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Precision engineering for localization, validation and modification of allergenic epitopes

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Abbreviations:

BLG	β-lactoglobulin
CDR	Complementary determining region/s
CRISPR	Clustered regularly interspaced short palindromic repeats
Cryo-EM	Cryo-electron microscopy
ELISA	Enzyme-linked immunosorbent assay/s
Fab	Fragment antigen-binding
HAT	Hypoxanthine-aminopterin-thymidine
HDX	Hydrogen-deuterium (^{1}H-^{2}H) exchange
hIgE mAb	Human IgE monoclonal antibodies
IMGT	ImMunoGeneTics
mAb	Monoclonal antibody/ies
NMR	Nuclear magnetic resonance
PBMC	Peripheral blood mononuclear cell/s
RT-PCR	Reverse transcription polymerase chain reaction
scFv	Single-chain variable fragment
scRNAseq	Single cell RNA sequencing

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Abstract

The allergen-IgE interaction is essential for the genesis of allergic responses, yet investigation of the molecular basis of these interactions is in its infancy. Precision engineering has unveiled the molecular features of allergen-antibody interactions at the atomic level. High resolution technologies, including X-ray crystallography, nuclear magnetic resonance spectroscopy, and cryo-electron microscopy determine allergen-antibody structures. X-ray crystallography of an allergen-antibody complex localizes in detail amino acid residues and interactions that define the epitope-paratope interface. Multiple structures involving murine IgG monoclonal antibodies have recently been resolved. The number of amino acids forming the epitope broadly correlates with the epitope area. The production of human IgE monoclonal antibodies from B cells of allergic subjects is an exciting recent development which has for the first time enabled an actual IgE epitope to be defined. The biologic activity of defined IgE epitopes can be validated *in vivo* in animal models or measuring mediator release from engineered basophilic cell lines. Finally, gene editing approaches using CRISPR to either remove allergen genes or make targeted epitope engineering at the source are on the horizon. This review presents an overview of the identification and validation of allergenic epitopes by precision engineering.

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Introduction

Allergens constitute one of the most well-defined groups of biomedically important proteins, yet analysis of IgE epitopes on allergens is in its infancy (**Table I**).[1-4] The very low levels of allergen specific IgE antibodies in sera, even from highly allergic patients, together with the polyclonality of IgE responses, have been persistent barriers to epitope analysis. Until recently, investigators were limited to indirect methods for identifying conformational epitopes. These epitopes involve residues that are close in space due to protein folding but are non-contiguous in the primary amino acid sequence. Evidence of the validity of conformational epitopes was largely circumstantial.

The term “engineering” in the context of allergens was introduced in a 1997 paper by Takai *et al.* who used site directed mutagenesis to disrupt intramolecular disulfide bonds on Der f 2. The engineered mutant Der f 2 showed a reduced skin test reactivity and basophil histamine release in mite allergic patients yet retained T cell stimulatory epitopes.[5] Similar data were reported for Der p 2 with cysteine variants showing ~100-fold reduction in IgE antibody binding.[6] The rationale was that allergen molecules engineered to reduce IgE reactivity with point mutations or deletions, or as multimeric proteins, would be safer for allergen immunotherapy. The approach was widely adopted to develop hypoallergens from many other allergens including Bet v 1, Phl p 5, Fel d 1 and Art v 1.[7-9] While many genetically engineered allergen variants have been constructed, they are of limited value for identifying IgE epitopes. In most cases, especially when loss of IgE binding to unfolded allergens is observed, these studies provide evidence that IgE antibodies to inhaled allergens are directed against conformational epitopes, rather than sequential or linear epitopes, made of consecutive amino acid residues.[10] Recent determination of the molecular structures of allergen-mAb complexes by X-ray crystallography and nuclear magnetic resonance spectroscopy has identified the contact amino acid residues that form conformational epitopes on several allergens (**Table II**).[11]

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For food allergens, it has long been considered that IgE antibody responses are directed against linear epitopes on 'small' peptides that are derived from digestion of food proteins in the gut. These peptides were successfully identified by screening overlapping peptides derived from allergen sequences for IgE reactivity, when bound to SPOTS membranes or other solid phases.[12, 13] Some of these IgE-binding peptides have disease associations. For example, persistent egg allergy is associated with IgE reactivity to four peptides on Gal d 1.[14] Selected peptides from food allergens are now being used to develop multiplex diagnostic tests for food allergy.[12, 15, 16] An important limitation of food epitope analyses is that they are based almost exclusively on testing IgE binding to peptides bound to solid phase. Few studies have investigated the capacity of peptides either alone or in combination to inhibit IgE responses to native allergen.[17] The relative importance of anti-peptide IgE to the repertoire of IgE responses to native food allergens is not known and there is growing evidence that some food allergen epitopes are conformational.[17-19]

For inhaled allergens, food allergens and other allergens, there are many unknowns concerning the localization, structure and repertoire of IgE B cell epitopes (**Table I**). With a few exceptions (noted in Table I) there is a paucity of data on allergen epitopes from trees, grass and weed pollens, molds, venoms, fish and shellfish. For allergens for which multiple epitopes are known to occur (e.g. Bla g 2, Der p 1, Der p 2, Ara h 2, Fel d 1), the structural information at present is limited in most cases to one to three epitopes (for IgG antibodies). Typically, small globular proteins (MW 10-20 kDa) contain 3-5 non-overlapping epitopes, each of which covers an area of 700-900 Å^2. The estimation of the number of non-overlapping epitopes for small allergens is facilitated by several reported structures of an allergen in complex with an antibody (e.g. Bla g 2, Der p 1[11]), and structures of an allergen in complex with two or three different antibodies simultaneously (**Figure 1**).[20, 21] This appears to be the case for epitopes on allergens that have been defined, but detailed mapping of allergenic surfaces is lacking.

