patients showed IL-4 treatment induced STAT6 phosphorylation in all the patient-derived cell cultures analyzed (despite high heterogeneity between samples) (*547*). Moreover, authors showed *in vitro* that a STAT6 inhibitor (AS1517499) prevented the IL-4-activated STAT6-mediated increase of the clonogenic potential of primary prostate cancer cells. The majority of the preclinical studies identified negative effects of both IL-13 alone (*548*) and in combination with IL-4 (*543*) in malignancy that could potentially be abrogated through therapeutic pathway inhibition opening new possibilities in targeting the IL-4/IL-13 signaling even beyond allergic diseases. However, there were also a small number of studies that demonstrated protective effects of these pathways (*549, 550*), which could potentially be lost following IL-4/IL-13-targeted treatment. The conflicting results may result from differences in the models used in each study but can also reflect differences between types of cancer. Interestingly, a study suggested that inhibiting the

IL-4/IL-13 pathways could have either beneficial or deleterious effects depending on the stage of cancer progression (*551*). These results suggested that it is probably important to determine the risk/benefit balance of targeting IL-4/IL-13 pathway (specially in patients at risk), but at the same time, that targeting IL-4/IL-13 could be beneficial impeding cancer progression.

6. Final conclusions

Altogether, in this thesis we have provided the first steps of the development of a novel strategy to target allergic diseases:

- We have shown that it is possible to break B cell tolerance, inducing neutralizing antibodies against self-proteins using Kinoid technology. We proposed vaccination as a valuable strategy to target allergic disorders.
- We have confirmed that IL-4, IL-13 and IgE are valuable targets to control allergic diseases. Depending on the specific allergic condition, we would suggest the development of one or the other kinoid. Our results in asthma models confirmed that dual IL-4/IL-13 vaccination is needed. In addition, it would be possible that some other allergic diseases such as atopic dermatitis could be treated with IL-4-K or IL-13-K alone or would also benefit more from a combined vaccine. Finally, we think that the IgE-K could be suggested for the treatment of food allergies and anaphylaxis.
- Our very preliminary results suggested that kinoids present a good safety profile. However, further work is required in order to support their clinical development.

Allergies represent a major health problem affecting hundreds of million people worldwide. Focusing on the underlying pathway driving disease rather than apparent tissue manifestations can lead to tailored therapies that curtail the root cause of the pathology. The availability of a vaccine strategy to promote long-term protection against allergic reaction would directly benefit people suffering from asthma, food allergy and potentially other allergic diseases. Taken together, our positive preclinical results open the possibility to test our vaccines in the clinics in the following 2 or 3 years, if the safety profile of the vaccine is previously validated.

7. References

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8. Annex

Review-

Approaches to target IgE antibodies in allergic diseases

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Approaches to target IgE antibodies in allergic diseases

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ABSTRACT

IgE is the antibody isotype found at the lowest concentration in the circulation. However IgE can undeniably play an important role in mediating allergic reactions; best exemplified by the clinical benefits of anti-IgE monoclonal antibody (omalizumab) therapy for some allergic diseases. This review will describe our current understanding of the interactions between IgE and its main receptors FcεRI and CD23 (FcεRII). We will review the known and potential functions of IgE in health and disease: in particular, its detrimental roles in allergic diseases and chronic spontaneous urticaria, and its protective functions in host defense against parasites and venoms. Finally, we will present an overview of the drugs that are in clinical development or have therapeutic potential for IgE-mediated allergic diseases.

Contents

Abbreviations: AD, atopic dermatitis; Ag, antigen; ADAM10, a disintegrin and metalloprotease 10; ADCC, antibody-dependent cell-mediated cytotoxicity; AECs, airway epithelial cells; ASST, autologous serum skin test; Cε, constant epsilon domain of IgE; C_L, constant region of an antibody's light chains; CSU, chronic spontaneous urticaria; DARPins, designed ankyrin repeat proteins; DC, dendritic cell; Fab, fragment antigen-binding region; Fc, fragment crystallizable region of an antibody; HRF, histamine releasing factor; IECs, intestinal epithelial cells; Ig, immunoglobulin; IL, interleukin; ITAM, immunoreceptor tyrosine-based activation motif; mIgE, membrane-bound IgE; PCA, passive cutaneous anaphylaxis; PLA2, phospholipase A2; PSA, passive systemic anaphylaxis; Tg, transgenic; T_H2, T cell helper type 2; TPO, Thyroperoxidase; V_H, variable region of an antibody's heavy chains; V_L, variable region of an antibody's light chains; WT, wild type.

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1. Introduction

Immunoglobulin E (IgE) was discovered about 50 years ago. In 1966, the Ishizakas' group in Japan described an immunoglobulin different from the known immunoglobulin classes, that could induce allergic reactions in the skin, and which they called γE antibody (Ishizaka & Ishizaka, 1967). During the same period, the group of Johansson and Bennich in Sweden isolated a new immunoglobulin class, which they called IgND (Johansson & Bennich, 1967). It soon turned out that γE and IgND belong to the same and unique antibody class, and the official name IgE was given in 1968 (Bennich et al., 1968). The story behind this discovery has been the subject of many reviews, including two recent reviews by the discoverers themselves (Ishizaka & Ishizaka, 2016; Johansson, 2016). IgE is the isotype found at the lowest concentration in the circulation (50–200 ng/ml IgE in healthy individuals *vs.* ~10 mg/ml for IgG) (Dullaers et al., 2012). However, IgE levels can increase dramatically in individuals with allergic diseases (Galli & Tsai, 2012; Platts-Mills, Schuyler, Erwin, Commins, & Woodfolk, 2016). Indeed, the importance of IgE in allergy was demonstrated at the time of its discovery, when the investigators identified that purified IgE was capable of transferring skin reactivity from sensitized human subjects to naive hosts (Ishizaka & Ishizaka, 2016; Johansson, 2016). This discovery has had great importance for both the diagnosis and treatment of allergic disorders: quantification of allergen-specific IgE is one of the main diagnostic criteria for allergies (Hamilton, MacGlashan Jr., & Saini, 2010), and the anti-IgE therapeutic antibody omalizumab is now approved for the treatment of moderate to severe persistent allergic asthma, and shows great potential for the treatment of other allergic diseases (Humbert et al., 2014; Kawakami & Blank, 2016; Pelaia et al., 2015). Omalizumab has also been approved for the treatment of chronic spontaneous urticaria (CSU), demonstrating that the pathologic functions of IgE extend beyond allergy (Chang et al., 2015; Maurer et al., 2013; Zhao et al., 2016).

IgE antibodies exist in two forms: a membrane-bound form (mIgE) expressed by B cells that have undergone class switching to IgE, and a secreted form produced by plasma B cells. mIgE serves as a B cell receptor involved in antigen uptake and presentation. The structure and functions of mIgE, as well as the regulation of IgE synthesis, have been extensively reviewed elsewhere (Geha, Jabara, & Brodeur, 2003; Gould & Sutton, 2008; Wu & Zarrin, 2014). This review will focus mainly on the effector functions of secreted IgE (hereafter referred to as 'IgE').

IgE exerts its biological functions by binding to two main receptors: FcεRI and CD23 (FcεRII). The high affinity IgE receptor, FcεRI, is expressed on the surface of blood basophils and tissue resident mast cells; and on other cell types in humans (but not in mice), including neutrophils, eosinophils, platelets, monocytes and dendritic cells (Kraft & Kinet, 2007). The low affinity receptor CD23 is expressed mainly by B cells (Sutton & Davies, 2015), but also by several other cell populations including neutrophils, eosinophils, follicular DCs and intestinal epithelial cells (IECs) (Acharya et al., 2010). CD23 on B cells serves mainly as a negative regulator of IgE synthesis (Acharya et al., 2010). Crosslinking of FcεRI-bound IgE can initiate allergic reactions by inducing the activation of mast cells and basophils, the immediate release of preformed granule-stored mediators such as histamine and proteases, and the *de novo* production of lipid mediators (*e.g.* prostaglandins, leukotrienes), cytokines and chemokines (Galli et al., 2005; Voehringer, 2013; Wernersson & Pejler, 2014).

In this review, we will describe our current understanding of the interactions between IgE and its receptors FcεRI and CD23. We will review the known and potential functions of IgE antibodies in health and disease, in particular their detrimental roles in allergic diseases and chronic spontaneous urticaria, as well as their protective functions in host defense against parasites and venoms. Finally, we will present an overview of the drugs that are in clinical development or have therapeutic potential for IgE-mediated allergic diseases.