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The known unknowns make the investigation of the molecular basis of IgE epitopes a ripe field for future study (**Table I**). A sophisticated array of molecular tools can be used for manipulating allergen sequences. Most importantly, human IgE antibody sequences are rapidly becoming available through the development of phage display technology to construct combinatorial IgE libraries, and panels of natural human IgE monoclonal antibodies (hIgE mAb) from allergic patients. The first structure of a human IgE antibody-allergen complex has recently been published.[22] Promising steps towards gene editing of allergen sequences using CRISPR technology have been reported.[23] This review focuses on current strategies for effectively identifying allergenic epitopes; for localization and definition of contact residues; and for validation of the biologic importance of IgE epitope(s) by genetic engineering and gene editing techniques.

Strategies for the identification of allergenic epitopes

Conformational epitopes

Conformational epitope mapping strategies consider the three-dimensional structure of the molecules involved in the allergen-IgE antibody interaction. Indirect ways to define conformational epitopes have mostly been used (including the use of chimeras, the identification of mimotopes, etc.), and the limitations of these indirect methods have been recently reviewed.[11, 24] In contrast, direct methods such as X-ray crystallographic analyses of allergen-antibody complexes, which provide comprehensive data of the interactions involved in antibody recognition (**Table II**), will be addressed here. Although structures of an antigen (egg lysozyme, Gal d 4) in complex with murine IgG mAb fragments were first reported in 1985 [25], the first structure of an allergen-antibody complex using murine IgG mAb known to interfere with IgE antibody binding was determined with the birch allergen Bet v 1.[11, 26] The allergen-antibody structures determined to date revealed the number of residues on the allergen (up to ~25) that participate in epitope-paratope recognition, and the nature of the interactions involved

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(hydrogen bonds, hydrophobic, etc.) (**Table II**). Selective mutations of a few residues in the epitope diminished murine mAb binding. Testing of mutants for their capacity to bind IgE also led to identification of potential IgE antibody binding sites.[27-30] Therefore, these studies also revealed that only a few amino acid residues were essential for mAb binding. As proof of principle, an epitope was created *de novo* on the mite allergen Der f 1 to confer specificity for the mAb 10B9 that is Der p 1-specific and does not bind Der f 1.[28] Seven rDer f 1 mutants were expressed, each containing 1-4 amino acid substitutions within the corresponding area of the mAb 10B9 epitope in Der p 1. As more substitutions were made in the Der f 1 mutants, more mAb 10B9 binding was observed in dose-response curves, with increased saturation levels of Ab binding, and increased affinities of the mutants for binding mAb 10B9. The mutation of three or four residues created an epitope with an affinity within the same order of magnitude as the full epitope in Der p 1. The newly engineered epitope also conferred to Der f 1 a higher capacity to bind IgE antibodies. Interestingly, the creation of a new mAb 10B9 binding site on Der f 1 showed that additional hydrogen bonds involving CDR other than the heavy chain CDR3, were required for mAb 10B9 binding.[28] This study is an example of precision engineering to generate an epitope with only few residues, and indicates that the CDR3 from the heavy chain, which is considered to be mostly responsible for antibody specificity, is not required for recognition by all mAb.[28, 31]

These crystallographic studies required pure and homogeneous protein preparations of the complex and used murine IgG mAb as surrogate antibodies for human IgE. Ideally, human IgE mAb should be used to identify IgE epitopes, but those have not been available until recently thanks to hybridoma technology, which led to the determination of the first structure of an IgE Fab in complex with the mite allergen Der p 2 (**Table II, Figure 2**).[22]

Loss of IgE binding to denatured allergens indicates that conformational IgE epitopes, especially from aeroallergens, are common.[10] Food allergens are processed (e.g. by heat denaturation) and cleaved by gastrointestinal digestion, which leads to changes in the native

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structure of the allergen and, potentially, to the induction of IgE against linear epitopes. The assumption is that digestion of the allergen is extensive, and short peptides mimic the fragments resulting from food processing or cleavage of the allergens through the digestive tract. However, it is difficult to imagine how short peptides could contain two epitopes that cross-link IgE. Complete digestion of allergens during food processing and/or digestion may not occur [32-34], and could result in IgE antibodies directed against fragments of allergens with defined three-dimensional structure, in addition to linear peptides. Strong evidence for the existence of conformational epitopes in food allergens was reported by Albrecht *et al.* who showed that unfolded Ara h 2 and peptides from Ara h 2 and Pen a 1, representing the identified sequences of linear epitopes, had no capacity to inhibit IgE antibody binding to the native allergen in ELISA or basophil activation tests.[17] Unfolded rAra h 2 showed reduced IgE-binding capacity compared with folded rAra h 2 and failed to elicit mediator release.[17] Therefore, there was little or no contribution of sequential epitopes to the IgE binding to this allergen in fluid phase antibody/target interactions. Additional evidence of conformational epitopes in food allergens exists.[18, 19, 35-38] For milk and egg allergens, for example, the known linear epitopes are not sufficient to explain IgE reactivity to the allergens from both sources. Predominance of IgE against conformational epitopes could explain the observed tolerance to cooked milk and egg.[35-38] In contrast, infants reactive to baked milk have a more severe phenotype to cow's milk allergy, with a higher risk of severe anaphylaxis and a more protracted course of disease.[39]

Linear epitopes

Most of the epitope mapping studies have been restricted to the identification of linear or sequential epitopes by design. In the 1990s, epitope mapping studies focused on testing the capacity of IgE antibodies to bind overlapping peptides covering the full length of the allergen, or to bind enzyme digests or recombinant allergen fragment bound to a solid phase (e.g. nitrocellulose or Sepharose).[40, 41] While these studies identified some IgE antibody binding

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residues, a possible drawback of synthesized overlapping peptides is that they might lack post-translational modifications that could be essential for IgE antibody binding (although peptides may also be synthesized with such modifications) (e.g. hydroxyprolines in peanut allergens).[42] These approaches to map linear epitopes evolved over time into the development of peptide microarrays[12, 13, 15] and bead-based assays [16, 43-49], which facilitated measurement of IgE antibody binding to peptides, bound to other solid phases (e.g. glass slides or polystyrene beads). Recently, extensive libraries of peptides have been generated to cover a large set of allergens: a) AllerScan, a programmable phage display library, covering all the protein sequences present in the Allergome database [50, 51], and b) a global microarray platform covering all possible linear overlapping 16-mer epitopes (> 17,000) of 731 allergens listed in the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature database.[52] These strategies allow patterns of peptide recognition by different isotype repertoires (IgG, IgG4 and IgE) to be analyzed, as well as the evolution of linear epitope recognition with age and at different times during immunotherapy, and show promise for allergy diagnosis and prediction of food tolerance.[15, 16, 43-52] On the other hand, cross-reactivity among food allergen epitopes complicates diagnosis and management of food allergies.[53] We suggest that the relative ease and low cost of studying peptide epitopes has led to a systematic bias in the literature favoring linear epitopes over the more difficult to determine conformational epitopes.