2. IgE structure

IgE antibodies are composed of two identical heavy chains (each comprising a variable V_H domain and four constant Cε domains) and two identical light chains (composed of a variable V_L domain and a constant C_L domain) with a total molecular weight of 190 kDa (Gould & Sutton, 2008; Wu & Zarrin, 2014) (Fig. 1). Similar to other antibody classes, the Fab region of IgE is responsible for antigen recognition and binding, while the effector function of IgE is determined by the carboxy-terminal Fc portion (Gould & Sutton, 2008, Wu & Zarrin, 2014). IgE shares a similar overall structure with IgG, with the exception of an additional domain in the heavy chain (Cε2). As detailed in Section 3.1.3, this additional Cε2 domain corresponds to the location of the flexible hinge region found in IgG, and plays a major role in enhancing the stability of the interaction between IgE and its high affinity receptor FcεRI (McDonnell et al., 2001). The FcεRI binding site is located in the Cε3 domain and in the Cε2-Cε3 linker region (Garman, Wurzburg, Tarchevskaya, Kinet, & Jardetzky, 2000) (described in more detail in Section 3.1.3). The binding site to the low affinity IgE receptor CD23 is also primarily located within the Cε3 domain, with contributions from the Cε4 domain (described in more detail in Section 3.2.3) (Fig. 1). The crystal structure of the human Cε3-Cε4 domains revealed that, by rotating relatively to Cε4, Cε3 can adopt either 'open' or 'closed' conformations. This conformational flexibility regulates the binding of IgE to both FcεRI and CD23 (Garman et al., 2000; Wurzburg, Garman, & Jardetzky, 2000). These features are discussed in more detail in Sections 3.1.3 & 3.2.3. Several intra- and inter-domain disulphide bridges control the structure and activity of IgE, which is also regulated by glycosylation at various sites (Fig. 1). In particular, disruption of the glycosylation site found in the Cε3 domain at asparagine-394 (N394) in humans, and N384 in mouse, abrogates the binding of IgE to FcεRI, highlighting the importance of glycosylation modifications in IgE biology (Shade et al., 2015).

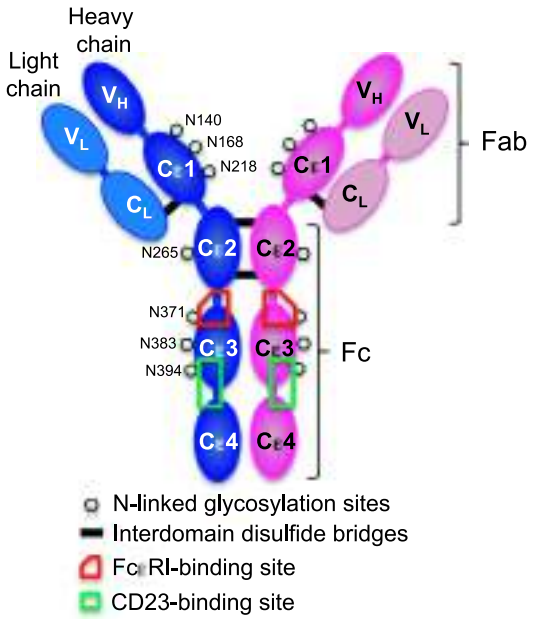

Fig. 1. IgE structure. IgE antibodies consist of two identical heavy chains (composed of a variable V_H domain and four constant Cε domains) and two identical light chains (composed of a variable V_L domain and a constant C_L domain). 'Fab': region responsible for antigen recognition and binding. 'Fc': portion responsible for IgE effector functions. The positions of interdomain disulfide bridges, N-linked glycosylation sites (in human IgE), FcεRI- and CD23-binding sites are indicated.

3. IgE receptors

3.1. The high affinity IgE receptor FcεRI

3.1.1. FcεRI structure and expression

FcεRI is the high affinity receptor for IgE (K_d of ~10^{-9} to 10^{-10} M). It is constitutively expressed at high levels on both human and rodent mast cells and basophils as a tetramer formed of one α subunit, one β subunit, and a dimer of disulfide-linked γ subunits (Blank et al., 1989). The α subunit (FcεRIα) belongs to the immunoglobulin (Ig) superfamily, with an extracellular portion composed of two Ig-like domains (D1 and D2), containing the IgE binding sites, a transmembrane domain and a short cytoplasmic domain which is thought to have no signaling function (Kraft & Kinet, 2007) (Fig. 2). Human FcεRIα is glycosylated at seven sites, and these glycosylations appear to be required for proper interactions with the folding machinery in the endoplasmic reticulum, rather than for binding to IgE (Letourneur, Sechi, Willette-Brown, Robertson, & Kinet, 1995; Sutton & Davies, 2015). FcεRIβ has a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM), which acts as signal amplifier. The FcεRIγ homodimer also contains two ITAM domains, which are responsible for signal transduction (Dombrowicz et al., 1998; Lin, Cicala, Scharenberg, & Kinet, 1996).

In humans, but not in rodents, FcεRI is also constitutively expressed as a αγ2 trimer at the surface of monocytes (Maurer et al., 1994; Takenaka, Tanaka, Anan, Yoshida, & Ra, 1995), dendritic cells (DCs) (Maurer et al., 1996), Langerhans cells (Bieber et al., 1992), neutrophils (Gounni et al., 2001), eosinophils (Gounni et al., 1994) and platelets (Hasegawa et al., 1999; Joseph et al., 1997). It was reported that expression of the αγ2 trimer is increased in peripheral blood monocytes from atopic patients, as compared to healthy controls (Maurer et al., 1994).

A circulating soluble form of FcεRI (sFcεRI) of about 40 kDa, and which contains an intact IgE binding site, has been described in human serum (Dehlink et al., 2011). However, the cell types that release or shed this protein in humans, and the physiological role of sFcεRI, remain to be identified (reviewed in (Platzer, Ruiter, van der Mee, & Fiebiger, 2011).

3.1.2. FcεRI functions

FcεRI plays a key role in mediating the biological functions of IgE *in vivo*, which is best exemplified by the fact that FcεRI-deficient mice are fully resistant to IgE-mediated passive cutaneous anaphylaxis (PCA) and passive systemic anaphylaxis (PSA) (Dombrowicz, Flamand, Brigman, Koller, & Kinet, 1993). These findings are most likely attributable to the αβγ2 FcεRI tetramer expressed on the surface of mast cells, since mast cell-deficient mice are also resistant to IgE-mediated PCA and PSA (Feyerabend et al., 2011; Lilla et al., 2011; Miyajima et al., 1997). Studies using transgenic mice expressing the human FcεRIα chain under the control of its own promoter have also given significant insight into the functions of human FcεRI (Dombrowicz et al., 1996; Dombrowicz et al., 1998; Greer et al., 2014). *hFcεRIα*^{Tg} mice (bred on a mouse FcεRI-deficient background) express a 'humanized' FcεRI receptor with a similar cellular distribution as that found in humans (Dombrowicz et al., 1996; Dombrowicz et al., 1998; Greer et al., 2014; Mancardi et al., 2008). *hFcεRIα*^{Tg} mice can develop PSA reactions upon sensitization with antigen-specific human or mouse IgE and challenge with the same antigen (Dombrowicz et al., 1996; Dombrowicz et al., 1998). Notably, mouse IgE is able to bind both human and mouse FcεRI, while human IgE does not bind the mouse receptor (Conrad, Wingard, & Ishizaka, 1983). PCA reactions can even be induced in *hFcεRIα*^{Tg} mice by intradermal transfer of plasma from allergic patients followed by challenge with the relevant allergen (Liu et al., 2013; Zhu et al., 2005). The αβγ2 tetramer on mast cells is also probably the main trigger of IgE-mediated systemic and cutaneous anaphylaxis in *hFcεRIα*^{Tg} mice, although, to the best of our knowledge, this has not yet been unequivocally demonstrated.

The biological functions of the αγ2 trimer of FcεRI are less well understood. Greer and collaborators recently used *hFcεRIα*^{Tg} mice to demonstrate that internalization of human FcεRI by conventional DCs and monocytes (which express the αγ2 trimer) contributes to serum IgE clearance (Greer et al., 2014). They injected human IgE into *hFcεRIα*^{Tg} mice and control mice (deficient for both human and mouse FcεRI), and found that serum IgE clearance was markedly accelerated in the transgenic animals. They subsequently demonstrated that human IgE was rapidly endocytosed by conventional DCs and monocytes, and that this endocytosis was associated with the rapid clearance of circulating IgE observed in *hFcεRIα*^{Tg} mice (Greer et al., 2014). While these findings appear convincing, it remains to be determined the extent to which trapping of circulating IgE by human FcεRI expressed on mast cells also contributes to its clearance. It was recently reported that perivascular mouse mast cells can 'sample' circulating IgE directly in the blood by extending cell processes across the vessel wall (Cheng, Hartmann, Roers, Krummel, & Locksley, 2013). However, the role of FcεRI in serum IgE clearance seems to be a specific feature of the human receptor, and not the mouse receptor, as mice deficient in FcεRI clear serum IgE to the same extent as WT mice (Cheng, Wang, & Locksley, 2010).

It has also been suggested that human peripheral blood DCs use the αγ2 FcεRI trimer for allergen uptake and presentation to naive T cells (Maurer et al., 1996). Using transgenic mice expressing human FcεRIα under the dependency of the CD11c promoter, in an attempt to restrict expression to DCs, these authors found that hFcεRI-expressing DCs can efficiently prime naive T cells for T_H2 differentiation, and amplify antigen-specific T_H2 responses *in vivo* (Sallmann et al., 2011).

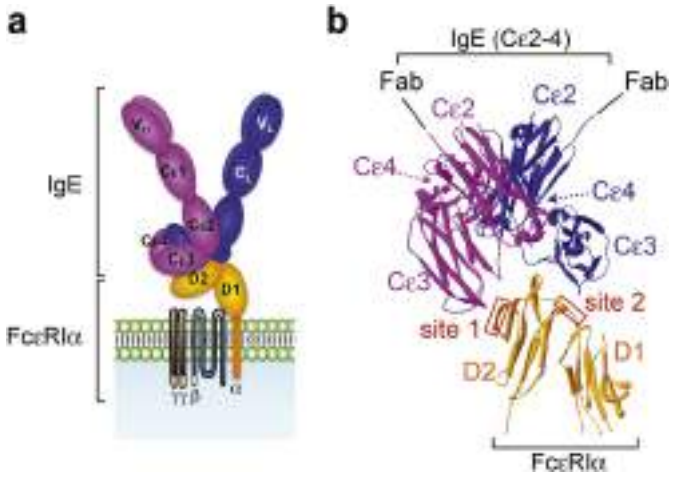

Fig. 2. Structure of FcεRI and its interaction with IgE. a. FcεRI is expressed on mast cells and basophils as a tetramer formed with one α subunit, one β subunit and a dimer of disulfide-linked γ subunits. IgE binds the receptor via surface loops in Cε3, with contributions from the Cε2–Cε3 linker region. **b.** The two Cε3 domains of IgE bind distinct sites on FcεRIα, one site found in the D2 domain (site 1), and a second site formed by a cluster of four surface-exposed tryptophan residues in the D1-D2 interface (site 2) (Protein Data Bank ID: 2Y7Q).

3.1.3. Binding of IgE to FcεRI

Mutagenesis studies have helped define the FcεRI binding epitope on IgE. Schwarzbaum and colleagues generated a mutant form of mouse IgE with a deletion of 45 amino acids in the carboxy end of Cε3: this mutant IgE was unable to bind FcεRI (Schwarzbaum et al., 1989). Nissim and collaborators produced several chimeric IgE containing the Cε2, Cε3 and Cε4 domains of human IgE (hereafter named Cε2-4), in which various domains were replaced by their murine counterparts. This work confirmed that the FcεRI binding site mapped to the Cε3 domain of IgE (Nissim, Jouvin, & Eshhar, 1991). In 2000, Garman et al. determined the crystal structure of the IgE Cε3–4 dimer bound to the extracellular part of FcεRIα (Garman et al., 2000). Analysis of this crystal structure confirmed that each of the two chains of the IgE Cε3–4 dimer could

bind the receptor using surface loops in Cε3, and revealed contributions of the Cε2–Cε3 linker region (Garman et al., 2000).

Analysis of the crystal structures of the extracellular portion of human FcεRIα alone (Garman, Kinet, & Jardetzky, 1998) or in complex with a dimeric Cε3–4 fragment (Garman et al., 2000) have also provided invaluable insight into how IgE interacts with FcεRI. The extracellular part of FcεRIα is formed of two immunoglobulin domains of about 85 amino acids each (D1 and D2), with a heavily bent D1–D2 interface forming an overall structure of an inverted V shape (Garman et al., 1998; Garman, Kinet, & Jardetzky, 1999) (Fig. 2). The two Cε3 domains of IgE bind distinct sites on FcεRIα, one site found in the D2 domain, and a second site formed by a cluster of four surface-exposed tryptophans in the D1–D2 interface (Garman et al., 2000). The presence of these two binding sites explains the 1:1 stoichiometry of the IgE-FcεRIα complex, which is essential to ensure that receptor crosslinking and activation occurs only upon multivalent antigen binding to IgE (Garman et al., 2000).

A unique feature of the FcεRI receptor, as compared to other Fc receptors, is the distinctly slow dissociation rate of the IgE-FcεRIα complex ($k_{off} \approx 10^{-5}\ s^{-1}$). This translates into a half-life of about two weeks for IgE bound to FcεRI (compared to only hours for IgG complexes bound to Fcγ receptors), and ensures that tissue mast cells and basophils remain saturated with IgE (Geha, Helm, & Gould, 1985; McDonnell et al., 2001). McDonnell and collaborators showed that full human IgE molecules and dimeric IgE fragments comprising the Cε2, Cε3 and Cε4 domains (Cε2–4) have identical kinetics of dissociation with FcεRIα, while Cε3–4 displays a markedly enhanced dissociation kinetic (~20-fold), indicating that Cε2 plays a major role in enhancing the stability of the IgE-FcεRIα complex (McDonnell et al., 2001). More recently, Holdom et al. published the crystal structure of human Cε2–4 bound to the extracellular domain of FcεRIα, and confirmed that the Cε2 domain contributes to the slow dissociation rate of IgE-FcεRIα complexes through conformational changes rather than direct interactions with the receptor (Holdom et al., 2011).

Analysis of the crystal structures of free *vs*. receptor-bound IgE Fc domains have revealed that the Cε3 domains of IgE undergo a large conformational rearrangement upon binding to FcεRI (Holdom et al., 2011; Wan et al., 2002; Wurzburg et al., 2000; Wurzburg & Jardetzky, 2009). The free IgE Fc portion was observed in a 'closed' conformation in which the FcεRIα binding site in Cε3 is masked (Wan et al., 2002; Wurzburg et al., 2000; Wurzburg & Jardetzky, 2009). This masking is achieved as the Cε2 domains in the free Fc fragment are folded back asymmetrically onto the Cε3 and Cε4 domains, locking the Cε3 domains in a 'closed' conformation (Wan et al., 2002) (Fig. 3). The authors suggest that free 'bent' IgE may first engage FcεRI through only one Cε3 domain, followed by an important conformational change involving Cε2, whereby Cε3 would adopt an 'open' conformation, leading to engagement of the second Cε3.

3.2. The low affinity IgE receptor CD23 (FcεRII)

3.2.1. CD23 structure and expression

CD23, also known as FcεRII, is the low affinity receptor for IgE ($K_d = 10^{-5}$ M) (Wurzburg, Tarchevskaya, & Jardetzky, 2006). The structure of CD23 and its interaction with IgE have been reviewed in detail (Sutton & Davies, 2015). CD23 self-associates as trimer, and is composed of an IgE-binding 'head domain' (which belongs to the C-type lectin superfamily) linked to the membrane by an extracellular coiled-coil stalk region, and a small cytoplasmic N-terminal domain (Fig. 4). CD23 exists in a membrane-bound form of 45 kDa (mCD23), as well as in soluble forms of various sizes (sCD23) which are released by proteolytic cleavage at several sites in the stalk region (Sutton & Davies, 2015). ADAM10 ('a disintegrin and metalloprotease 10') is considered to be the main endogenous protease responsible for cleavage and generation of sCD23 (Lemieux et al., 2007; Weskamp et al., 2006). The exogenous house dust mite cysteine protease *Der p I* is also able to cleave mCD23 at two sites (Schulz et al., 1997). mCD23 (hereafter referred to as CD23) is expressed by B cells (Sutton & Davies, 2015), and several other cell populations including neutrophils (Yamaoka et al., 1996), eosinophils (Capron et al., 1992), follicular DCs (Johnson, Hardie, Ling, & Maclennan, 1986) and IECs (Yang, Berin, Yu, Conrad, & Perdue, 2000; Yu et al., 2003). Human CD23 exists as two isoforms (CD23a and CD23b), which differ in the first seven (CD23a) or six (CD23b) amino-acid residues of the cytoplasmic N-terminal part (Sutton & Davies, 2015; Yokota et al., 1992).

3.2.2. CD23 functions

CD23 is expressed on the surface of B cells, where it serves as a negative regulator of IgE synthesis. Several publications show increased levels of IgE in mice deficient for CD23 (Haczku et al., 2000; Lewis et al., 2004; Riffo-Vasquez et al., 2000; Stief et al., 1994; Yu, Kosco-Vilbois, Richards, Kohler, & Lamers, 1994). Conversely, transgenic mice overexpressing CD23 in B (and T) cells have markedly reduced levels of circulating IgE after immunization (Payet, Woodward, & Conrad, 1999). The regulation of IgE production seems to require the oligomerization of CD23, since serum IgE levels are also increased in mice treated with an antibody that binds to the stalk region of CD23 and thus blocks receptor oligomerization (Ford et al., 2006; Kilmon et al., 2001). It is possible that CD23 on B cells plays an additional role(s) in regulating serum IgE levels, independently of its effects on IgE production. This was suggested by a study showing that exogenous IgE injected into mice deficient for B cells or treated with an anti-CD23 antibody can be detected in the blood one hour later at levels two-fold higher than in the corresponding control mice (Cheng et al., 2010). The mechanism through which CD23 regulates serum IgE levels is still unclear, and appears to be independent on B cells, since the administered IgE had similar rates of clearance in B cell-deficient and -sufficient mice (Cheng et al., 2010).

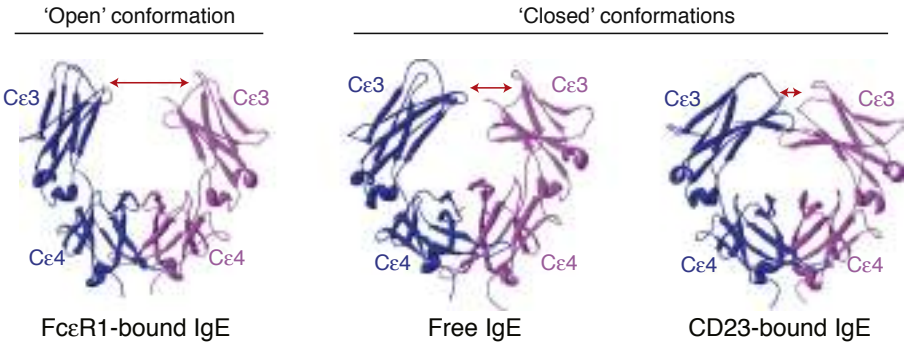

Fig. 3. Conformational changes in IgE Fc portion upon binding to FcεRI or CD23. The Cε3 domains of free IgE are found in a 'closed' conformation in which the FcεRIα binding site in Cε3 is masked (middle; Protein Data Bank [PDB] ID: 2WQR). Cε3 adopts an 'opened' conformation upon binding to FcεRI, which is incompatible with CD23 binding (left; PDB ID: 1F6A-2). By contrast, Cε3 adopts a 'closed' conformation upon binding to CD23, which is incompatible with FcεRI binding (right; PDB ID: 4GKO).