Definitive epitope identification using hIgE mAb

Despite the great advancements in understanding epitopes bound by human IgE antibodies, significant gaps in our knowledge remain due in part to the uncertainty of the indirect methodologies outlined above (**Table 1**). The 'ideal' approach is to use naturally occurring allergen-specific human IgE mAb obtained from allergic subjects, which are representative of the IgE antibody repertoire. Recent technological advances have allowed human IgE mAb to be

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produced using two alternative approaches: single cell RNA sequencing (scRNAseq) and human hybridoma generation.[54, 55]

Single B cell RT-PCR was originally used to obtain allergen-specific IgG antibody pairings[56], but this is not an efficient approach for IgE due to the low frequency of B cells encoding IgE in peripheral blood.[57] Heavy chain variable gene sequences of IgE antibodies were obtained from deep sequencing of peripheral blood mononuclear cells (PBMC), but did not result in the production of natural pairs of allergen-specific heavy and light chains.[58] With great progress in sequencing and the development of single cell technologies, scRNAseq can now capture naturally occurring IgE antibodies from allergic donors. Human IgE mAb against peanut allergens isolated using this approach were cloned from single B cell transcriptomes.[54] Convergent evolution was identified in which IgE antibodies underwent identical gene rearrangements in unrelated individuals.[54, 59] The scRNAseq approach has several advantages over the labor intensive hybridoma method, the greatest of which is that it can both capture IgE encoding memory B cells and plasmablasts. The disadvantages, including cost and the challenges associated with analyzing large transcriptomic datasets, are diminishing as sequencing technologies become cheaper and analytical tools more readily available.

Human hybridoma technology has been successfully employed to engineer allergen specific human IgE and to identify for the first time actual epitopes recognized by IgE antibodies.[22] This approach has previously been used to generate antigen specific IgG, IgA, and IgE antibodies to study human immunity to pathogens.[55, 60-64] It is highly versatile, scalable, and capable of identifying ultra-rare populations of circulating antibody encoding memory B cells. The ability to study the precise epitopes bound by the human IgE antibodies has been sought for decades. There are three major reasons why IgE mAb have not been made until now. The first is that techniques for efficiently making full-length human mAb by hybridoma technologies were not in place until a decade ago.[60, 61] The second, and most important reason, is that the frequencies of IgE producing B cells in the peripheral blood are exceedingly low. Even in the

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most allergic individuals, frequencies are often < 5 memory B cells per 10 mL of blood. Because these IgE B cells are so rare, the numbers that can be captured with a single blood draw are often not sufficient for previous recombinant mAb technologies, which include single cell sorting of antibody-secreting plasma cells or Epstein-Barr virus transformation of human B cells.[65-67] The third reason is the poor surface expression of membrane IgE (B cell receptor) on circulating IgE encoding plasmablasts (which grow poorly in culture) and memory B cells.[54, 68-70] Plasmablasts primarily secrete antibody, making it difficult for flow cytometry-based methods involving labeling of the B cell receptor to identify IgE encoding B cells.[68-70] These features compound the difficulty of detecting the ultra-rare populations of IgE producing B cells within PBMC samples. Studies describing the identification of IgE producing B cells may inadvertently use methods that destroy them, including staining intracellular IgE by fixation and permeabilization of cell membranes.[71, 72] For this reason, studies of IgE expressing B cells in culture have only involved artificially class-switched polyclonal cultures using the cytokine IL4.[73-75] Since IL4 class-switches B cells in a nonpredictive way (regarding origin/destination of isotype), allergen-specific human antibodies are not made.

Allergen-specific human IgE mAb have been generated from highly allergic symptomatic patients with rhinitis, asthma atopic dermatitis and eosinophilic esophagitis.[76] These patients have high levels of total and allergen specific serum IgE, and tend to have relative enrichment of allergen specific memory B cells.[77] Careful donor selection allows for allergen specific IgE mAb to be generated in an allergen unbiased manner, without the use of allergen at any time in the process. Following PBMC isolation, without cell depletion or selection, all memory B cells are expanded without concern for isotype or specificity, to produce sufficient cells to overcome the inefficiency of the myeloma fusion step. Culture wells containing an IgE isotype expressing B cell are identified using highly sensitive ELISA screening, again without regard for allergen specificity. In a typical highly allergic donor, IgE will be detected in approximately one out of every 100 culture wells (each plate containing ~2 million PBMC). IgE producing cells are fused

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to a myeloma cell partner using electrical cytofusion to create immortal memory B cell clones. The IgE secreting hybridoma is cloned by limiting dilution and single cell sorting by flow cytometry. Typically, it is only after an IgE mAb is generated that its specificity is determined. The number of IgE mAb obtained from an allergic donor depends on several factors, including the number, severity, and duration of the donor's allergies, their age, the volume of blood drawn, and the number of PBMC isolated.

The IgE mAb can be manufactured in serum free culture medium and purified using anti-IgE affinity chromatography. IgE antibody variable gene sequences can be amplified by RT-PCR and analyzed using the ImMunoGeneTics (IMGT) database to determine germline gene usages (V/D/J), the CDR sequences, variable gene mutation rates, and the presence of insertions and/or deletions (indels). Finally, recombinant mammalian expression of these variable sequences can be used to produce isotype switched variant IgG mAb, engineered antibody constructs, and Fab for epitope analyses (see Stone *et al.* review on antibody engineering in the same issue).