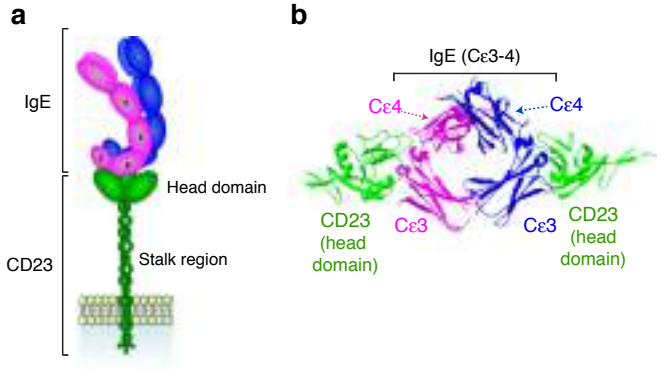

Fig. 4. Structure of CD23 and its interaction with IgE. a. CD23 self-associates as a trimer, and is composed of an IgE-binding 'head domain' (which belongs to the C-type lectin superfamily) linked to the membrane by an extracellular coiled-coil stalk region, and a small cytoplasmic N-terminal domain. **b.** The IgE binding site of CD23 is located in the C-terminal head domain (in green), with some additional contributions from the stalk region (not shown). Two CD23 molecules bind to each IgE heavy chain, primarily to the Cε3 domains but with a contribution from Cε4 (Protein Data Bank ID: 4GKO).

In B cells, CD23 has also been implicated in IgE-dependent antigen uptake and presentation to T cells. *In vitro* experiments showed that mouse and human B cells incubated with antigen-specific IgE were up to 100-fold more efficient than untreated B cells at presenting low concentrations of the respective antigen, and this phenomenon was markedly reduced by a CD23 blocking antibody (Kehry & Yamashita, 1989; Pirron, Schlunck, Prinz, & Rieber, 1990).

CD23 is expressed on IECs, and such expression is enhanced upon antigen sensitization in rodents (Yang et al., 2000; Yu et al., 2001), or exposure to the T_H2 cytokine IL-4 in humans (Tu et al., 2005). Studies using CD23 blocking antibodies or mice deficient for CD23 have demonstrated that CD23 in IECs is involved in the transepithelial transport of IgE and IgE/antigen complexes into the intestinal lumen (Tu et al., 2005; Yang et al., 2000; Yu et al., 2001). This phenomenon is potentially important for food allergy, since it could explain how IgE and allergens are delivered to mast cells located in the lamina propria beneath the epithelial lining of the gut (Gould & Sutton, 2008; Tu et al., 2005). Similarly, CD23 is expressed on human airway epithelial cells (AECs), where it is also subject to upregulation by IL-4, and *ex vivo* experiments suggest that CD23 in AECs is involved in transepithelial transport of IgE and IgE/antigen immune complexes (Palaniyandi, Tomei, Li, Conrad, & Zhu, 2011). A more recent study using CD23-deficient mice confirmed that CD23 expressed by AECs is involved in IgE and IgE/antigen transport, and showed that expression of CD23 in lung structural cells is important for the development of allergic airway inflammation (Palaniyandi et al., 2015).

The soluble form of CD23 (sCD23) can also regulate IgE synthesis. sCD23 exists in several isoforms of different sizes. All isoforms can interact with IgE, but the shorter sCD23 remains monomeric while the longer isoforms associate in trimers (reviewed in detail in (Platzer et al., 2011)). sCD23 isomers can have divergent effects on B cells. Trimeric sCD23 can upregulate IgE synthesis through the co-ligation of CD21 and membrane IgE on B cells (Aubry, Pochon, Graber, Jansen, & Bonnefoy, 1992; Cooper et al., 2012; Hibbert et al., 2005; McCloskey et al., 2007), whereas monomeric sCD23 inhibits IgE synthesis in human B cells (McCloskey et al., 2007).

3.2.3. Binding of IgE to CD23

Early mutagenesis studies mapped the IgE binding site of CD23 to discontinuous epitopes between residues 160-287 in the C-terminal head domain (Bettler, Maier, Ruegg, & Hofstetter, 1989; Bettler, Texido, Raggini, Ruegg, & Hofstetter, 1992). These mutagenesis studies also suggested that binding of IgE requires six out of eight extracellular cysteine residues of CD23, which are likely involved in the formation of intramolecular disulfide bridges (Bettler et al., 1992). The head domain of CD23 is involved in IgE binding, since its proteolytic cleavage by the house dust mite protease *Der p I* abrogates binding (Schulz et al., 1997). Nevertheless, one mutagenesis study suggested that the stalk region of CD23 is also involved in IgE binding (Chen et al., 2002); a finding that was recently confirmed, indicating that the IgE-CD23 interaction is more complex than previously anticipated (Selb et al., 2017). Interestingly, the latter study also demonstrated that mutation of the N-glycosylation site of CD23 (N63) alone is sufficient to enhance binding of IgE (Selb et al., 2017).

Vercelli et al. first demonstrated, using a bank of peptides spanning the IgE Cε2-4 domains, that CD23 recognizes a motif in the Cε3 domain of IgE (Vercelli et al., 1989). This was confirmed in a study using chimeric IgE molecules in which the human Cε3 domain was replaced by mouse Cε3: these chimeric molecules bound to mouse CD23 and concomitantly lost their ability to bind the human receptor (Nissim, Schwarzbaum, Siraganian, & Eshhar, 1993). Thereafter, the CD23 binding site on IgE was more precisely mapped to the A-B loop of the Cε3 domain (residues 341–356), with a key role for lysine 352 (Sayers, Housden, Spivey, & Helm, 2004). More recently, the crystal structure of the soluble head domain of CD23 bound to a Cε3–4 IgE dimer was resolved by Dhaliwal et al. (2012). These authors found that one CD23 molecule binds to each IgE heavy chain, principally via the Cε3 domains but with a contribution from Cε4 (Dhaliwal et al., 2012) (Fig. 4). Although the binding sites for FcεRI and CD23 are at opposite ends of the Cε3 domain, binding of the two receptors to IgE is mutually exclusive. Indeed, binding of IgE to CD23 induces conformational changes in Cε3, leading to a highly 'closed' conformation incompatible with FcεRI binding (Borthakur et al., 2012; Dhaliwal et al., 2012). Similarly, the 'opened' conformation adopted by Cε3 upon binding to FcεRI is incompatible with CD23 binding (Borthakur et al., 2012, Dhaliwal et al., 2012) (Fig. 3). Finally, the crystal structure of CD23 bound to a complete IgE Fc fragment was reported, revealing that the IgE Cε2 domain also contributes to CD23 binding, in addition to the known contributions of the Cε3 and Cε4 domains (Dhaliwal et al., 2017).

3.3. Other IgE or FcεRI binding molecules

Mast cells and basophils can be activated by the cytokine-like protein histamine-releasing factor (HRF) (reviewed in (Kawakami, Kashiwakura, & Kawakami, 2014)). It was shown that HRF could bind to a subset of IgE antibodies via their Fab regions, thereby inducing antigen-independent cross-linking of FcεRI-bound IgE molecules, and that this process could amplify inflammation in mouse models of cutaneous anaphylaxis or allergic airway inflammation (Kashiwakura et al., 2012). Similarly, the protein Galectin-3 (formerly known as ε binding molecule), which is released by several cell types, can bind to both IgE and FcεRI and induce mast cell and basophil activation via antigen-independent crosslinking of FcεRI (Frigeri, Zuberi, & Liu, 1993; Zuberi, Frigeri, & Liu, 1994). Galectin-3 is also directly produced by mast cells (it is found in the cytoplasm and nucleus of mast cells (Craig et al., 1995)), and it was shown that mast cells derived from the bone marrow of galectin-3 deficient mice displayed reduced activation by IgE and antigen *in vitro* as compared to WT mast cells (Chen et al., 2006).

Takizawa and collaborators reported that IgE immune complexes can bind to the mouse IgG receptors FcγRIIB and FcγRIII expressed on mast cells and macrophages, with an affinity similar to that of IgG immune complexes (Takizawa, Adamczewski, & Kinet, 1992). They further demonstrated that such binding to FcγRs can induce mast cell activation independently of FcεRI (Takizawa et al., 1992). IgE immune complexes were also found to bind and activate mouse FcγRIV, expressed on monocytes, macrophages and neutrophils (Hirano et al., 2007; Mancardi et al., 2008). Confirming that FcγRIV can act as a low-affinity receptor for mouse IgE, treatment of mice with an anti-FcγRIV antibody inhibited late phase reactions in a model of IgE-mediated passive cutaneous allergic inflammation (Hirano et al., 2007). In addition,

experiments performed in mice deficient for FcεRI, CD23 and all FcγRs except FcγRIV suggested that the *in vivo* engagement of FcγRIV by IgE immune complexes can synergize with mediators released by IgE-activated mast cells to induce lung inflammation (Mancardi et al., 2008).

4. Roles of IgE in health and disease

4.1. Pathologic roles of IgE

4.1.1. Immediate hypersensitivity reactions

IgE antibodies are probably best known for their critical role in acute allergic reactions. In allergic individuals, mast cells and basophils have antigen-specific IgE bound to FcεRI expressed on the cell surface (Galli & Tsai, 2012). Antigen-mediated IgE/FcεRI crosslinking initiates a complex signaling cascade (Reber & Frossard, 2014; Sibilano, Frossi, & Pucillo, 2014), leading to the eventual activation of these effector cells and the immediate and rapid release of preformed granule-stored mediators (Wernersson & Pejler, 2014) (*e.g.*, histamine, serotonin, proteoglycans, proteases and cytokines) and *de novo* production and release of an impressive range of lipid mediators (*e.g.*, prostaglandins, leukotrienes), cytokines and chemokines (Galli et al., 2005; Voehringer, 2013). These mediators can act locally or systemically, leading to the clinical features of immediate hypersensitivity, such as bronchoconstriction, urticaria, diarrhea (when acting locally in the airways, the skin and the gut, respectively) (Fig. 5).

4.1.2. Anaphylaxis

Anaphylaxis is the most extreme manifestation of an allergic reaction. In humans, anaphylaxis can be attributed to an IgE- and mast cell-dependent immediate hypersensitivity reaction in individuals previously sensitized to that allergen (Burton & Oettgen, 2011; Galli & Tsai, 2012; Lieberman et al., 2006). Indeed, quantification of specific IgE levels is used to identify potential triggers of anaphylaxis in patients with a personal history of anaphylaxis (Hamilton et al., 2010). IgE-dependent anaphylactic reactions can also be recapitulated in mice, in which a local or systemic injection of antigen one day after passive injection of antigen-specific IgE induces features of anaphylaxis (Dombrowicz et al., 1993; Oka, Kalesnikoff, Starkl, Tsai, & Galli, 2012; Wershil, Mekori, Murakami, & Galli, 1987).

IgE-mediated anaphylaxis is abrogated in mice lacking the high affinity IgE receptor FcεRI (Dombrowicz et al., 1993), as well as in mast cell-deficient mice (Feyerabend et al., 2011; Lilla et al., 2011; Oka et al., 2012), highlighting the importance of IgE-mediated mast cell activation in this reaction. Mast cells likely also play a key role in human anaphylaxis. Indeed, elevated levels of the mast cell specific protease tryptase have been detected during anaphylactic reactions in humans (Schwartz, Metcalfe et al. 1987, Schwartz, 2006, Brown et al., 2013). Moreover, an increased incidence of anaphylaxis was reported in patients with mastocytosis, a disease characterized by increased numbers of mast cells (Schuch & Brockow, 2017). By contrast, the role of basophils in anaphylaxis is more debated. So-called "Basophil activation tests" are used to confirm allergen sensitization in human patients. In these tests, which are performed on blood samples *ex vivo*, IgE-mediated activation of basophils is monitored by measuring up-regulation of surface markers such as CD63 and CD203c (Giavina-Bianchi, Galvao, Picard, Caiado, & Castells, 2017; Kim et al., 2016; Santos et al., 2015). Recently, Korosec and colleagues also reported an increase of CD63 expression on circulating basophils, as well as a marked reduction in the absolute number of circulating basophils, during anaphylactic reactions to *Hymenoptera venom* in humans (Korosec et al., 2017). While these data suggest that basophils are activated in human anaphylaxis, they do not however demonstrate a significant contribution to anaphylaxis pathophysiology. Even in mice, the role of basophils in IgE-mediated anaphylaxis remains contentious. Different reports indicate that depletion of basophils does not reduce IgE-mediated local or systemic passive anaphylaxis (Sawaguchi et al., 2012; Wada et al., 2010). Mukai and colleagues reported that intravenous injection of antigen-specific IgE in mice, followed one day later by subcutaneous challenge with the antigen, can induce a triphasic response (Mukai et al., 2005). The 'immediate' and 'late-phase' (6 to 10 h after challenge) responses were dependent on mast cells. However, the third-phase, beginning one to two days after challenge, was independent of mast cells and was abrogated upon depletion of

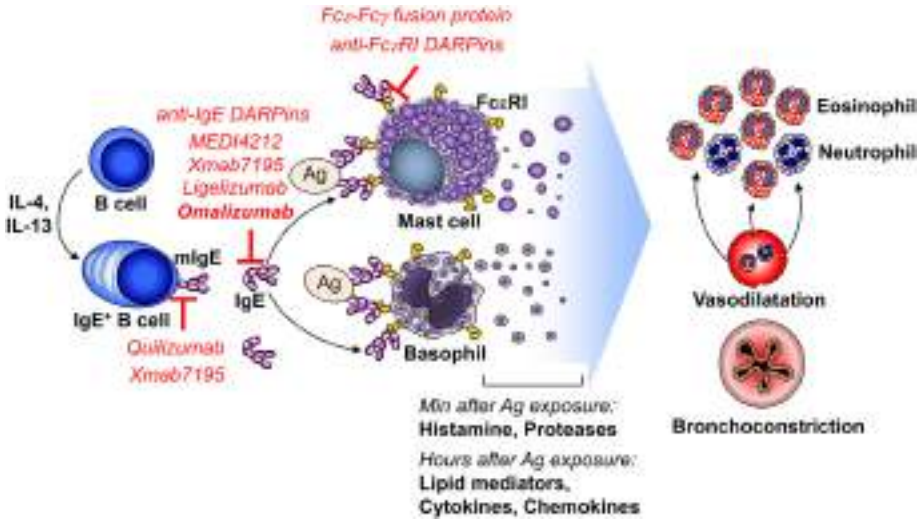

Fig. 5. Key role of IgE in allergic reactions. Stimulation with the T_H2 cytokines IL-4 and IL-13 induces class-switching of B cells into IgE-producing cells. IgE binds to its high-affinity receptor FcεRI on the surface of tissue mast cells and blood basophils. Upon exposure to an allergen, in allergic patients, allergen recognition by allergen-specific IgE on the surface of mast cells and basophils induces crosslinking of FcεRI, leading to degranulation and the immediate release of histamine, proteases and other preformed mediators, as well as *de novo* synthesis of lipid mediators (prostaglandins, leukotrienes, …), cytokines and chemokines. These mediators can act locally or systemically, leading to the clinical features of immediate hypersensitivity, such as bronchoconstriction, urticaria, diarrhea (when acting locally in the airways, the skin and the gut, respectively) and vasodilatation. These mediators are also responsible for late-phase allergic responses, entailing the recruitment of leukocytes, mainly eosinophils and neutrophils. Several drugs have been developed to counteract the effects of IgE. These drugs either target IgE production, block free IgE or compete with IgE for binding to FcεRI. The only FDA-approved anti-IgE drug is Omalizumab, a humanized anti-IgE mAb that blocks free IgE, and which is approved for the treatment of moderate to severe persistent allergic asthma, and chronic spontaneous urticaria (CSU). Ag: antigen.

basophils (Mukai et al., 2005; Obata et al., 2007; Sawaguchi et al., 2012). This third-phase delayed response was also absent in mice lacking FcRγ (a signaling subunit shared by FcεRI and activating IgG Fcγ receptors), and was restored upon engraftment of these mice with basophils purified from WT mice but not from *FcRγ*$^{-/-}$ mice (Mukai et al., 2005). Since this passive model relies on specific IgE antibodies, and not on IgG, these results strongly suggest that direct activation of basophils through FcεRI is responsible for the delayed allergic skin inflammation observed this model. Using a similar model of IgE-mediated chronic allergic inflammation, Cheng et al. also reported markedly reduced eosinophilic dermatitis in basophil-deficient mice as compared to control mice three days after cutaneous challenge with the relevant antigen (Cheng et al., 2015).

The presence of allergen-specific IgE alone does not explain an individual's susceptibility to allergy and anaphylaxis. Allergen-specific IgE can be detected in subjects who do not develop clinical symptoms when exposed to the corresponding allergen (Sicherer & Sampson, 2010). Conversely, some patients can experience near fatal anaphylaxis despite having low or undetectable levels of circulating allergen-specific IgE (Simons et al., 2007), which suggests (but does not prove) the existence of IgE-independent pathways of anaphylaxis in humans (recently reviewed in (Finkelman, Khodoun, & Strait, 2016) and (Reber, Hernandez, & Galli, 2017)). More definitive evidence for IgE-independent pathways of anaphylaxis has been obtained using mouse models of active systemic anaphylaxis (ASA), in which mice are sensitized with an antigen (to produce antigen-specific antibodies) and re-exposed later on to the same antigen to induce anaphylaxis (Finkelman et al., 2016; Munoz-Cano, Picado, Valero, & Bartra, 2016). Mice deficient for IgE or for FcεRI can still partially (Arias et al., 2011; Balbino et al., 2017; Sun et al., 2007) or fully (Dombrowicz et al., 1997; Jonsson et al., 2011; Oettgen et al., 1994) develop features of anaphylaxis in these ASA models. Other studies have subsequently shown that mouse IgG antibodies can trigger anaphylaxis in ASA models, through activation of IgG receptors (FcγRs) on the surface of various myeloid cells, including basophils, macrophages and neutrophils (Balbino et al., 2017; Finkelman et al., 2016; Jonsson et al., 2011; Khodoun et al., 2013; Miyajima et al., 1997).