Localization and structure of IgE epitopes

Allergen-antibody interfaces have been analyzed in detail primarily by crystallography as shown in **Table II**. The analysis of the available epitopes identified on allergens shows that on average they have an area of approximately 800 Å^2, which can vary significantly from below 600 Å^2 for the smallest epitopes, to close to 1,000 Å^2 for the largest ones (**Figure 3**). Most often the epitopes are formed by approximately 20 residues (**Table II**), but this number can vary from slightly more than 10 residues to almost 30 residues (**Figure 3**). Usually, the heavy chain of the antibody is responsible for most interactions with the allergen, contributing on average almost 70% of the paratope-epitope area (**Table E1**). However, this can vary significantly. In Api m 2 and Hev b 8 complexes, the light chains contribute over 50% of the area.[78, 79] It is worth mentioning that in the complex of Bet v 1 with REGN5715, the heavy chain is responsible for

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over 90% of the area between paratope and epitope (**Figure 1, Table E1**).[20] Generally, the epitopes on the same allergen can differ significantly in their size and number of residues. In some cases, even a relatively small number of residues can form a large epitope.

A detailed description of IgE-allergen interactions allows for a rational design of modified versions of allergens that potentially can be used for immunotherapy. For example, the mutated version of Der p 2, which was designed based on the analysis of a complex formed between the allergen and an hIgE mAb (2F10), prevented anaphylaxis in a mouse model.[22] The 2F10 Fab is currently the only example of an hIgE mAb construct to have its structure determined (**Figure 2**). The heavy and light chains of 2F10 are properly paired, whereas there is no such certainty in the case of other IgE fragments derived from combinatorial libraries, where heavy and light chains are randomly paired. Therefore, the use of hIgE mAb has become not only the best approach to get accurate information on allergy relevant epitopes but it is also the most direct and efficient strategy for engineering allergens for future use in allergy treatment. Human IgE mAb can be used for X-ray crystallography, cryo-electron microscopy (cryo-EM) and nuclear magnetic resonance (NMR) spectroscopy studies. An additional application is their use in protection assays combined with mass spectrometry (MS). In such cases, the complex can be modified by using the hydrogen-deuterium (^{1}H-^{2}H) exchange (HDX) technique. The hydrogens protected in the antigen-antibody interface have a lower ^{1}H-^{2}H exchange rate. After exposure to ^{2}H, the complex is cleaved, the ratio of ^{1}H:^{2}H in the cleavage products is measured and the binding area is identified using MS. The HDX-MS technique can be successfully used for identification of not only epitopes and paratopes, but also sites that are responsible for other protein-protein interactions.[80-82]

Other techniques: NMR and Cryo-EM

X-ray crystallography provides the highest resolution chemical detail of allergen-antibody complexes. Both NMR spectroscopy and cryo-EM can also generate atomic level detail of

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molecular interactions. NMR has been primarily utilized to determine the structures of small individual allergens, as compared to complexes with antibodies.[83-85] Larger complexes with antibodies or antibody fragments are more difficult because signal to noise suffers and information content drops as molecular weight increases using NMR. Nevertheless, atomic information about epitopes can be gleaned from a variety of specialized NMR techniques.[11] Focusing on Der p 2, epitope regions were proposed by NMR for murine IgG and human IgE antibodies prior to subsequent verification by crystallography.[22, 86] In this case, the epitope data provided by NMR relied on the methyl resonances of isoleucine, leucine, and valine residues that were examined before and after forming the complex with IgG or IgE mAb. Using different probes like uniform ^{15}N labeling, more complete models of epitopes can be deduced for smaller complexes, like that of an Fab with Blo t 5 or an scFv with Gad m 1.[87, 88] Note that in all these cases the data represents a limited set of atoms for binding information compared to the all-atom models of crystallography or cryo-EM. Hence, defining the epitope is likely to be less precise. Complete atomic models of scFv and Fab complexes with antigens have been determined by NMR,[89] however to the best of our knowledge this has not been reported for an allergen.

In contrast to NMR, cryo-EM has difficulty with small molecules, but excels at structures of very large complexes. Indeed, the only reported cryo-EM structure of an allergen complex is Bet v 1 with 3 Fab fragments attached simultaneously, making the complex about 140 kDa (**Figure 1**).[20] Currently, complexes around 100 kDa structures are feasible,[90] which is approximately 2 Fab + 1 allergen. Images of the complexes in vitrified ice are compared and signal averaged to create all-atom models of the complex. In the Bet v 1_1-Fab_3 complex, the epitope regions were clearly delineated. However, compared to the crystallographic structures, the cryo-EM structure provided less precise information of the details of the molecular interactions. For example, in the Bet v 1 structure there are several lysine side chains close to the Fab that could be making important interactions. However, the side chains are modeled in

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awkward conformations pointing away from possible stronger interactions likely due to the lack of definitive data. This means that if one is trying to engineer hypoallergens, it may require making more mutants to find key residues using cryo-EM or NMR as opposed to crystallography.

Precision engineering in allergy using CRISPR

Recent innovations in genome engineering, particularly Clustered Regularly Interspaced Short Palindromic Repeats technology (CRISPR), present exciting opportunities in allergy, such as removing allergen genes from the source or editing allergenic epitopes to produce hypoallergens. CRISPR systems employ small, modifiable guide RNAs that direct CRISPR-associated proteins to cleave a DNA target sequence (**Figure 4**).[91, 92] The high specificity of CRISPR systems, which is determined by the short guide RNA sequences, enables editing a genomic target with remarkable precision.[93]

To date, CRISPR has been applied for the targeted deletion of allergens with the aim of better understanding allergen proteins and providing improved, alternative treatment options for allergic disease.[23] Several recent applications have demonstrated the feasibility of knocking out inhaled allergens using CRISPR-Cas9, such as the development of pollen-free Japanese cedar trees.[94] CRISPR-Cas9 was used to knock out the major allergen produced by domestic cats, Fel d 1, in a non-expressing cell line.[95] *In vitro* CRISPR knockouts of the Fel d 1 genes, *CH1* and *CH2*, resulted in editing efficiencies of up to 55%, confirming that the allergen is a viable target for gene deletion in an approach to create Fel d 1-free cats. Accompanying comparative sequence analyses of Fel d 1, of which the biologic function in cats remains unknown, revealed that the allergen genes may not be conserved or biologically essential for cats.[95] Deleting Fel d 1 in cats using CRISPR technology could significantly benefit cat allergy sufferers by removing the allergen from the animal, and may provide further insight into the definitive function of the Fel d 1 protein.

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Major food allergens from hen's egg, milk, soybean, peanut, and wheat have also been targeted with CRISPR gene editing (**Figure 4**). CRISPR-Cas9 demonstrated editing efficiencies of up to 90% in knockouts of egg white proteins in chicken primordial germ cells.[96] Targeting β-lactoglobulin (BLG), a milk whey protein allergen, with CRISPR-Cas9 in goat embryos resulted in editing efficiencies of ~25% and knockout goats with significantly less BLG protein expression in milk.[97] Preliminary knockouts of two allergenic soybean proteins demonstrated proof-of-principle for developing hypoallergenic soybean plants, while proposed CRISPR knockouts of several major allergens in peanut highlight the therapeutic potential of CRISPR in tackling life-threatening allergic reactions to peanut.[98, 99] Alternatively, knocking out immunodominant epitopes in α-gliadin gluten proteins using CRISPR-Cas9 resulted in editing efficiencies of up to 75% in an approach to produce bread and durum wheat lines that lack epitopes associated with celiac disease.[100]

Though the above applications demonstrate the utility of CRISPR-Cas9 in knocking out allergens with targeted DNA double-strand breaks, alternative CRISPR systems such as base or prime editors will enable precise, single nucleotide edits at specified DNA targets for the modification of critical IgE epitopes and the subsequent development of hypoallergens (**Figure 4**).[101-103] In contrast with the production of hypoallergens by removing specific IgE epitopes using conventional recombinant technologies[104], the latest CRISPR systems will allow their production directly from the allergen source. Precision engineering with CRISPR base or prime editors could also be applied to impair IgE recognition of allergen epitopes while preserving the underlying function of the protein, similar to a recent approach to render immune cells unrecognizable by anti-CD45 CAR-T cells.[105] While CRISPR base or prime editing have yet to be applied in allergy, these systems have shown therapeutic promise in preclinical and clinical studies of several monogenic disorders, including sickle cell disease, T cell acute lymphoblastic leukemia, and Duchenne muscular dystrophy.[106, 107] In the future, CRISPR editing in allergy could further inform the allergen-IgE interaction or reveal essential functions of allergen

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proteins, and CRISPR-engineered (hypo)allergens may serve as valuable therapeutic alternatives for managing and treating allergic disease.

Limitations and Challenges

Limitations and challenges for identifying IgE antibody epitopes in structural studies are: 1) obtaining the hIgE mAb of the desired specificity, 2) the need for multiple screening allergens to determine IgE mAb specificity, and 3) the need for homogenous and properly folded allergen and hIgE mAb molecules. Recombinant allergens might not always fold correctly or might lack post-translational modifications that occur in the natural counterpart and that might be needed for IgE antibody binding (e.g. glycosylation or hydroxylation of prolines as in peanut Ara h 2).[42, 108] Proof of IgE mAb binding to the allergen indicates proper folding of both molecules, which can be confirmed by CD spectra or NMR analyses. Another potential challenge that could impair or prevent crystallization, is that natural allergens might either have molecular impurities (including other allergens) or several variants or isoforms. Nevertheless, this last possibility did not prevent the determination of the structure of group 1 mite allergens with Fab from murine IgG mAb. Fortunately, the crystals contained a major isoform (Der f 1.0101 and Der p 1.0105 in complex with the Fab from the IgG mAb 4C1), despite the large number of isoforms listed for these allergens in the WHO/IUIS Allergen Nomenclature database.[109]

The structures of allergen-antibody complexes provide information on how the human humoral immune system interacts with allergens. There are potential limitations of focusing on a few antibodies given the fact that IgE responses are polyclonal and vary among patients. However, once a group of IgE mAb recognizing different epitopes on an allergen have been identified, they can be pooled and compared with polyclonal IgE responses in allergic patients (which has not previously been possible). In addition, mutagenesis analyses of each epitope allows to identify the relevance of that epitope versus the full allergen-specific IgE repertoire and

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its degree of dominance in a population of subjects sensitized to that allergen, as it has been reported for the dominant IgE mAb 2F10 epitope on Der p 2 (**Figure 2**).[22]

A primary challenge of CRISPR engineering is the potential for off target editing in unintended genomic sites.[110] This risk is mitigated by employing bioinformatics tools to predict or detect genome-wide, CRISPR-mediated DNA breaks, or by using alternative CRISPR systems with reduced off target potential.[101-103, 111-113] Polyploid genomes pose the additional challenge of targeting several alleles simultaneously to achieve a successful knockout.[114] Nevertheless, the high efficiency and versatility of CRISPR systems will undoubtedly improve polyploid genome engineering compared to preceding technologies, with successful CRISPR editing already reported in several plant species including wheat.[100, 114]

Focused Conclusions

Despite the worldwide increase in allergies in the last 30 years, and the continuous discovery of new allergens from different sources (www.allergen.org), a knowledge gap remains regarding how specific interactions between allergens and IgE antibodies occur. Epitope mapping studies identify specific residues on allergens, which are recognized by IgE antibodies. Key elements of these studies involve high resolution analyses of allergen-antibody complexes by X-ray crystallography, NMR and cryo-EM, coupled with the use of hIgE mAb derived from subjects with allergic disease to define allergenic epitopes. Large panels of hIgE mAb to inhaled allergens, food allergens, alpha-gal and parasite antigens have been produced to facilitate analysis of IgE B cell epitopes. Clinically, IgE epitopes allow the specificity and cross-reactivity of homologous allergens to be defined, which is useful for diagnostic purposes. Potential therapeutic applications include engineered modified allergens, with reduced IgE reactivity, to reduce side effects from immunotherapy. Finally, recent clinical trials showed that non-overlapping IgG monoclonal antibodies to Fel d 1 can block IgE antibody binding to the allergen.