4.1.3. Allergic asthma

Asthma is a chronic inflammatory disease of the airways with continual increasing prevalence (Busse & Lemanske Jr., 2001; Subbarao, Mandhane, & Sears, 2009). In many patients, the asthmatic condition is associated with allergic reactivity to environmental allergens and elevated levels of IgE antibodies (Busse & Lemanske Jr., 2001). In these allergic patients, IgE is thought to contribute to the asthmatic manifestations (Galli & Tsai, 2012). Following antigen exposure in the airways, rapid local IgE/FcεRI-dependent mast cell activation and the immediate hypersensitivity reaction can lead to increased vascular permeability, bronchoconstriction and increased mucus production. A large array of cytokines, growth factors and chemokines secreted by activated mast cells can influence airway remodeling (Galli, Tsai, & Piliponsky, 2008; Moiseeva & Bradding, 2011). Finally, IgE can also act on other cell types that express FcεRI or CD23, such as DCs, B cells, basophils or (in humans) eosinophils, which may potentially affect several biological responses associated with the asthmatic response (Galli et al., 2008; Galli & Tsai, 2012). Supporting the important role of IgE in asthma, the anti-IgE antibody omalizumab has been shown to reduce asthma symptoms in several clinical trials involving patients with moderate-to-severe and severe allergic asthma (reviewed in (Humbert et al., 2014)) (for more detail see Section 5.1, below).

4.1.4. Atopic dermatitis

Eczema, or atopic dermatitis (AD), is a pruritic inflammatory skin disease with dramatically increased incidence over the last decades (Bieber, 2008; Dharmage et al., 2014). AD manifestations are characterized by pruritus (itching), skin inflammatory lesions associated with cellular infiltration and histopathological changes, and atopy. Indeed, the majority of AD patients exhibit increased serum levels of total and antigen-specific IgE (Laske & Niggemann, 2004; Leung & Bieber, 2003; Oyoshi, He, Kumar, Yoon, & Geha, 2009). The function of IgE in development of AD is supported by the beneficial effect of anti-IgE therapy in a number of clinical studies (Belloni, Andres, Ollert, Ring, & Mempel, 2008; Liu, Goodarzi, & Chen, 2011).

Abboud, Staumont-Sallé et al. used a mouse model of AD induced by repeated epicutaneous sensitizations with ovalbumin. They reported that several features of this model (including T_H1 and T_H2 skin responses, mast cell recruitment into draining lymph nodes and IgE production) were reduced in *FcεRI*$^{-/-}$ mice. In this model, T_H2 skin response as well as T cell proliferation and IgG1 production were also reduced in mice lacking the IgG receptor FcγRIII (Abboud et al., 2009). In addition, symptoms of AD were completely absent in mice deficient for FcRγ, a subunit shared by FcεRI and FcγRIII (and several other FcR). The authors therefore concluded that in this model, FcεRI and FcγRIII both contribute to AD but differentially regulate immune responses associated with the disease (Abboud et al., 2009). Ando and colleagues developed a mouse model of AD in which eczematous skin lesions are induced by repeated epicutaneous applications of house dust mite extract and staphylococcal enterotoxin B (Ando et al., 2013; Kawakami, Yumoto, & Kawakami, 2007). The global skin gene expression pattern in this model was very similar to that observed in human AD skin. Mast cell-deficient mice had markedly reduced skin inflammation; and FcεRI expression was required to attain maximal clinical scores in this AD model (Ando et al., 2013). However, some features of the model were reduced in mast cell-deficient mice but not in *FcεRI*$^{-/-}$ mice, which suggests that mast cells can amplify inflammation in the context of AD model though both IgE-dependent and IgE-independent pathways (Ando et al., 2013).

4.1.5. Chronic spontaneous urticaria

Chronic spontaneous urticaria (CSU; also known as chronic idiopathic urticaria) is defined as itchy wheals, angioedema, or both that reoccur for more than 6 weeks without a specific trigger (Zuberbier, Aberer et al., 2014). Antihistamines show clinical benefit for many (but not all) CSU patients, and it is therefore believed that skin mast cells, which are a major source of histamine, play an important role in CSU (Vonakis & Saini, 2008). CSU patients often have high levels of total IgE (Kessel et al., 2010). However, CSU may not be triggered by specific external antigens. By contrast, most CSU patients exhibit autoimmune responses in the form of serum IgE to autoantigens or IgG autoantibodies to IgE or FcεRI (reviewed in (Kolkhir et al., 2017)). 35-45% of adults with CSU develop a wheal when injected intradermally with their own serum, a test called autologous serum skin test (ASST) (Metz et al., 2009). Such positive ASSTs responses have been linked to IgG autoantibodies directed against the high-affinity IgE receptor FcεRI, or less commonly against IgE (Auyeung, Mittag, Hodgkin, & Harrison, 2016; Chang et al., 2015; Hide et al., 1993). Both types of autoantibodies can trigger activation of mast cells (and other FcεRI-bearing cells) through cross-linking of FcεRI. In a recent study, autoreactive T cells specific for FcεRI were also detected in the blood of a large proportion of patients with CSU (Auyeung et al., 2016). The authors therefore proposed that, as for other autoimmune diseases, activation of autoreactive T cells is likely one of the initial events in CSU (Auyeung et al., 2016). Moreover, some CSU patients have high titers of autoreactive IgE directed against dsDNA or thyroid antigens, such as thyroperoxidase (TPO) (Altrichter et al., 2011; Hatada et al., 2013). It was also recently reported that IL-24 is a common autoantigen in patients with CSU (Schmetzer et al., 2017). Such IgE autoantibodies could mediate skin reactions in CSU by inducing mast cell degranulation in response to autoantigens (Altrichter et al., 2011; Chang et al., 2015; Hatada et al., 2013). It should be noted, however, that the presence of

IgE against autoantigens is also documented in diseases other than CSU, such as atopic dermatitis (reviewed in (Hradetzky, Werfel, & Rosner, 2015)), and a direct link between autoantibodies and the clinical manifestations of CSU has not yet been demonstrated. Some reports also indicate the presence of IgE against exogenous antigens, such as *Staphylococcus aureus* enterotoxins, in some CSU patients, which could contribute to the pathogenesis of CSU in a subpopulation of patients (Altrichter et al., 2018; Ye et al., 2008).

In support of a key role of IgE and FcεRI in CSU, the anti-IgE therapeutic antibody omalizumab is now approved for the treatment of CSU (Chang et al., 2015; Maurer et al., 2013; Zhao et al., 2016). Moreover, most patients with CSU who stop omalizumab treatment relapse within a few months, and a recent study indicates that total IgE serum levels before omalizumab treatment correlate negatively with the time to relapse in these patients (Ertas et al., 2017). As reviewed in detail by Chang et al. (2015), the clinical benefits of omalizumab are likely due to a direct blockade of IgE antibodies before they can bind FcεRI and activate mast cells (especially in patients with autoreactive IgE), and/or a downregulation of FcεRI on the surface of mast cells and other effector cells (Chang et al., 2015).

4.2. Protective roles of IgE

IgE and the main FcεRI-expressing effector cells, mast cells and basophils, do not only play roles in pathology, but also critically contribute to host defense. This has been convincingly demonstrated using mouse models of host defense against certain parasites and venoms.

4.2.1. Host defense against parasites

Helminth infections are generally associated with a “type 2” immune response, characterized by helper type 2 T (T_H2) cells that typically produce IL-4, IL-5 and IL-13, increased numbers of tissue mast cells and eosinophils, and elevated levels of antigen-specific and unspecific IgE (Anthony, Rutitzky, Urban Jr., Stadecker, & Gause, 2007; Finkelman et al., 1997; Grencis, Humphreys, & Bancroft, 2014). Data from epidemiological studies in humans point towards a protective role for IgE in helminth infections, as increased levels of helminth-specific IgE correlate with host resistance (Faulkner et al., 2002; Hagan, Blumenthal, Dunn, Simpson, & Wilkins, 1991; Rihet, Demeure, Bourgois, Prata, & Dessein, 1991). Remarkably, anti-IgE antibody treatment of human patients at high risk of helminth infections did modestly increase parasite infection risk, albeit an effect that did not reach statistical significance (Cruz et al., 2007). Increased IgE levels might, however, simply reflect a strong T_H2 cell response in infected individuals, the latter being of unquestionable importance in host defense against parasites. Indeed, the actual contributions of non-specific *vs*. specific IgE antibodies in host defense and parasite clearance are still unclear and numerous experimental studies aiming at addressing this question have led to different, sometimes opposing, conclusions (recently reviewed in (Mukai, Tsai, Starkl, Marichal, & Galli, 2016)). Also, protective *vs*. detrimental roles of IgE antibodies in anti-parasite immunity appear to be parasite-dependent. For instance, data from experiments with IgE-deficient mice indicate beneficial functions for IgE in models of *Trichinella sprialis* (Gurish et al., 2004), *Schistosoma mansoni* (King et al., 1997), *Brugia Malayi* (Spencer, Porte, Zetoff, & Rajan 2003), *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* (Schwartz et al., 2014). On the other hand, experiments with IgE- or FcεRIα-deficient mice in other studies showed no effect or decreased parasite burden in infections with *H. polygyrus* (McCoy et al., 2008), *Strongyloides venezuelensis* (Matsumoto et al., 2013) or *S. mansoni* (Jankovic et al., 1997). Among the factors potentially contributing to these discrepancies, one could cite differences in experimental approaches (transgenic [IgE- or FcεRIα-deficient mice] or pharmacological [anti-IgE treatments]), the experimental model and/or the genetic background of the mice (Mukai et al., 2016).