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The two anti-Fel d 1 IgG antibodies reduced symptoms in patients with cat allergy and prevented cat allergen–induced early asthmatic responses in an environmental exposure unit.[115, 116] Similar trials showed that three Bet v 1-specific IgG antibodies blocked the birch pollen allergic response.[20] The use of IgE-blocking IgG antibodies is another interesting option for future therapies.

Suggestions for future work

i) Continued structural analysis of the epitopes recognized by hIgE mAb on inhalant allergens and food allergens, with the goal of mapping the entire allergenic surfaces of these molecules. This will be especially important for identifying the actual IgE epitopes on food allergens and whether they are linear or conformational.

ii) While current epitope mapping techniques provide spectacular atomic detail, there is significant effort involved in every single complex structure. Development of higher throughput approaches is needed to elucidate the repertoire(s) of IgE epitopes from allergic patients and, possibly, patient-specific epitope repertoires. If certain epitopes are common, they could be targets for hypoallergen design, or markers of disease severity or therapeutic success.

iii) Applying the latest CRISPR technologies, base and prime editors, as a more efficient approach to traditional mutagenesis for the identification and development of hypoallergens at the source, or other beneficial mutations in allergen molecules.

iv) Clinical studies that will be designed to compare the repertoires of IgE epitopes in children and adults with different allergic conditions.

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Table I.

Allergenic Epitopes: What is Unknown

- Except for Bet v 1 and Phl p 2, very little is known about the nature of allergenic epitopes on tree, grass and weed pollen allergens.
- The structure of natural heterodimeric cat allergen Fel d 1 and the location of epitopes on this important allergen.
- Precise mapping and structural analysis of IgE epitopes on allergens for which multiple non-overlapping human IgE mAb are available (e.g. Der p 2, Ara h 1, Ara h 2, Ara h 3, Ara h 6).
- The repertoire and epitope diversity of natural human IgE responses and whether there are immunodominant IgE binding sites.
- For food allergens, validation of the relative importance and biologic activity of linear epitopes; and structural analysis of putative conformational epitopes.
- The structure of epitopes on mold allergens, venoms, fish, shellfish tropomyosin allergens, tree nuts, and important mammalian allergens, such as the lipocalins.
- Whether IgE (or IgG) antibodies to specific epitopes are biomarkers of either disease severity or therapeutic importance?

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Table II. Structures of allergen-antibody complexes

Allergen in complex with murine IgG antibody			
Allergen/Source	**Antibody**	**PDB code[a]**	**# residues in epitope/# H bonds[b]**
Api m 2/Honeybee	Fab; mIgG1 mAb 21E11	2j88[c79]	12/13
Bet v 1/Birch	Fab′; mIgG1 mAb BV16	1FSK[c26]	20/10
Bet v 1/Birch	Fab; mIgG4 mAb REGN5713[e]	7N0U[20]	20/6
Bet v 1/Birch	Fab; mIgG4 mAb REGN5715[e]	7N0V[20]	13/11
Bet v 1/Birch	Fab; mIgG4 mAb REGN5713 [e] Fab; mIgG4 mAb REGN5714 [e] Fab; mIgG4 mAb REGN5715 [e]	7MXL[20] (cryo-EM)	21/4 28/16 17/9
Bla g 2/German cockroach	Fab′, mIgG1 mAb 7C11	2NR6[c117]	23/17
Bla g 2/German cockroach	Fab, mIgG1 mAb 4C3	3LIZ[c118]	23/11
Der f 1/House dust mite	Fab; mIgG1 mAb 4C1	5VPL[109] (3RVV)[cd]	18/7
Der p 1/House dust mite	Fab; mIgG1 mAb 4C1	1) 5VPG[109](3RVW)[cd] 2) 5VPH[109](3RVX)[cd]	1) 16/11 2) 20/12
Der p 1/House dust mite	Fab; mIgG1 mAb 5H8	5VCN[119] (4PP1)[cd]	21/14
Der p 1/House dust mite	Fab; mIgG1 mAb 10B9	5VCO[119] (4PP2)[cd]	20/11
Der p 2/House dust mite	Fab; mIgG1 mAb 7A1	6OY4[c30]	17/7
Fel d 1/Cat	Fab; mIgG4 mAb REGN1909[e]	5VYF[116]	17/9

Gal d 4[g]/Chicken		1) 3HFM[120] 2) 1FDL[121] 3) 1MLC[122] 4) 1YQV[123]	1) 22/13 2) 10/13 3) 16/10 4) 19/13
Allergen in complex with human IgG antibody			
Allergen/Source	**Antibody**	**PDB code**	**# residues in epitope/# H bonds[b]**
Ara h 2/Peanut	rFab; 22S1 mAb[f] rFab;13T1 mAb[f]	8DB4[21]	14/14 22/14
Gal d 4[g]/Chicken	Human V_H domain (VH H04)[h]	1) 4PGJ[124] 2) 4U3X[124]	1) 21/11 2) 22/19
Phl p 7/Timothy grass	Fab; hIgG1 mAb 102.1F10[i]	5OTJ[125]	17/9
Allergen in complex with IgG antibody containing human IgE variable regions from phage display combinatorial libraries			
Allergen/Source	**Antibody**	**PDB code**	**# residues in epitope/# H bonds[b]**
Bos d 5/Cow	Fab; hIgG1 mAb D1[j]	2R56[126]	25/11
Phl p 2/Timothy grass	Fab; hIgG1 mAb huMab2[j]	2VXQ[c127]	21/15
Allergen in complex with murine IgE antibody			
Allergen/Source	**Antibody**	**PDB code**	**# residues in epitope/# H bonds[b]**
Hev b 8/Latex	rFab; mIgE mAb 2F5	1) 7SBD[78] 2) 7SBG[78]	23/11 21/10
Allergen in complex with human IgE antibody			
Allergen/Source	**Antibody**	**PDB code**	**# residues in epitope/# H bonds[b]**
Der p 2/House dust mite	rFab; hIgE mAb 2F10[k]	7MLH[22]	15/10

Footnotes to Table II

[a] References for all the structures in the table are indicated as a superscript to the PDB code and most were reviewed at[11]. In 7MXL, three anti-Bet v 1 IgG4 antibodies were co-crystallized with the allergen in a quaternary complex.

[b] PDBePISA was used to calculate the number of residues and H-bonds.[128] Residues contributing at least 2.0 Å^2 to the epitope area were included, and 3.30 Å distance cutoff was used for identification of H-bonds.

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[c] Manuscripts that report inhibition of IgE antibody binding by the antibody used in the X-ray crystal structure (or *vice versa*).

[d] Original published PDB code in parenthesis was replaced by the definitive one shown.

[e] Fully human Bet v 1 and Fel d 1 specific IgG4 mAb were generated using the VeloImmune human antibody mouse platform.[20] Human antibodies were made by a mouse immune system and do not necessarily target the same distribution of epitopes that may be seen in humans.

[f] Recombinant IgG1 antibodies were obtained from paired H and L chains that were amplified, sequenced and cloned from single cell sorted Ara h 2 specific B cells from allergic subjects. The two mAb were co-crystallized with the allergen in a 22S1Fab–13T1Fab–Ara h 2 ternary complex.

[g] Only selected complexes with lysozyme are listed. For example, complexes of human VH domains with lysozyme were chosen to compare them with complexes formed by Fabs.

[h] VH H04: Phage displayed

[i] hIgG1 mAb 102.1F10 was expressed based on a hIgG4 that was generated from matched heavy and light chain sequences by single B cell cloning from allergic individuals.

[j] CK and CH1 of IgG1 cloned with IgE VH/VL isolated from human IgE derived from a combinatorial library.

[k] hIgE mAb 2F10 obtained by hybridoma technology using B cells from allergic subject.

Figure Legends

Figure 1. A. Crystal structure (PDB: 8DB4) of Ara h 2 in complex with two Fab fragments of antibodies 13T1 and 22S1. **B.** Cryo-EM structure (PDB: 7MXL) of Bet v 1 in complex with three different Fab fragments of antibodies REGN5713, REGN5714 and REGN5715. Molecules are shown in space filling representation. Heavy chains are marked using darker colors. The figure shows that there is a limited number of antibodies that can simultaneously bind to an allergen due to steric restrictions.

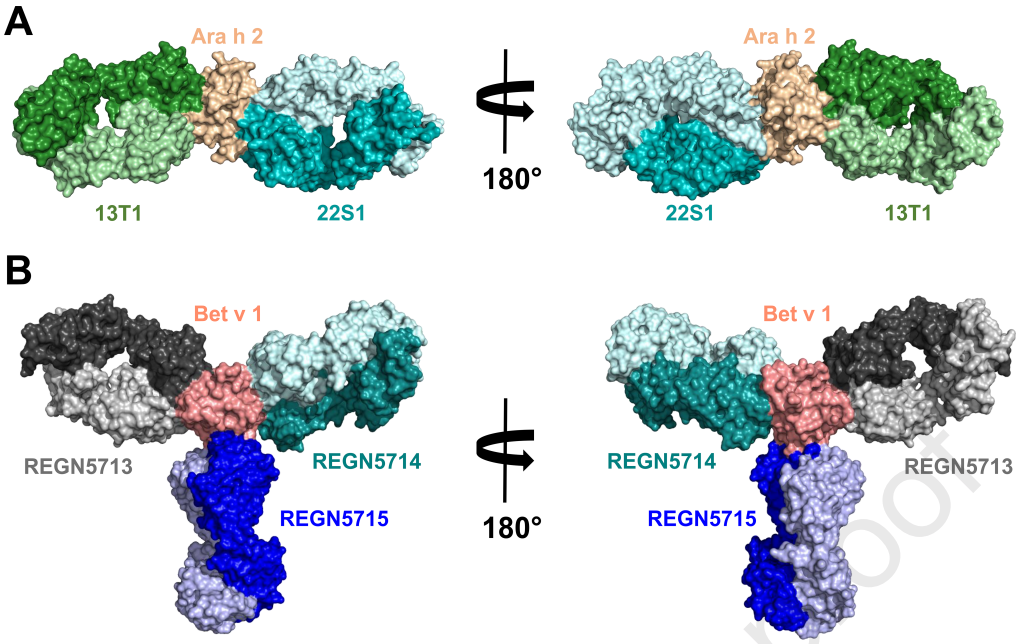
Figure 2. A. Crystal structure (PDB: 7MLH) of Der p 2 in complex with Fab of the IgE mAb 2F10. Der p 2 is shown in space filling representation with 2F10 epitope colored in pink. The Fab is presented using ribbon representation. Water molecules mediating the allergen antibody

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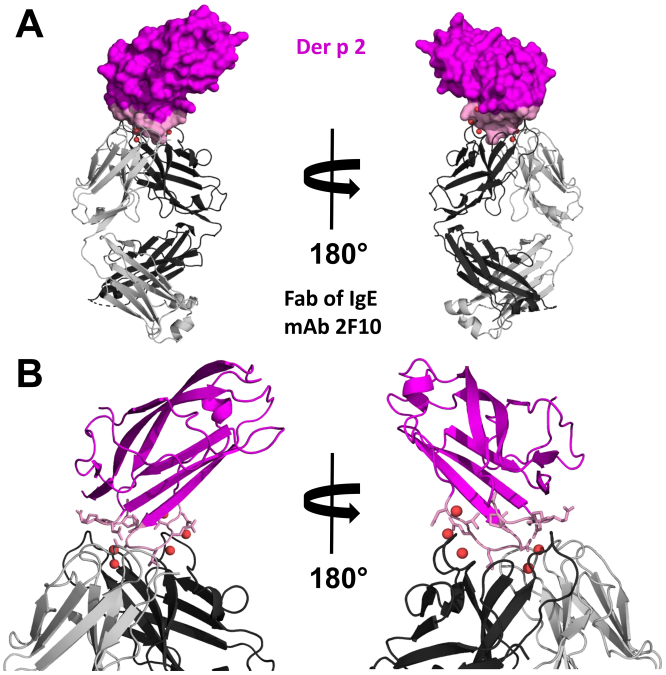
interactions are shown as red spheres. **B.** Close view of the allergen-antibody interface. Der p 2 is shown in ribbon representation with residues forming the epitope presented in stick representation and colored pink.