4.2.2. Host defense against venoms

Toxic substances, such as venoms, represent an obvious threat for mammals, against which defense mechanisms are needed. In 1991, Margie Profet proposed a theory known as the "toxin hypothesis", suggesting that allergic immune responses (*i.e.*, IgE-associated type 2 immune responses and effector cell-mediated allergic reactions) represent an immunological defense against toxins (Profet, 1991). According to this theory, the purpose of an acute allergic reaction (manifested by, *e.g.*, scratching, vomiting, diarrhea, and, in extreme cases, anaphylaxis) is to respond rapidly and avoid, eliminate and/or neutralize toxic substances indicative of life-threatening situations (Palm, Rosenstein, & Medzhitov, 2012; Profet, 1991).

Recently, Profet's hypothesis was supported by experimental evidence demonstrating that IgE antibodies could contribute to acquired resistance against honeybee and snake venoms (Marichal et al., 2013; Palm et al., 2013; Starkl et al., 2016). Marichal, Starkl et al. characterized the immune response of mice following subcutaneous injection of whole bee venom to mimic bee stings (Marichal et al., 2013). The venom induced a robust adaptive type 2 immune response associated with development of venom-specific T_H2 cells and IgE, and this acquired immune response was associated with increased resistance of mice (quantified by survival and body temperature) against a subsequent challenge with bee venom. Experiments involving passive immunization and transgenic animals deficient in IgE or FcεRI demonstrated that IgE antibodies and IgE effector mechanisms played a crucial role in mediating acquired host resistance against bee venom (Marichal et al., 2013). In a complementary study, Palm, Rosenstein et al. provided experimental evidence that a type 2 immune response directed against the bee venom component phospholipase A2 (PLA2) was able to confer protection against a subsequent near lethal dose of PLA2, and that such protection was dependent on FcεRI (Palm et al., 2013). Subsequently, Starkl, Marichal et al. found that IgE effector mechanisms also played a critical role in acquired host defense against the venom of the Russell's viper (Starkl et al., 2016).

The strong evidence for the important protective function of IgE and IgE effector cells in immune defense against venoms in mice challenges the current view of the function of IgE in (venom-) allergic humans (Artis, Maizels, & Finkelman, 2012). Therefore, future investigations are needed to determine whether IgE-associated responses can enhance resistance to other toxins, and to understand why, in some species or individuals, exposure to the same venom or venom component may induce either a protective IgE-dependent adaptive immune response, as in the mouse studies described above (Marichal et al., 2013, Palm et al., 2013, Starkl et al., 2016), or a deleterious and potentially fatal allergic reaction (*i.e.*, anaphylaxis) (Charavejasarn, Reisman, & Arbesman, 1975; Saelinger & Higginbotham, 1974). This question is of great interest and relevance for basic and clinical allergy research.

5. Targeted anti-IgE therapies

5.1. Anti-IgE antibodies

5.1.1. Omalizumab

Omalizumab is a recombinant humanized IgG1 monoclonal antibody directed against human IgE sold by Novartis and Genentech under the trade name Xolair® (Presta et al., 1993). It binds to the Cε3 domain of free IgE, and thereby impairs binding of IgE to both FcεRI and CD23 (Chang et al., 1990; Davies et al., 2017; Selb et al., 2017) (Fig. 5). Importantly, omalizumab does not recognize IgE already bound to FcεRI or CD23, and therefore cannot induce cell activation by crosslinking of IgE receptors (Chang et al., 1990; Davies et al., 2017).

The IgE binding site of omalizumab has been characterized recently by molecular modeling and crystallography (Davies et al., 2017; Pennington et al., 2016; Wright et al., 2015; Zheng et al., 2008). Omalizumab binds to symmetric sites on the two IgE Cε3 domains: it does not directly mask the FcεRI binding site on IgE, but rather induces

major conformational changes in the Cε3 domains that inhibit interaction with FcεRI (Davies et al., 2017, Pennington et al., 2016, Wright et al., 2015, Zheng et al., 2008). Davies and colleagues reported that, furthermore, IgE binding to CD23 is sterically hindered by Omalizumab due to overlapping binding sites on each Cε3 domain (Davies et al., 2017). While omalizumab is alleged to be unable to bind IgE already bound to FcεRI, *in vitro* data suggest that omalizumab could also facilitate the dissociation of FcεRI-bound IgE (Eggel et al., 2014).

The first randomized, double blind, placebo controlled trials were conducted in 1996 to assess the tolerability and efficiency of omalizumab in patients with allergic asthma (Boulet et al., 1997; Fahy et al., 1997). These trials showed a reduction of free serum IgE levels (but an increase in total serum IgE, *i.e.* free IgE and IgE complexed with omalizumab), and improved responses to inhaled allergens following omalizumab therapy (Boulet et al., 1997, Fahy et al., 1997). In addition to the reduction of free serum IgE levels, treatment with omalizumab also induced a decrease in the expression of FcεRI on the surface of basophils, DCs and mast cells (Lin et al., 2004; Prussin et al., 2003; Saini et al., 1999). In 2003, Xolair® was approved for the treatment of moderate to severe persistent allergic asthma, and is now also approved for the treatment of chronic spontaneous urticaria (CSU) (Chang et al., 2015; Maurer et al., 2013; Zhao et al., 2016). In addition, more than 150 clinical trials of omalizumab are now listed on the website clinicaltrials.gov, in various diseases including food and venom allergies (in combination with allergen-specific immunotherapy), allergic rhinitis or mastocytosis. It is, however, important to note that, although Xolair® is generally well tolerated, it can induce side effects ranging from skin inflammation (at the site of subcutaneous injection) to systemic anaphylaxis (in 0.1–0.2% of patients) (Harrison, MacRae, Karsh, Santucci, & Yang, 2015; Lieberman, Umetsu, Carrigan, & Rahmaoui, 2016).

5.1.2. Ligelizumab

Ligelizumab (QGE031) is a more recent humanized anti-IgE antibody developed by Novartis. It is also directed against Cε3, but is designed to achieve improved IgE suppression, with an equilibrium dissociation constant (K_D) of 139 pM (as compared to the K_D of omalizumab, ~6–8 nm) (Arm et al., 2014) (Fig. 5). The first clinical results of ligelizumab treatment indicated that this antibody can reduce free-IgE and basophil FcεRI with an efficiency superior to that of omalizumab (NCT01716754). Although the authors did not observe serious adverse events in this study, one patient treated with ligelizumab developed systemic symptoms (Arm et al., 2014). In 2016, ligelizumab was tested in patients with mild allergic asthma, and was shown to have greater efficacy than omalizumab on inhaled and skin allergen responses in these patients (NCT01703312) (Gauvreau et al., 2016). However, in a more recent phase II field study of asthma patients, ligelizumab was not seen to be superior to omalizumab (NCT01716754), and further development for asthma has been discontinued.

5.1.3. Quilizumab

Quilizumab (MEMP1972A) is a humanized monoclonal antibody developed by Genentech targeting the M1′ epitope which is present on membrane IgE (mIgE) but not on serum IgE (Fig. 5). Brightbill and colleagues demonstrated, using genetically modified mice that contained the human M1′ domain inserted into the mouse IgE locus, that quilizumab could reduce serum IgE and deplete IgE-producing plasma cells *in vivo*, without affecting other immunoglobulin isotypes (Brightbill et al., 2010). Quilizumab has been tested in clinical trials in patients with allergic rhinitis (NCT01160861) and mild allergic asthma (NCT01196039) (Gauvreau et al., 2014). In both studies, reductions in total and allergen-specific serum IgE were observed, as well as improved clinical responses to allergen, suggesting that targeting mIgE can reduce IgE production in humans (Gauvreau et al., 2014). In a subsequent trial (NCT01582503), treatment with quilizumab also reduced total and allergen-specific IgE in patients with allergic asthma uncontrolled by standard therapy. However, treatment with quilizumab had no impact on asthma exacerbations, lung functions, or patient-reported symptoms in this trial (Harris et al., 2016). Similarly, quilizumab reduced IgE levels by about 30% in CSU patients, but it did not lead to clinical improvements in patient's self-reported itch-severity scores (NCT01987947) (Harris et al., 2016).

5.1.4. XmAb7195

XmAb7195 is a monoclonal anti-IgE antibody developed by Xencor through humanization, affinity maturation, and Fc engineering of the murine parental antibody of omalizumab (MaE11) (Chu et al., 2012). XmAb7195 has an IgE-binding affinity 5.3-fold higher than that of omalizumab. In addition, two point mutations in the IgG1 Fc portion of the mAb (G236R and L328R) increase the binding affinity to inhibitory IgG receptor FcγRIIB by 400 times compared to omalizumab (Chu et al., 2012). The authors demonstrated that XmAb7195 could block free IgE and inhibit IgE production in B cells through co-engagement of mIgE and FcγRIIB (Chu et al., 2012) (Fig. 5). In a first-in-human phase 1a trial in healthy volunteers (NCT02148744), XmAb7195 decreased IgE levels below the limit of detection in 90% of subjects that had detectable IgE levels at baseline. Transient thrombocytopenia was observed at a dose of 3.0 mg/kg, but no other major adverse events were reported (Gershman, Goldwater, & Forster, 2016). A phase 1b study on the safety, tolerability and bioavailability of a subcutaneous formulation of XmAb7195 has been recently completed (NCT02881853), but the results of this study have not yet been reported.