Figure 3. Plot showing epitope area versus number of residues in the epitopes from structures in Table II. There is a statistically significant correlation between both variables ($r = 0.68$, $p < 0.001$). Stars indicate that there are two different experimental models for a particular allergen-antibody pair (Bet v 1 (REGN5713) and Bet v 1 (REGN5713)* correspond to the PDB structures 7N0U and 7MXL, respectively; Bet v 1 (REGN5715) and Bet v 1 (REGN5715)* correspond to the PDB structures 7N0V and 7MXL, respectively; Der p 1 (4C1) and Der p 1 (4C1)* correspond to the PDB structures 5VPG and 5VPH, respectively; Gal d 4 (VH H04) and Gal d 4 (VH H04)* correspond to the PDB structures 4UX3 and 4PGJ, respectively, and Hev b 8 (2F5) and Hev b 8 (2F5)* correspond to the PDB structures 7SBD and 7SBG, respectively). IgE epitopes are highlighted in color (green: murine IgE mAb; blue: human IgE mAb derived from combinatorial libraries; and yellow: human IgE mAb generated by hybridoma technology).

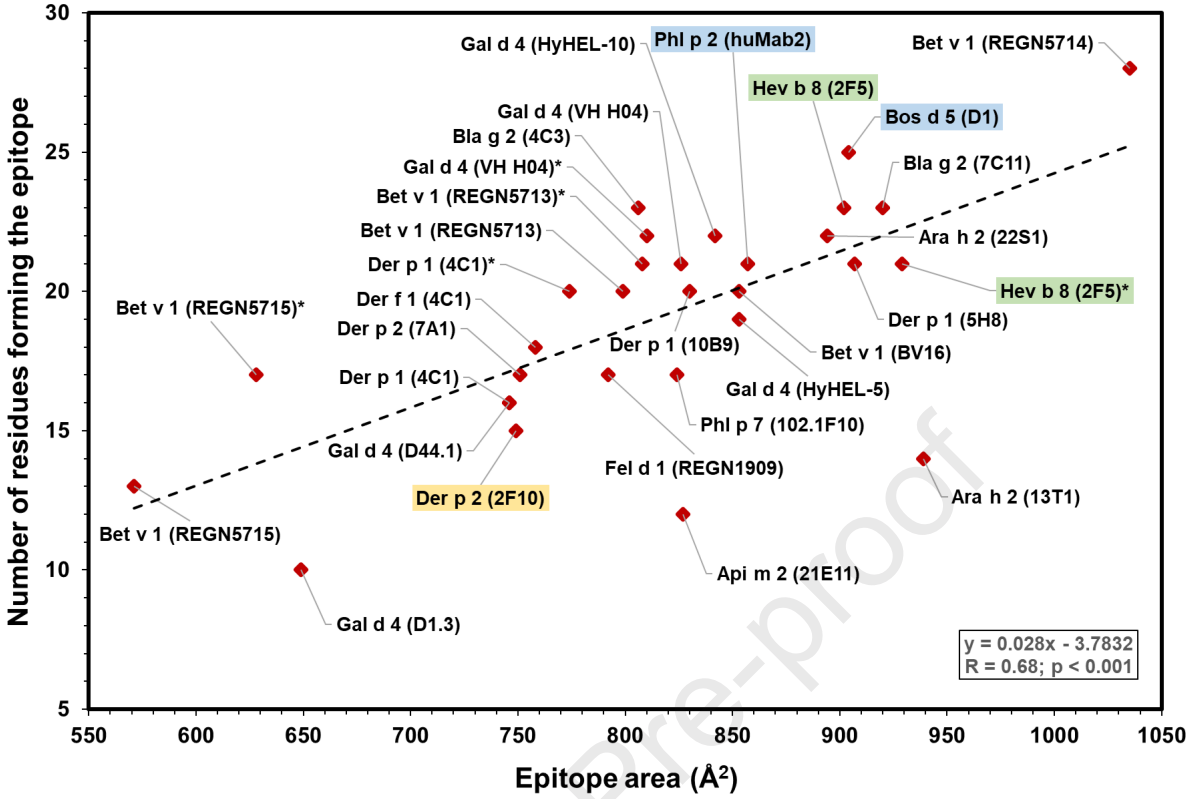
Figure 4. CRISPR engineering a genetic target using (top) traditional CRISPR-Cas9, resulting in gene knockout following DNA double strand break; or (bottom) CRISPR base or prime editors, which enable precision editing of a single target DNA nucleotide with the assistance of associated deaminase or reverse transcriptase enzymes. To date, CRISPR engineering has been applied to edit allergen genes in cat, goat's milk, soybean, peanut, hen's egg, and wheat. BLG: β-lactoglobulin. (© InBio, 2023)



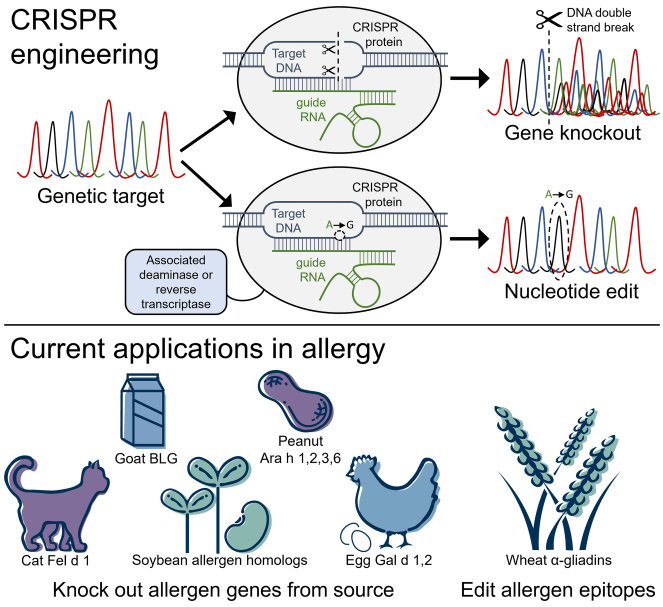
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