5.1.5. MEDI4212

MEDI4212 is a human IgG1 anti-IgE antibody developed by MedImmune. MEDI4212 was generated using phage display technology, combined with targeted mutagenesis of V_H and V_L sequences to increase its affinity for IgE (Cohen et al., 2014). Like omalizumab, MEDI4212 does not recognize IgE already bound to FcεRI, but the authors report that MEDI4212 binds free IgE with an affinity of 1.95 pM, more than 100-fold higher than omalizumab (Cohen et al., 2014) (Fig. 5). Analysis of the crystal structure of IgE Cε3-4 domains in complex with MEDI4212 Fab portion revealed that MEDI4212 recognizes residues in the Cε3 and Cε4 domains, and targets critical residues in Cε3 also involved in binding to FcεRI. This suggests that MEDI4212 directly competes with FcεRI for IgE binding (Cohen et al., 2014).

Since MEDI4212 recognizes residues in the IgE Cε3-4 domains, it can also bind mIgE on the surface of B cells. MEDI4212 was further engineered in order to increase its potential to eliminate IgE-expressing B cells through antibody-dependent cell-mediated cytotoxicity (ADCC) (Nyborg et al., 2016). The authors chose to insert mutations in the Fc portion of MEDI4212 in order to improve its affinity for the IgG receptor FcγRIIIA, as ADCC can be performed by natural killer (NK) cells that express FcγRIIIA. Indeed, *in vitro* experiments revealed that, thus Fc-engineered, MEDI4212 could eliminate class-switched human IgE B cells more efficiently (Nyborg et al., 2016). A phase I study on the pharmacokinetics, pharmacodynamics, and safety of MEDI4212 in subjects with atopy was initiated in 2012 (NCT01544348); and demonstrated that MEDI4212 rapidly reduced free IgE to a greater extent than omalizumab. However, recovery of free IgE to baseline was much faster in patients receiving MEDI4212 as compared as omalizumab, which was attributed to a rapid decrease of serum MEDI4212. Since then, no other study has been initiated with this antibody.

5.2. Anti-IgE, anti-FcεRI and anti-CD23 DARPins

Designed ankyrin repeat proteins (DARPins) are engineered small proteins that can recognize targets with high specificity and with affinity in the low nanomolar range (Binz et al., 2004; Pluckthun, 2015). In 2009, Eggel and collaborators reported identification of two monovalent DARPins, termed B-A4-85 and C-A3-30, displaying high affinity for two

different epitopes on human FcεRIα (Eggel, Baumann, Amstutz, Stadler, & Vogel, 2009). They further produced a bispecific anti-FcεRIα DARPin (designated 30/85) by linking sequences of the two monovalent DARPins with a $[Gly_4–Ser]_4$ linker. Remarkably, this bispecific DARPin showed greater affinity than IgE for FcεRIα, and was able to inhibit IgE-FcεRIα interaction and IgE-mediated degranulation of rat basophilic leukemia cells expressing human FcεRIα (RBL-2H3-huα cells), with an effect similar to that of omalizumab (Eggel et al., 2009) (Fig. 5).

Using a similar strategy, the same group reported identification of several DARPins binding human IgE (Fig. 5). Among these, the DARPins E2_79 and E3_54 were able to inhibit binding of IgE to either FcεRIα or omalizumab, and inhibit IgE-mediated activation of RBL-2H3-huα cells with higher efficacy than omalizumab (Baumann, Eggel, Amstutz, Stadler, & Vogel, 2010). It was further demonstrated that E2_79 not only prevented binding of free IgE to FcεRI, but also actively disrupted pre-formed IgE:FcεRI complexes (Kim et al., 2012). Such facilitated IgE dissociation was observed both *in vitro*, *ex vivo* in primary human basophils, and *in vivo* in human FcεRI transgenic mice (Eggel et al., 2014; Kim et al., 2012), suggesting that anti-IgE DARPins might be suitable drug candidates to desensitize allergic patients.

Another DARPin (E3_53) can recognize both free IgE and IgE bound to FcεRI. This DARPin was linked to the Fc portion of human IgG1 (using a $[Gly_4–Ser]_3$ linker) to produce a fusion protein capable of cross-linking FcεRI-bound IgE with the inhibitory receptor FcγRIIB. This molecule, termed DE53-Fc, was able to reduce allergen-induced basophil activation *ex vivo* using whole blood samples from allergic patients (Eggel et al., 2011). Furthermore, by using blocking antibodies against FcγRIIB, the authors demonstrated that binding of DE53-Fc to FcγRIIB was required for full inhibitory properties of the fusion molecule (Eggel et al., 2011). Confirming this mode of action, it was later reported that mutant forms of DE53-Fc displaying enhanced affinity for FcγRIIB also have greater capacity to inhibit basophil activation (Buschor, Eggel, Zellweger, Stadler, & Vogel, 2014). However, while mouse basophils and mast cells and human basophils express high levels of FcγRIIB, it is still ambiguous whether human mast cells also express this inhibitory receptor (Zhao et al., 2006). Therefore, whether cross-linking of FcεRI-bound IgE to FcγRIIB could inhibit IgE- and mast cell-mediated responses in humans remains an open question.

More recently, two DARPins (D86 and D89), which specifically recognize CD23, were also identified. These anti-CD23 DARPins inhibited binding of IgE to CD23 (which suggests that they share a similar binding epitope to IgE), and could inhibit IgE synthesis in human peripheral B cells (Fellmann, Buschor, Rothlisberger, Zellweger, & Vogel, 2015).

5.3. Fcε-Fcγ fusion proteins

The human Fcγ-Fcε bifunctional fusion protein consists of the Fc region of human IgG1 (hinge-Cγ2-3) linked to the Fc portion of human IgE (Cε2-4) by a 15 amino acid linker $(Gly_4Ser)_3$ (Zhu, Kepley, Zhang, Zhang, & Saxon, 2002). As first described by Zhu et al., this fusion protein (called GE2) was able to compete with IgE for the binding to FcεRI, and could thereby be used to 'desensitize' mast cells and basophils (Fig. 5). It could also bind to IgG FcγRs through its Cγ2-3 domains, and it was therefore proposed that GE2 could block IgE-mediated mast cell and basophil activation through co-engagement of FcεRI with the inhibitory receptor FcγRIIB (Zhu et al., 2002). Indeed, the authors demonstrated that GE2 was able to inhibit histamine release in primary human blood basophils sensitized with IgE, and could also block IgE-mediated passive cutaneous anaphylaxis (PCA) in transgenic mice expressing human FcεRI (Zhu et al., 2002). In addition to its effect on mast cells and basophils, it was proposed that the fusion protein could also inhibit allergic inflammation through effects on FcεRI-expressing Langerhans cells (Kepley, Zhang, Zhu, & Saxon, 2003), and inhibit IgE class switch recombination in B cells by co-aggregating CD23 and FcγRII (Yamada, Zhu, Zhang, & Saxon, 2003). Several attempts were subsequently made to improve the efficiency of the fusion protein, such as removal of the $(Gly_4Ser)_3$ linker, or mutations in the Cγ portion to improve binding to FcγRIIB and/or decrease binding to FcγRIII (Allen, Kepley, Saxon, & Zhang, 2007). However, most of these modifications altered the effectiveness of the fusion protein to inhibit FcεRI-mediated functions (Allen et al., 2007). Nevertheless, and as described above (Section 5.2), while basophils undoubtedly express FcγRIIB, it is still unclear whether human mast cells express FcγRIIB *in vivo* (Zhao et al., 2006).

The effects of GE2 were also tested in non-human primates. Rhesus monkeys have been reported to exhibit skin test reactivity and serum IgE directed against dust mites (Schelegle et al., 2001; Zhang et al., 2004). Taking advantage of this, Zhang and collaborators showed that GE2 was able to inhibit dust mite allergen-induced skin reactivity in rhesus monkeys in a dose-dependent manner (Zhang et al., 2004). In a subsequent study, GE2 demonstrated efficacy in a model of house dust mite-induced allergic asthma in cynomolgus monkeys (Van Scott et al., 2008). The effects of GE2 lasted for 4 weeks and were associated with reduced numbers of circulating basophils and reduced FcεRI expression on basophils. However, repeated injections of GE2 induced the production of serum antibodies against the fusion protein, and increased occurrence of serious adverse events, including anaphylaxis (Van Scott et al., 2008).

6. Concluding remarks

Discovered some 50 years ago, IgE continues to be the focus of extensive academic and industrial research. The clinical benefits of the anti-IgE antibody omalizumab best exemplify the key role of IgE in allergic diseases and chronic spontaneous urticaria. Besides omalizumab, several new anti-IgE therapies are now at various stages of clinical development, with some promising early results. Recent insights from crystallographic studies have also shed light on the mechanisms by which IgE antibodies recognize their main receptors FcεRI and CD23; findings that should help in the design of additional therapeutic approaches aimed at blocking these interactions.

While IgE can undeniably trigger allergic reactions, it is also now clear that not all allergies are IgE-mediated, and evidence from mouse models suggests that IgE may have protective functions in host defense against parasites and venoms. An ongoing effort is therefore necessary to clearly identify the full spectrum of IgE-mediated diseases, but also to address the potential limitations of targeted anti-IgE therapies.

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Conflict of interest statement

E.C. is an employee of Neovacs SA. L.L.R. reports serving as consultant for Neovacs SA. All other authors have declared no conflicts of interest.

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